HEPATOCYTE AQUAPORINS IN BILE PHYSIOLOGY AND DISEASE

Raúl A. Marinelli[1*], Julieta Marrone[1] and Leandro R. Soria[2]

[1]Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2000 Rosario, Santa Fe, Argentina.

[2]L.R. Soria's present address is: Telethon Institute of Genetics and Medicine, Naples, Italy.

*** Correspondence to:**
Raúl A. Marinelli, Instituto de Fisiología Experimental. Facultad de Ciencias Bioquímicas y Farmacéuticas. Universidad Nacional de Rosario. Suipacha 570, 2000 Rosario, Santa Fe, Argentina. PHONE: 54-341-4305799. FAX: 54-341-4399473. E-mails: rmarinel@unr.edu.ar / marinelli@ifise-conicet.gov.ar

ABSTRACT

Bile formation by hepatocytes is an osmotic secretory process resulting from the canalicular secretion of water in response to osmotic gradients created by the active transport of solutes, primarily bile salts, and other organic anions. Thus bile secretion would be ultimately dependent on the canalicular expression of bile salt and organic anion transporters as well as the osmotic water permeability of the canalicular plasma membrane domain, mainly determined by aquaporin-8 (AQP8) water channels. Compelling experimental evidence suggests that canalicular AQP8 facilitates the osmotically-coupled transport of solute and water during the formation of bile. Downregulation of AQP8-mediated hepatocyte canalicular water permeability is found in rat models of hepatocellular cholestasis suggesting that defective hepatocyte AQP8 expression is involved in the molecular mechanisms of bile secretory failure. The study of AQP function in liver has provided new insights into normal bile physiology and disease mechanisms, and may yield novel therapies to improve certain cholestatic conditions.

Keywords: aquaporin water channels; hepatocytes; bile secretion; cholestasis

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Aquaporins (AQPs) are a family of membrane channel proteins that facilitate the osmotically driven movement of water molecules [1, 2]. Some AQPs also display permeability to certain small uncharged molecules. AQPs assemble into homotetrameric functional units (Figure 1). Thirteen mammalian AQP proteins, numbered 0 to 12, have been identified thus far. Each subunit is composed of around 270 amino acids with cytoplasmic carboxy- and amino-terminal ends. They consist of six bilayer-spanning domains connected by three extracellular (A, C, and E) and two intracellular loops (B and D). Two of these loops enclose the conserved motif Asn-Pro-Ala (NPA) which is part of the aqueous pore [1] (Figure 1). Several recent studies have revealed the high-resolution structures of the water channel family of proteins that explain their selectivity [3]. The three key features for channel selectivity are: (i) size restriction. The pore narrows to a diameter of 2.8 Å (approximately the diameter of a water molecule); (ii) electrostatic repulsion. The residue Arg-195 imposes a barrier to cations, including protonated water (H_3O^+); (iii) water dipole reorientation. Positively charged dipoles reorient water molecules and prevent the formation of a proton conductance.

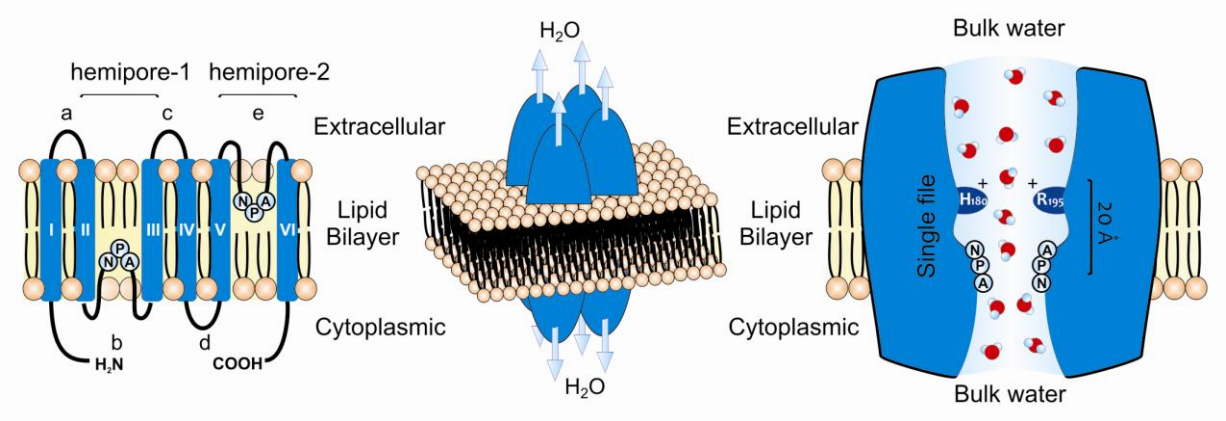

Figure 1. Membrane Topology and Organization of the AQP Molecule. ***Left:*** A single AQP monomer is composed of six transmembrane domains connected by five loops (a-e) with two NPA boxes shaping the water pore and the amino and carboxy termini oriented intracellularly. ***Middle:*** AQPs are arranged in tetramers. The water pore is formed by connecting loops b and e in each subunit allowing bidirectional water movement. ***Right:*** The channel consists of an extracellular and intracellular vestibule containing water in bulk solution joined in the center by a constriction of 20 Å in length where water molecules pass in single file. The a/R constriction delimited by arginine in the position 195 (R195) and histidine in the position 180 (H180) provides fixed positive charges which prevent proton passage. The second constriction is bounded by two asparagine residues from the highly conserved NPA motif. The single water molecule passes through the constriction with no resistance as it forms transient hydrogen bonds with the nearby asparagines.

Hepatocyte Aquaporins

Hepatocytes, as polarized epithelial cells, are characterized by two definite plasma membrane domains: a basolateral domain, in contact with the sinusoidal blood and a bile canalicular domain, defining a sealed apical compartment. Two members of the AQP family, i.e., AQP8 and AQP9 proteins were demonstrated to be expressed in rodent and human hepatocytes [4-6].

AQP8 is localized to pericanalicular vesicles and the canalicular plasma membrane domain of hepatocytes as an N-glycosylated protein of about 34 kDa [4]. In its non-glycosylated form of about 28 kDa, AQP8 was found to be expressed in mitochondria, specifically in the inner mitochondrial membrane [7, 8]. Hepatocytes are able to hormonally regulate their AQP8-mediated canalicular water permeability. Thus, the choleretic hormone glucagon

induces AQP8 trafficking from an intracellular vesicular compartment to lipid raft microdomains in canalicular plasma membranes which is accompanied by an increase in membrane water permeability [9-11]. Glucagon-induced hepatocyte AQP8 trafficking requires activation of cAMP-PKA and PI3K signaling pathways. Since the AQP8 molecules lack consensus PKA and PI3K phosphorylation sites [9, 11] and therefore may not be phosphorylated, the effect of glucagon is believed not to be directed towards AQP8 protein itself but rather to microtubule-associated proteins involved in vesicle trafficking. In fact, an intact microtubular network is required for AQP8 trafficking [9, 12]. Hepatocyte AQP8 can also be modulated on the long term basis by modifying its gene expression. Thus glucagon upregulates AQP8 protein expression. Although the precise mechanisms by which glucagon exerts its action have not been determined, they seem to involve inhibition of calpain- mediated AQP8 protein degradation, a process that also involves cAMP-PKA and PI3K activation [13]. There is compelling experimental evidence for the significance of canalicular AQP8 in modulating membrane water permeability and to promote osmotically-driven water secretion [14]. However, mitochondrial AQP8 did not appear to have major relevance in mediating the transport of water across mitochondrial membranes [15, 16]. Based on the fact that AQP8 is also able to facilitate the transmembrane diffusion of ammonia [17] and hydrogen peroxide [18], there is recent evidence suggesting the involvement of hepatocyte mitochondrial AQP8 in ammonia detoxification via ureagenesis [8, 19, 20] and in H_2O_2 dependent cellular signaling [21].

Hepatocyte AQP9 is an approximately 32 kDa protein specifically localized on the sinusoidal plasma membrane domain of hepatocytes (6, 22). In addition to water, AQP9 displays selectivity to a broad variety of small uncharged solutes including glycerol and urea [23]. Thus AQP9 is thought to facilitate the basolateral movement of water as well as the cellular uptake or exit of metabolites with minimal osmotic perturbation. Hepatocyte expression or subcellular localization of AQP9 does not seem to be regulated by glucagon [9, 13].

Significance of Aquaporins in Bile Physiology

Water transport across hepatocytes plays a significant role in bile production because bile is a complex fluid composed of more than 95% water. Current concepts of hepatocyte bile formation encompass the following general features. Osmotically active substances, primarily bile salts, and other organic anions are actively transported into and concentrated within the canalicular lumen, resulting in the passive entry of water. Thus canalicular bile formation is an osmotic secretory process. The excretion of bile salts via the bile salt transporter Bsep/ABCB11, glutathione via the organic anion transporter Mrp2/ABCC2 and HCO_3^-, via the Cl^-/HCO_3^- exchanger AE2, are thought to be the major driving forces for water movement from the sinusoidal blood to the bile canaliculus [24].

Theoretically, water can flow through the hepatocyte epithelial barrier either across tight junctions between adjacent hepatocytes (paracellular route) or across hepatocyte plasma membrane domains (transcellular route). Current experimental evidence from water transport studies in isolated polarized hepatocyte couplets, basolateral and canalicular membrane vesicles and hepatic cell lines, suggests that the transcellular pathway accounts for most of the water entering the bile canaliculus, with minimal paracellular contribution [14, 24]. Besides, the studies demonstrated that the canalicular membrane has lower water permeability than the basolateral membrane, and thus it is rate-limiting for transcellular water transport in hepatocytes [25]. AQPs can account for the water permeability of both hepatocyte plasma membrane domains, suggesting their key role in canalicular bile formation. Experimental evidence for the role of AQP8 in canalicular bile secretion comes

from chemical inhibitory studies in rat hepatocyte couplets [5] as well as AQP8 knockdown studies in human bile canaliculi-forming HepG2 cells [26]. AQP8 would modulate the canalicular, rate-limiting water flow, whereas AQP9 its basolateral uptake [14]. Moreover, hormones able to stimulate choleresis, such as endothelins and glucagon, increase canalicular AQP8 expression [27, 9]. Thus AQP8 would provide a molecular mechanism for the efficient coupling of osmotically active solutes and water transport during agonist-stimulated hepatocyte bile formation (Figure 2).

Significance of Aquaporins in Liver Cholestatic Disease

Cholestasis is characterized by impairment of bile formation and it is caused by several liver diseases. Chronic cholestasis can progress towards biliary cirrhosis and liver failure requiring liver transplantation (28). As detailed above, cumulative evidence indicates that AQPs play a physiological role in canalicular bile formation. Hence, it is conceivable that

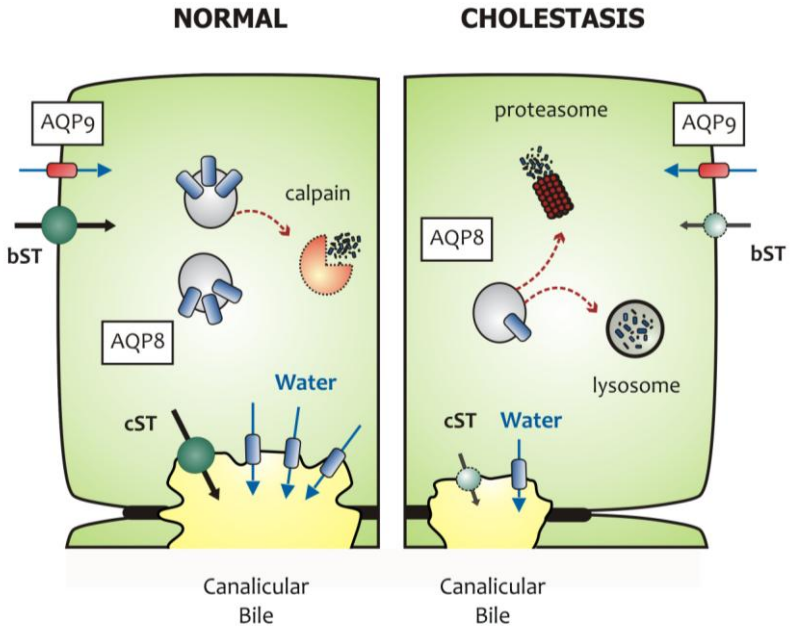

Figure 2. Hepatocyte AQPs in Bile Formation and Cholestasis. On the **left**, it is illustrated a normal hepatocyte. Bile salts and other endogenous anions are secreted into the bile canaliculus via canalicular solute transporters (cST), i.e. the bile salt export pump Bsep/ABCB11, the organic anion transporter Mrp2/ABCC2, and the Cl-/HCO3-exchanger AE2. AQP8 facilitates the canalicular, rate-limiting osmotic water flow whereas AQP9 contributes to the sinusoidal water uptake. Calpains are involved in modulating the hepatocyte degradation of endogenous AQP8 protein. On the **right**, a cholestatic hepatocyte is illustrated. Canalicular AQP8 expression is down-regulated by anomalous lysosomal and proteosomal protein degradation. Hepatocellular cholestasis results from a mutual occurrence of impaired biliary solute secretion via downregulated cST and reduced AQP8-mediated canalicular osmotic water transport.

defective canalicular AQP expression may lead to alterations of normal bile physiology. Defective expression of canalicular AQP8 protein was found to be present in experimental pathological conditions in which altered bile secretion occurs, i.e. in extrahepatic (obstructive) cholestasis [29] and in intrahepatic (hepatocellular) cholestasis, such as estrogen-induced [30] or sepsis-associated cholestasis [31, 32]. Although the mechanism for AQP8 protein reduction has not been fully elucidated yet, it seems to be posttranscriptional and it involves an increase lysosomal and proteosomal protein degradation. The finding that canalicular AQP8 expression is defective in different rat models of cholestasis suggests that AQP water channels are involved in the development of bile secretory dysfunction (Figure 2).

Aquaporin Gene Therapy to Cholestasis

Adenoviruses are commonly used vectors for gene therapy. The gene transfer of human water channel AQP1 via the adenoviral vector Ad*h*AQP1 has been successfully used to restore normal salivary flow to irradiated hypofunctional salivary gland of experimental animals [33] and humans [34]. We designed and performed studies to evaluate whether the administration of the Ad*h*AQP1 vector can promote AQP1-mediated canalicular water secretion and improve hepatocyte bile secretory failure in a cholestatic condition. As estrogens are well known to cause liver cholestatic disease, we made use of an established experimental model of estrogen-induced cholestasis, i.e., the administration of a synthetic estrogen ethinylestradiol [30]. Ad*h*AQP1, administered by retrograde bile ductal infusion, induced hepatocyte canalicular *h*AQP1 expression and a concomitant increased in canalicular osmotic water permeability. Bile flow as well as choleretic efficiency of endogenous bile salts (i.e., volume of bile per micromol of excreted bile salt) were significantly augmented. Thus indicating that the bile flow was improved to some extent by *h*AQP1-mediated canalicular water transport [35]. An unexpected finding in *h*AQP1-transduced cholestatic rats was a noteworthy improvement in the biliary output of bile salts caused by increased activity of the canalicular bile salt transporter Bsep/ABCB11 [36]. This effect was absent in normal rats, indicating its specificity for cholestatic rats. Our data suggest that the adenoviral transfer of *h*AQP1 gene to livers of estrogen-induced cholestatic rats improves bile secretory failure by increasing both biliary output and choleretic efficiency of bile salts [35, 36]. This finding may have potential as novel treatment for certain liver cholestatic diseases.

REFERENCES

[1] Preston GM, Carroll TP, Guggino WB, Agre P. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science* 1992; 256: 385-387.

[2] King LS, Kozono D, Agre P. From structure to disease: the evolving tale of aquaporin biology. *Nat Rev Mol Cell Biol* 2004; 5: 687-698.

[3] Tani K, Fujiyoshi Y. Water channel structures analysed by electron crystallography. *Biochim Biophys Acta.* 2014; 1840: 1605-1613.

[4] García F, Kierbel A, Larocca MC, Gradilone SA, Splinter P, LaRusso NF, Marinelli RA. The water channel aquaporin-8 is mainly intracellular in rat hepatocytes, and its plasma membrane insertion is stimulated by cyclic AMP. *J Biol Chem* 2001; 276: 12147-12152.

[5] Huebert RC, Splinter PL, García F, Marinelli RA, LaRusso NF. Expression and localization of aquaporin water channels in rat hepatocytes. Evidence for a role in canalicular bile secretion. *J Biol Chem* 2002; 277: 22710-22717.

[6] Elkjaer ML, Vajda Z, Nejsum LN, Kwon TH, Jensen UB, Amiry-Moghaddam M, Frokiaer J, Nielsen S. Immunolocalization of AQP9 in liver, epididymis, testis, spleen, and brain. *Biochem Biophys Res Commun* 2000; 276: 1118-1128.

[7] Calamita G, Ferri D, Gena P, Liquori GE, Cavalier A, Thomas D, Svelto M. The inner mitochondrial membrane has aquaporin-8 water channels and is highly permeable to water. *J Biol Chem* 2005; 280: 17149-17153.

[8] Soria LR, Marrone J, Calamita G, Marinelli RA. Ammonia detoxification via ureagenesis in rat hepatocytes involves mitochondrial aquaporin-8 channels. *Hepatology* 2013; 57: 2061-2071.

[9] Gradilone SA, García F, Huebert RC, Tietz PS, Larocca MC, Kierbel A, Carreras FI, LaRusso NF, Marinelli RA. Glucagon induces the plasma membrane insertion of functional aquaporin-8 water channels in isolated rat hepatocytes. *Hepatology* 2003; 37: 1435-1441.

[10] Mazzone A, Tietz P, Jefferson J, Pagano R, LaRusso NF. Isolation and characterization of lipid microdomains from apical and basolateral plasma membranes of rat hepatocytes. *Hepatology* 2006; 43: 287-296.

[11] Tietz P, Jefferson J, Pagano R, LaRusso NF. Membrane microdomains in hepatocytes: potential target areas for proteins involved in canalicular bile secretion. *J Lipid Res* 2005; 46: 1426-1432.

[12] Gradilone SA, Carreras FI, Lehmann GL, Marinelli RA. Phosphoinositide 3-kinase is involved in the glucagon-induced translocation of aquaporin-8 to hepatocyte plasma membrane. *Biol Cell* 2005; 97: 831-836.

[13] Soria LR, Gradilone SA, Larocca MC, Marinelli RA. Glucagon induces the gene expression of aquaporin-8 but not that of aquaporin-9 water channels in the rat hepatocyte. *Am J Physiol Regul Integr Comp Physiol* 2009; 296: R1274-R1281.

[14] Marinelli RA, Lehmann GL, Soria LR, Marchissio MJ. Hepatocyte aquaporins in bile formation and cholestasis. *Front Biosci (Landmark Ed)* 2011; 17: 2642-2652.

[15] Calamita G, Gena P, Meleleo D, Ferri D, Svelto M. Water permeability of rat liver mitochondria: A biophysical study. *Biochim Biophys Acta* 2006; 1758: 1018-1024.

[16] Gena P, Fanelli E, Brenner C, Svelto M, Calamita G. News and views on mitochondrial water transport. *Front. Biosci.* 2009; 1: 352-361.

[17] Saparov SM, Liu K, Agre P, Pohl P. Fast and selective ammonia transport by aquaporin-8. *J Biol Chem* 2007; 282: 5296-5301.

[18] Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem* 2007; 282: 1183-1192.

[19] Soria LR, Fanelli E, Altamura N, Svelto M, Marinelli RA, Calamita G. Aquaporin-8-facilitated mitochondrial ammonia transport. *Biochem Biophys Res Commun* 2010; 393: 217-221.

[20] Soria LR, Marrone J, Molinas SM, Lehmann GL, Calamita G, Marinelli RA. Lipopolysaccharide impairs hepatocyte ureagenesis from ammonia: involvement of mitochondrial aquaporin-8. *FEBS Lett.* 2014; 588: 1686-1691.

[21] Marchissio MJ, Francés DEA, Carnovale CE, Marinelli RA. Mitochondrial aquaporin-8 knockdown in human hepatoma HepG2 cells causes ROS-induced mitochondrial depolarization and loss of viability. *Toxicol Appl Pharmacol*. 2012; 264: 246-254.

[22] Carbrey JM, Gorelick-Feldman DA, Kozono D, Praetorius J, Nielsen S, Agre P: Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. *Proc Natl Acad Sci U S A* 2003; 100: 2945-2950.

[23] Tsukaguchi H, Weremowicz S, Morton CC, Hediger MA. Functional and molecular characterization of the human neutral solute channel aquaporin-9. *Am J Physiol* 1999; 277: F685-F696.

[24] Boyer JL. Bile formation and secretion. *Compr Physiol*. 2013; 3: 1035-1078.

[25] Marinelli RA, Tietz PS, Caride AJ, Huang BQ, LaRusso NF. Water transporting properties of hepatocyte basolateral and canalicular plasma membrane domains. *J Biol Chem* 2003; 278: 43157-43162.

[26] Larocca MC, Soria LR, Espelt MV, Lehmann GL, Marinelli RA: Knockdown of hepatocyte aquaporin-8 by RNA interference induces defective bile canalicular water transport. *Am J Physiol Gastrointest Liver Physiol* 2009; 296: G93-G100.

[27] Rodriguez MR, Soria LR, Ventimiglia MS, Najenson AC, Di María A, Dabas P, Fellet A, Marinelli RA, Vatta MS, Bianciotti LG. Endothelin-1 and -3 induce choleresis in the rat through ETB receptors coupled to nitric oxide and vagovagal reflexes. *Clin Sci (Lond)* 2013; 125 :521-532.

[28] Lee J, Boyer JL. Molecular alterations in hepatocyte transport mechanisms in acquired cholestatic liver disorders. *Semin Liver Dis* 2000; 20: 373-384.

[29] Carreras FI, Gradilone SA, Mazzone A, García F, Huang BQ, Ochoa JE, Tietz PS, LaRusso NF, Calamita G, Marinelli RA. Rat hepatocyte aquaporin-8 water channels are down-regulated in extrahepatic cholestasis. *Hepatology* 2003; 37: 1026-1033.

[30] Carreras FI, Lehmann GL, Ferri D, Tioni MF, Calamita G, Marinelli RA. Defective hepatocyte aquaporin-8 expression and reduced canalicular membrane water permeability in estrogen-induced cholestasis. *Am J Physiol Gastrointest Liver Physiol* 2007; 292: G905-G912.

[31] Lehmann GL, Carreras FI, Soria LR, Gradilone SA, Marinelli RA. LPS induces the TNF-alpha-mediated downregulation of rat liver aquaporin-8: role in sepsis-associated cholestasis. *Am J Physiol Gastrointest Liver Physiol* 2008; 294: G567-G575.

[32] Lehmann GL, Marinelli RA. Peritoneal sepsis downregulates liver expression of Aquaporin-8: a water channel involved in bile secretion. *Liver Int* 2009; 29: 317-318.

[33] Delporte C, O'Connell BC, He X, Lancaster HE, O'Connell AC, Agre P, Baum BJ. Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. *Proc Natl Acad Sci U S A* 1997; 94:3268-3273.

[34] Baum BJ, Alevizos I, Zheng C, Cotrim AP, Liu S, McCullagh L, Goldsmith CM, Burbelo PD, Citrin DE, Mitchell JB *et al.* Early responses to adenoviral-mediated transfer of the aquaporin-1 cDNA for radiation-induced salivary hypofunction. *Proc Natl Acad Sci USA* 2012; 109: 19403-19407.

[35] Marrone J, Lehmann GL, Soria LR, Pellegrino JM, Molinas S, Marinelli R. Adenoviral transfer of human aquaporin-1 gene to rat liver improves bile flow in estrogen-induced cholestasis. *GeneTher* 2014; 21:1058-1064.

[36] Marrone J, Soria LR, Danielli M, Lehmann GL, Larocca MC, Marinelli RA. Hepatic gene transfer of human aquaporin-1 improves bile salt secretory failure in rats with estrogen-induced cholestasis. *Hepatology (in press).*

About the Authors

Raúl A. Marinelli received his Ph.D. in Animal Physiology from the National University of Rosario, Argentina. He undertook his postdoctoral training in Cellular Physiology at Mayo Clinic and Foundation, MN, U.S.A. He is currently Principal Investigator from CONICET and Professor of Physiology at the National University of Rosario. His major research interest is the physiology of aquaporin channels in liver cells.

Julieta Marrone is a postdoctoral fellow from CONICET.

Leandro R. Soria is a postdoctoral fellow at Telethon Institute of Genetics and Medicine, Naples, Italy.