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Review

Mammalian aquaglyceroporin function in metabolism

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ABSTRACT

Aquaglyceroporins are integral membrane proteins that are permeable to glycerol as well as water. The movement of glycerol from a tissue/organ to the plasma and vice versa requires the presence of different aquaglyceroporins that can regulate the entrance or the exit of glycerol across the plasma membrane. Actually, different aquaglyceroporins have been discovered in the adipose tissue, small intestine, liver, kidney, heart, skeletal muscle, endocrine pancreas and capillary endothelium, and their differential expression could be related to obesity and the type 2 diabetes.

Here we describe the expression and function of different aquaglyceroporins in physiological condition and in obesity and type 2 diabetes, suggesting they are potential therapeutic targets for metabolic disorders.

Contents

1. Glycerol metabolism

Glycerol, chemically a polyol, is the backbone of triglycerides (TG) and a precursor of phospholipids; it is an important intermediate in carbohydrate and lipid metabolism, and functions as a shuttle of electrons from cytosol to mitochondria by regenerating NAD + from NADH [1].

The digestion of dietary TG by pancreatic lipase produces mono- and diacylglycerols, which are absorbed by the small intestinal mucosa. A lesser amount of glycerol is also absorbed in the free form (see section 5, "Small Intestine") due to the low concentration of glycero-kinase (GK) within the enterocyte. Monoglycerides and fatty acids undergo TG resynthesis. TG enter the bloodstream as chylomicrons and VLDL. A lipoprotein lipase attached to the capillary endothelial cells of adipose tissue, muscles and the heart hydrolyzes TG to free fatty acids and glycerol. Fatty acids are actively transported into tissues whereas glycerol is mainly converted to glycerol-3-phosphate (glycolysis intermediate) by GK in the liver and the kidney. Glycerol needs to be activated by a phosphorylation reaction before entering the carbohydrate and lipid metabolism.

Glycerol plasma concentration is determined primarily by the amount released by adipose tissue lipolysis, but also by the amount

Abbreviations: TG, triglycerides; GK, glycerol kinase; AQP, aquaporin; GlpF, glycerol facilitator; FDA, Food and Drug Administration; WT, wild type; AMPK, AMP-activated protein kinase; NAFLD, non-alcoholic fatty liver disease; NASH, steatohepatitis; VRAC, volume-regulated anion channels.

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Table 1
Aquaglyceroporins: main organ/tissue expression and localization, and their related functions.

Aquaporin	Localization	Main physiological functions
AQP3	Gastrointestinal tract*	Water and small solutes absorption and secretion
	Kidney*	Contribute to urinary concentration
	Adipose tissue*	Glycerol metabolism
	Skin [139,140]	Skin barrier, hydration and elasticity, cell proliferation and migration (tumorigenesis, wound healing)
	Lymphocytes/Dendritic cells [141,142]	Activation, proliferation, migration
	Erythrocytes [143,144]	Glycerol transport in human erythrocytes and ROS cleaning system by primitive erythroid cells
	Female reproductive system [145]	Vaginal lubrication, cervical water balance in pregnancy and parturition, water and non-charged solutes in placenta
	Male reproductive system [145]	Maintenance of normal fertility
	Eye [146]	Corneal wound healing, involvement in the postnatal retina development
	Heart/skeletal muscle*	Glycerol transport to be used for energy production
	Respiratory tract [147]	Water homeostasis in the epithelia of the upper and lower respiratory tract
	Mammary gland [148,149]	Production of the aqueous component of the milk
	Articular cartilage [150]	Chondrocyte volume regulation and extracellular matrix
	Inner Ear [151,152,153]	Water homeostasis of perilymph and endolymph
AQP7	Gastrointestinal tract*	Water and small solutes absorption and secretion
	Kidney*	Involvement in glycerol reabsorption
	Adipose tissue*	Glycerol metabolism
	Heart/skeletal muscle*	Transport of glycerol to be used for energy production
	Endocrine pancreas*	Could be involved in insulin production/secretion and in the reduction of β-cell mass
	Female reproductive system [145,154]	Endometrial decidualization; fluid transport into antral follicles
	Male reproductive system [145]	Sperm maturation, storage and motility; seminiferous tubule fluid production.
	Eye [155]	May participate in water transport into the vitreous body
	Inner Ear [151,152,153]	Water homeostasis of perilymph and endolymph
AQP9	Gastrointestinal tract [156]	Involvement in the synthesis/secretion of a certain type of mucus
	Liver*	Glycerol uptake for gluconeogenesis
	Adipose tissue*	Glycerol metabolism
	Inner ear [151,152,153]	Water homeostasis of perilymph and endolymph, may be involved in the vestibular sensory transduction system
	Female reproductive system [145,157]	Endometrial decidualization, epithelial water transport in the oviduct and the movement of the embryo from the oviduct to the uterus, fluid transport into antral follicles, transport of water and/or non-charged solutes across the placenta
	Male reproductive system [145]	Water and small solutes transport in Leydig cells; epididymal and seminiferous tubules fluid formation
	Eye [146,155]	may serve to transport lactate, glycerol and metabolites in the retina
	Bone [158,159,160]	Osteoclast differentiation and cell fusion process (in stressed condition?)
	Erythrocytes [161]	AQP9 is the major pathway for glycerol and other small uncharged solutes transport in mouse erythrocytes
	Neutrophil leukocyte [162,163,164]	Cell motility
	Brain [165]	Possible involvement in glucose energy metabolism in astrocyte and in some catecholaminergic neurons
	Skin [80]	Involved in maintaining skin hydration.
AQP10	Gastrointestinal tract*	Water and small solutes absorption and secretion
	Adipose tissue*	Glycerol metabolism
	Male reproductive system [166]	Water balance maintenance
	Skin [140]	Involved in skin barrier

* See related section for references.

absorbed in the gastrointestinal tract, and by the variable amount reabsorbed in the kidney tubules [2,3,4,5,6].

In the interprandial state, in starvation and in exercise, the glycerol from lipolysis represents an important substrate for gluconeogenesis together with lactate, pyruvate and the amino acids alanine and glutamine [7,8,9]. In prolonged starvation, the amount of glycerol metabolized to glucose reached 76% in obese subjects [7]. Adipocyte lipolysis is a highly regulated process: it is activated by β-adrenergic agonists, melanocortins, thyroid-stimulating hormone and atrial natriuretic peptide, leptin, glucocorticoids (both directly and through permissive action) and growth hormone and it is inhibited by insulin, neuropeptide Y and peptide YY [10].

However, all the metabolic reactions described above can only occur intracellularly, after the glycerol has crossed the plasma membrane barrier. Glycerol transport across cell membranes requires specific proteins called aquaglyceroporins, a subfamily of the water channel proteins aquaporins (AQPs), [11]. For these reasons AQP expression and functioning play a key role be implicated in the control of fat accumulation and in the associated metabolic alterations.

2. Aquaporins

Aquaporins are integral membrane proteins that were initially thought to function only as bidirectional water-selective channels, but they have successively been found to play a role in important cellular functions, such as cell proliferation, cell differentiation, cell migration and cell adhesion [12,13,14]. Thirteen water channel proteins have been identified in mammals and they have been divided into three groups based on their structural and functional characteristics: (i) aquaporins (AQP1, 2, 4, 6 and 8) selectively permeable to water; (ii) aquaglyceroporins (AQP3, 7, 9 and 10) permeable to glycerol, urea and other small solutes in addition to water (see Table 1 for tissue localization and main physiological functions); (iii) S-aquaporins (AQP11 and 12), with peculiar intracellular localization and functions, currently under study [12,15,16]. AQPs are small proteins (26–34 kDa) assembled as homotetramers in the membrane, with each monomer containing a single aqueous pore. As demonstrated for AQP1, the monomer sequence consists of two repeated segments (tandem repeats), each formed by three α-helical transmembrane domains and five loops (three external and two internal to the membrane), with the amino-and carboxy-termini intracellularly localized. The two tandem repeats contain (in the second and the last loop) two highly conserved domains called NPA boxes (Asn-Pro-Ala) that play a crucial role in pore formation. In the "hourglass model", the six transmembrane helices form the walls of the channel, like two connected bulbs, while the two NPA motifs, folding in the center of the membrane, represent the narrowest part of the pore. This channel conformation allows a trickle of water molecules in single-file from one side of the membrane to the other, which depends exclusively on the presence of an osmotic gradient across the

membrane. The selectivity filter in the center of the pore is ensured by both electrostatic and steric factors. In particular, the specificity is determined by the constriction region; the target of mercury compounds, which are known inhibitors of almost all AQPs (AQP4 being the most studied exception) has been identified close to this region [17]. Point mutation experiments demonstrated that the Cys189 of AQP1, located in the narrowest site of the pore, is the residue that binds covalently the sulfhydryl-reactive heavy metal ions [18,19,20].

Aquaglyceroporins sequencing revealed a common additional conserved Asp residue near the second NPA box that enlarges the pore to permit glycerol to permeate [12,21].

Moreover, the selectivity filter (the constriction region) in the center of the pore is ensured by the diameter and composition of the aromatic arginine region (ar/R) through electrostatic and steric factors that form a barrier to protons and other cations. In the human AQP1 the pore diameter is about 2.8 Å, with the ar/R constriction region formed by the juxtaposition of hisidine/arginine residues. In human aquaglyceroporins and in the glycerol facilitator (GlpF), the aquaglyceroporin expressed in *Escherichia coli*, the presence of a smaller amino acid residue in the constriction region with the glycine substituting the histidine makes the pore larger (pore diameter of about 3.5 Å) and permeable also to glycerol [22,23,24,25,26].

Although the selectivity of AQPs and aquaglyceroporins to water and polyols like glycerol is well established, a potentially wider permeability also to gases and other small solutes has been suggested [27,28] and is currently being discussed [29]. AQP8 shows remarkable permeability to urea and ammonia, so that in the liver this AQP seems to be involved in the detoxification processes [27,30]. In addition, AQP8 and probably almost all AQPs to different extents, are able to transport hydrogen peroxide; this implies AQPs may have a role in mediating oxidative stress and in tuning intracellular signaling [24,31].

An interesting feature of aquaglyceroporins is their ability to facilitate arsenous acid and arsenite transmembrane transport, with AQP9 being the most efficient channel, followed by AQP7 [32,33]. The mechanism by which arsenic and other metalloids can permeate aquaglyceroporins is that at physiological pH they mimic the characteristics of the glycerol molecule: they are undissociated, similarly uncharged, and their volume is only slightly smaller than that of glycerol [34]. The presence of high levels of AQP9 protein in the liver could therefore play a role in arsenic detoxification by mediating metalloid inflow into hepatocytes [35]. Interestingly, AQP9 expression in leukocytes [36] has proved fundamental for the transport of arsenic trioxide, so that this compound can be used as an antineoplastic chemotherapy drug; in September 2000, the FDA approved arsenic reintroduction for the treatment of relapsed or refractory acute promyelocytic leukemia [34,37,38].

Unlike other aquaporins, AQP6 shows a surprisingly low water permeability in basal conditions, but its water channel function can be turned on by mercury treatment and by an acidic environment. Moreover, this activation of AQP6 water permeability is accompanied by a marked anion permeability with the following permeability order: $NO_3^- > I^- >> Br^- > Cl^- >> F^- >> SO_4^{2-}$ [39,40].

Finally, a permeability of AQPs to gases such as CO_2 and nitric oxide, and to a variety of non-charged solutes, like carbamides, purines and pyrimidines has also been proposed [27,36,41], but needs further validation.

Here we will consider the handling of glycerol by the adipose tissue, the liver, the gastrointestinal tract, the kidney, the endocrine pancreas, the heart/skeletal muscle and by microvessels.

3. Adipose tissue

AQP7 was the first glycerol channel identified in the adipose tissue of humans and rodents, and for fifteen years it was considered the only aquaglyceroporin in this tissue [42,43]. More recently, other aquaglyceroporins have been found in human adipocytes: AQP3 and AQP9 [44], AQP10 [45] and AQP11 [46]. Interestingly, AQP10 protein is expressed in humans, while in mice and in other animal species it is a nonfunctional pseudogene [12,47,48].

As for cellular and subcellular expression and localization, research yielded conflicting results. The first attempts to define AQP7 localization in adipocytes were performed in murine and human cell cultures, followed by immunohistochemical studies in human adipose tissue. The results supported an expression confined to adipocytes [49,50,51] with an intracellular localization of the protein, which migrated to the plasma membrane in response to β-adrenergic stimuli [44,45,49,51]. Other studies found instead that AQP7 is expressed in the endothelial cells of small vessels within the adipose tissue [52,53]. Laforenza et al. found that AQP7 is localized in both adipocyte and endothelial plasma membranes of human adipose tissue [45]. This double localization is now confirmed [54].

AQP3 and 9 were found in the human subcutaneous and omental adipose tissue with an mRNA concentration similar to that of AQP7, with the omental AQP3 even more expressed [44]. AQP3 and AQP7 are both localized in the membranes surrounding the lipid droplets and in the plasma membranes of adipocytes, while AQP9 is constitutively expressed in the plasma membranes [44]. Since other studies both in mice and in humans were unable to detect these AQPs [49,53,55], and given the absence of appropriate antibody controls, these results need to be confirmed. AQP10 was found at mRNA and protein level in human subcutaneous adipose tissue and localized both in the membranes surrounding the lipid droplets and in the plasma membranes [45]. Miranda et al., searching for more aquaglyceroporins in the adipose tissue, did not find traces of AQP10 transcript [51]. More recently, AQP11 has been identified in both subcutaneous and human mature visceral adipocyte cell models, localized in proximity of the lipid droplets; both hAQP11 silencing and overexpression demonstrated its water and glycerol permeability [46]. However, the intracellular localization of AQP11 and its unexpected glycerol permeability need to be further confirmed.

A study by Kishida et al. [49] provided the first evidence for the fundamental role of AQP7 in the release of glycerol from adipocytes. The results showed that AQP7 functions as a glycerol channel which is regulated negatively by insulin, positively by fasting and which is more abundantly expressed in obese mice with insulin resistance. Epinephrine, glucagon, and ACTH were ineffective at regulating AQP7 mRNA expression. However, epinephrine induced AQP7 translocation to the plasma membranes from the intracellular regions. Kuriyama et al. confirmed these findings and demonstrated that insulin coordinates the regulation of adipose AQP7 and liver AQP9 expression in fasting/refeeding [56]. Moreover, obese insulin-resistant db+/db+ mice showed higher AQP7 and 9 levels compared to controls. A similar increase in AQP7 mRNA was observed in adipose tissue from Otsuka Long-Evans Tokushima Fatty rats, an animal model of type 2 diabetes with obesity, compared to normal Long-Evans Tokushima Otsuka rats [57].

In addition to insulin, other hormones have been demonstrated to regulate aquaglyceroporin expression in the adipose tissue. Isoproterenol, tumor necrosis factor α and dexamethasone reduced the expression of AQP7 mRNA of cultured 3 T3-L1 adipocytes by 62, 60 and 39%, respectively, while angiotensin-2, growth hormone, and triiodothyronine had no effect [58]. The effects of leptin and ghrelin, two hormones involved in the regulation of food intake and body weight, were also analyzed. Acylated and desacyl-ghrelin were found to decrease AQP7 mRNA expression and to stimulate lipid accumulation in human visceral adipocytes after incubation for 48 h [59], while 30 min incubation with leptin increased AQP3 and decreased AQP7 and AQP9 protein in human cultured omental adipocytes [44].

Studies in two independent AQP7-knockout (KO) mouse lines [55,60,61] gave a boost to this topic. Initially, the first AQP7-KO mouse line did not show any change in either body weight or fat mass at least until 10 weeks of age [55]. However, the mice had lower plasma glycerol concentration, normal plasma FFA, and high glycerol content in adipocytes after fasting; in these AQP7-KO mice, the increase in

plasma glycerol induced by a β3-adrenergic agonist was also impaired. Similar results were obtained in AQP7-silenced 3 T3-L1 adipocytes. AQP7-KO mice also showed a slower increase in plasma glycerol and rapid reduction in plasma glucose during prolonged fasting. The same Authors one year later observed that the body weight of AQP7-KO mice significantly increases after 12 weeks of age and mice develop obesity [61]. AQP7-KO mice showed adipocyte hypertrophy, increased GK activity and susceptibility to obesity, and insulin resistance after being fed a high-fat/high-sucrose diet. Results obtained independently using a different AQP7-KO mice line evidenced a 3.7 fold increase in body fat mass after 16 weeks of age [60]. Adipocytes were hypertrophic, had elevated contents of glycerol, free fatty acids and triglycerides, and had an impaired glycerol uptake/release.

As a whole, AQP7 deficient mice show a marked reduction in glycerol release; the increased glycerol accumulation and the activation of GK enhances triacylglycerol synthesis, which in turn results in adipocyte hypertrophy and obesity. Moreover, glucose homeostasis was also impaired in AQP7-KO mice, which showed a tendency to develop insulin-resistance [60,61].

To complicate the picture, these results were not confirmed by subsequent studies using different AQP7-KO mouse models with different genetic backgrounds [52,62]. In particular, the AQP7-KO mouse model did not show any obesity onset in adulthood. However, Skowronski et al. found AQP7 expression in the adipose tissue capillaries but not in adipocytes; they found an increased urine glycerol level in 24 h starved AQP7 null mice and an upregulation of AQP7 in the adipose tissue of streptozotocin diabetic mice and in WT mice after prolonged fasting [52].

Surprisingly, Matsumura et al., using the most recent AQP7- KO mouse model, found no change in adiposity, but showed an increased islet glycerol concentration, increased GK and increased triglyceride content in pancreatic islets. This suggests the existence of a common alteration in different tissues of different AQP7-KO mouse models [62].

Differently from what observed in rodents, in humans defective AQP7 was not correlated with either obesity or type 2 diabetes. The first study looking for human AQP7 mutations found three missense mutations (R12C, V59L and G264V), and two silent mutations (A103A and G250G), with G264V being the only one that showed a loss of both water and glycerol permeability. Surprisingly, the subject, homozygous for the non-functional AQP7-G264V mutant, was neither obese nor diabetic and displayed only a defective exercise-induced plasma glycerol increase [63]. These findings were successively confirmed [64]. More recently, three unrelated children with the AQP7 G264V mutation were also found to be neither obese nor diabetic, with normal glycerolemia, but with hyperglyceroluria [65]. A mild platelet secretion defect and psychomotor retardation were also observed, but the association of these impairments with the AQP7 mutation has not been demonstrated.

The differences observed between AQP7 null mice developing obesity and humans lacking a functional AQP7 without an obese/diabetic phenotype have prompted the research community to look for other glycerol pathways in the human adipose tissue. As mentioned above, AQP3, 9 and 10 were found in human adipocytes; their function in glycerol transport and their regulation by lipogenic and lipolytic stimuli have been demonstrated [44,45]. Immunolocalization and hormone regulation experiments demonstrated that AQP3, AQP7 and AQP10 reside in both adipocyte plasma membranes and lipid droplets membranes, that insulin stimulation enhances AQP3,7,10 labeling around the lipid droplets and that isoproterenol induces AQPs translocation to the plasma membrane [44,45]. As above indicated, AQP9 appears to be constitutively present in the adipocyte plasma membranes [44].

Although not always consistently, AQP7 mRNA and protein expression in human adipose tissue have been shown to be altered in both obesity and insulin resistance [44,64,66,67,68]. Kuriyama et al. [56] found that AQP7 expression in the mesenteric fat is upregulated in obese insulin resistant db+/db + mice, but downregulated by insulin secreted in the feeding state. Subsequently, several studies in human obesity demonstrated an increased AQP7 expression in visceral adipose tissue [44,51,68] and a reduced [44,64,66,67] or an unmodified [51,69] AQP7 expression in the subcutaneous adipose tissue. In normal weight subjects, the visceral adipose tissue (usually a small mass compared to the subcutaneous) can store more dietary fats and release more free fatty acids under stimulation than the subcutaneous adipose tissue [70,71,72,73]. Moreover, visceral fat is more strongly associated with cardiometabolic risk factors than the subcutaneous fat depot [74].

As a whole, in obesity, the expression changes of AQP7 in the two kinds of adipose tissue seem to cause both an increase in fat accumulation in the subcutaneous depot and an increase in glycerol exit from the visceral depot.

As for the possible correlation between AQP7 expression and type 2 diabetes, no conclusive results have emerged. Two studies reported an increased AQP7 mRNA expression in visceral adipose tissue from type 2 diabetic patients [44,51] and one study reported a decreased AQP7 mRNA expression in the subcutaneous tissue [44]. Others observed no differences in either the visceral [68,69] or the subcutaneous [51,53,69] adipose tissue. Nevertheless, subjects with the -953G variant in the AQP7 promoter have a reduced AQP7 expression and an increased risk of obesity and/or type 2 diabetes [67]. As for other AQPs, differences in their expression were observed in obesity-associated type 2 diabetic patients: AQP3 expression was upregulated in both the visceral and the subcutaneous adipose tissue, while AQP9 was upregulated only in the visceral fat [44].

4. Liver

Multiple AQPs are expressed in the hepatobiliary system with the following distribution: liver (AQP3, 7, 8, 9, 11), quiescent perisinusoidal fat-storing cells (AQP 0, 1, 8 and 9), intrahepatic bile ducts (AQP1, 4), gallbladder (AQP1, 4, 8) and endothelia and small vessels (AQP1) [15,75,76]. In hepatocytes, AQPs show a specific localization: AQP8 in the apical and subapical membranes and in organelles, AQP9 in the basolateral sinusoidal surface membrane, AQP11 in the endoplasmic reticulum, AQP3 and 7 not yet clarified [2,15,75,76,77,78]. In the hepatobiliary system the aquaglyceroporins are involved in various physiological processes: 1) bile secretion, 2) detoxification and 3) glycerol utilization for gluconeogenesis by facilitating its entry into hepatocytes.

The process of bile secretion by hepatocytes and the subsequent modification in the bile duct by cholangiocytes requires a large amount of water movement. Water is secreted by hepatocytes and then is reabsorbed by cholangiocytes. Water basolaterally flows from the sinusoidal blood into the hepatocytes through AQP9 and then is transferred in the bile canaliculus through apical AQP8 (see scheme in [75]). Successively, under choleretic or cholestatic stimuli water can be secreted or reabsorbed by apical AQP1 and basolateral AQP4, respectively (see schematic representation in [75]). Water movement in the two directions is supported by endothelial AQP1.

The detoxification function is achieved by the AQP9 expressed in hepatocytes. Due to the wide permeability of the pore, AQP9 revealed an unexpected permeability to arsenite (As(OH)3) [32,35] and to NH3 and NH4 [79] in addition to water and small polyols. It was suggested that AQP9 transports arsenite (absorbed by the intestine or circulating) and ammonia (originated in the intestine) from the blood into the periportal hepatocytes, thus providing a route for their excretion and thereby partially reducing their toxicity [32,34,35,79].

The liver is the organ designated for glycerol metabolism; the glycerol released from adipose tissue through AQP7 enters the hepatocytes via AQP9. Here, it is phosphorylated to glycerol-3-phosphate, a precursor for both gluconeogenesis and TAG synthesis [4,44]. AQP9 is the main hepatic aquaglyceroporin and the prevalent route of entry for glycerol into the liver, both in the fed and in the fasting state [80]; any alteration in AQP9 abundance determines a modification in glycerol permeability of the basolateral plasma membrane [81]. Other aquaglyceroporins,

AQP3 and AQP7, have been identified in hepatocytes, but their physiological role has not been clarified [44,78].

The physiological role of AQP9 in glycerol metabolism emerged in a study in AQP9-KO mice [80]. After confirming the high expression of AQP9 in the liver with a plasma membrane localization, the authors observed an increase in both plasma glycerol and triglycerides, which is consistent with an impaired glycerol metabolism. Finally, in $Lepr^{db/db}$ mice, which developed obesity and type II diabetes after three to four weeks, concurrent AQP9 depletion ameliorated their diabetic state: a decreased hepatic uptake of glycerol decreases gluconeogenesis. Thus, AQP9 seems to play a role not only in glycerol and glucose metabolism, but also in the developing or worsening of diabetes mellitus.

The use of AQP9 as a possible therapeutic target has been suggested even if a deeper comprehension of its role in hepatic triglyceride synthesis is needed and these results still need to be confirmed in humans. It is noteworthy that the role of AQP9 in glycerol-glucose metabolism and in detoxification can be linked. During fasting or in diabetes, AQP9 mediates both the influx of glycerol used for gluconeogenesis and the efflux of urea from hepatocytes after ureagenesis [82,83].

The importance of AQP9 in glucose and glycerol metabolism and its regulation by insulin has been previously investigated by Kuriyama and coworkers [56]. These authors demonstrated a negative relationship between circulating insulin and hepatic AQP9 levels; in the liver of diabetic mice AQP9 is over-expressed whereas in rat H4IIE hepatoma cells AQP9 levels are downregulated by insulin treatment. The effect of insulin is due to the presence of a negative insulin response element (IRE) in the promoter region of the AQP9 gene, which determines the suppression of AQP9 transcription. This regulation by insulin resembles that of AQP7 in the fat tissue [50,63].

After a meal, insulin mediates the physiological reduction of lipolysis, which results in reduced glycerol release (AQP7 downregulation) and reduced glycerol uptake in the liver (AQP9 downregulation), thereby slowing the gluconeogenesis process [50,56,63]. Further, obese and insulin resistant $db+/db+$ mice showed abnormally high levels of adipose AQP7 and liver AQP9 despite high insulin, and high glycerol and high glucose plasma levels (in comparison to normal lean mice); this results in the stimulation of gluconeogenesis, which leads to a worsening of hyperglycemia in the diabetic state [56].

The downregulation of hepatic AQP9 by insulin seems to occur only in male rodents and to be prevented by estrogen in females [84].

The coordinated regulation of adipose and liver aquaglyceroporin expression described above could play an important role both in the normal physiological condition and in insulin-resistance. More recently, Frühbeck's group reported a downregulation of AQP9 mRNA in the liver of Type 2 diabetic patients, a control mechanism that limits the uptake of glycerol in the liver, to reduce gluconeogenesis and the further increase of hyperglycemia in these patients [68].

The interesting roles of AQP9 in glycerol and glucose metabolism both in normal conditions and in metabolic disorders, prompted the investigation of the regulation of AQP9 expression in the liver and possibly the pathogenesis of some diseases. To this regard, the involvement of p53 in enhancing gluconeogenesis has been studied by gene expression microarray in human liver-derived cells in different p53 statuses [85]. Results showed a p53-dependent induction of the catalytic subunit of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 2, but also the expression of glycerol kinase, glutamic-oxaloacetic transaminase 1, and of AQP3 and AQP9 [85]. Therefore, p53 is demonstrated to increase hepatic glucose production.

In human hepatoma HepG2 cells, AQP9 was downregulated by AMP-activated protein kinase (AMPK) stimulation through the Akt-FoxA2 pathway [86]. Notably, AMPK is an energy sensor in the homeostasis of carbohydrate and lipid metabolism, also stimulated by metformin, the oral antidiabetic drug belonging to the biguanide class. Moreover, FoxA2 activity is inhibited by insulin, so in insulin-resistant and Type-2 diabetic patients, this transcription factor is inactive, confined in the cytoplasm of hepatocytes [87]. As a consequence, FoxA2 cannot reduce AQP9 expression, which in turn cannot reduce glycerol uptake, thus promoting lipid metabolism and ketogenesis [87]. A similar role has been attributed to the transcriptional factor peroxisome proliferator-activated receptor alpha, which induces AQP9 expression during starvation [88] to promote glucose metabolism, fatty acid oxidation and ketogenesis [89].

Different results were obtained by Rodríguez et al., who found that insulin upregulates AQP3, 7 and 9 and that leptin upregulates AQP3 and downregulates AQP7 and AQP9 in both human omental adipocytes and HepG2 hepatocyte [44]. These different results were ascribed to species differences.

Glycerol plays a fundamental role in maintaining the balance between lipid accumulation and hepatic gluconeogenesis, so variations in AQP9 expression levels can weaken this delicate balance. For these reasons the role of AQP9 in steatosis in non-alcoholic fatty liver disease (NAFLD) and in steatohepatitis (NASH) has been examined [90,91,92]. In a NAFLD cell model transfected with an AQP9 recombinant plasmids, AQP9 overexpression significantly increased intracellular triglyceride, free fatty acid and glycerol contents and worsened the steatosis, while AQP9 suppression ameliorated the degree of steatosis. These results suggest a novel molecular target for therapeutic intervention in NAFLD [90].

In a different study conducted on human liver biopsies of obese and insulin-resistant or diabetic patients, a downregulation of AQP9 emerged in obese patients with type-2 diabetes, with NAFLD and NASH correlated to the degree of steatosis. AQP9 was further reduced in insulin-resistant individuals [92]. The reduced AQP9 expression and the subsequent lower glycerol influx into hepatocytes could be interpreted as a possible compensatory response by the liver to prevent further triglyceride, free fatty acid and glycerol accumulation and gluconeogenesis in NAFLD patients [92]. Conversely, other authors observed no correlation between AQP9 liver expression and hepatic steatosis or fibrosis in a group of morbidly obese patients [69]. A possible molecular explanation of the altered expression levels of AQP9 in hepatic steatosis is related to the presence of oleic acid inside the cells. Treatment with this fatty acid downregulates AQP3 and upregulates AQP9 in a concentration-dependent manner up to 500 μmol/L [91]. In particular, oleic acid reduces AQP3 protein levels by inducing the p38 MAPK pathway and increases AQP9 protein levels by inactivating the PI3K/Akt pathway [91].

However, the physiological meaning for the supposed redundancy of different aquaglyceroporins in human liver remains to be clarified.

5. Small intestine

It is established that triglycerides in the intestinal lumen are hydrolyzed to monoacylglycerols and free fatty acids. Pancreatic lipase further hydrolyzes monoacylglycerols to free fatty acids and glycerol. It has been previously demonstrated that about 75% of the glycerol present in the dietary triglycerides is absorbed as monoglycerides and about 25% as free glycerol [93]. Also, glycerol is assumed in the free form as food additive (E422) for its properties of sweetener, humectant, emulsifier, and as a support for other synthetic additives in several liqueurs, syrups and baked goods. Glycerol in the free form is absorbed mainly by the small intestine through AQPs, therefore contributing to the total amount of circulating glycerol. In fact, the very low GK levels in the small intestinal mucosa under basal, unstimulated conditions do not allow glycerol to be utilized for triglyceride synthesis during the absorption of fats [94].

The first glycerol gateway, AQP3, was identified thirty-five years ago in the gastrointestinal tract of the rat [95]; more members of the AQP family were later found. A total of AQP have been discovered in the human small intestine (AQP1, 3, 7, 10 and 11),. Excluding AQP1 expressed in the capillary endothelium of the intestinal mucosa and AQP11 whose transport properties have not been fully characterized, all others are members of aquaglyceroporins family (for a review, see [96]). In addition to the AQPs transport route, a carrier-mediated

glycerol transport has been identified and characterized in the rat small intestine [97].

Successively, AQP10 has been shown to have carrier features, being saturable and inhibited by structural analogs of glycerol [98], in addition to its previously known properties of a glycerol facilitator channel. The dual functional characteristic of AQP10 as a channel/carrier seems to be shared by hAQP9, another human aquaglyceroporin highly expressed in the liver: [99]; however, this is still controversial and needs to be confirmed.

Although there are still uncertainties about the exact localization of AQPs, their function as water channels in both normal and pathologic conditions, like infectious diarrhea, inflammatory bowel disease and celiac disease, has been documented [96,100,101,102]. Up- or down-regulation of AQPs is strictly related to altered gastrointestinal water absorption or secretion, but what about its involvement in glycerol homeostasis?

The first clue to the importance of aquaporins in the metabolism of glycerol appeared in a study in AQP3 null mice [103]. Analysis of serum parameters did not show any difference between AQP3 null and wild-type mice, except for triglyceride levels that were halved in the AQP3 null mice: the authors correlate the hypotriglyceridemia in AQP3 deficient mice with the glycerol transporting property of AQP3.

The second clue more directly indicates that, in Caco-2 cells, AQP3 gene expression, and thus glycerol intestinal absorption, is controlled by hormones and drugs (like insulin, the antidiabetic drugs troglitazone and tolbutamide and epinephrine) that regulate blood glucose [104]. This might suggest that the free glycerol absorbed by the small intestine could be implicated in glycemic homeostasis, although it is not clear how much glucose originates from the pool of glycerol absorbed.

6. Kidney

Animal studies have previously demonstrated that, at normal concentration, glycerol is almost completely reabsorbed in the proximal part of the nephron by a “conversion or reabsorption” mechanism [105]. Normal serum glycerol concentration in adults is about 0.065–0.1 mmol/L, and rarely exceeds 0.33–0.43 mmol/L under lipolytic conditions [1,106,107,108,109]. Glycerol is usually completely reabsorbed by kidney under physiological conditions and begins to appear in the urine when plasma glycerol concentrations exceed its saturation point [105]. In dogs and in cats, the saturation point for glycerol is significantly higher than in humans: 1.1–1.62 mmol/L [109,110]. However, a large amount of the glycerol that enters the kidney proximal tubule cells is used in the gluconeogenesis process, which contributes up to 20–25% of the whole-body glucose production [111,112].

There is no need to emphasize the importance of aquaporins in the urinary concentration function: the osmotic water transport across the tubule epithelium is an AQP-facilitated mechanism (for reviews, see [15,113,114]). So far, eight AQPs have been identified in the mammal kidney: AQP1, 2, 3, 4, 6, 7, 8, 11, each characterized by a specific cellular or subcellular localization [15].

As for aquaglyceroporins, AQP3 is abundantly expressed in the basolateral plasma membranes of the collecting duct principal cells, while AQP7 is expressed in the brush border plasma membranes of the proximal tubule cells of the S3 segment [15].

Transgenic mice studies have provided fundamental insight into AQPs functions in renal physiology.

Experiments with AQP3-KO mice demonstrate the involvement of AQP3 in the urinary concentration function, where AQP4 represents the exit pathway in transcellular water transport [103,114].

Experiments with AQP7 null mice demonstrated a small but significant reduction in water permeability of the proximal tubule brush border membranes concomitant with a drastic increase of urinary glycerol concentration, with a 340-fold increase in glycerol concentration in the KO vs. WT. Similar increases in urinary glycerol concentration were observed in another AQP7-KO mouse model [52]. However, it is not clear if renal glycerol excretion in AQP7-KO mice can affect serum glycerol concentration: while Maeda et al. show a significantly lower plasma glycerol in AQP7 null mice compared to controls, both in the fed and fasted state (about 53% and 38%, respectively) [55], other authors showed either a small, non significant reduction (about 15% decrease) [115] or no changes [52]. In humans, three unrelated homozygous G264V mutant children had normal plasma glycerol levels but a very high concentrations of glycerol in the urine, ranging from 500 to 2800 mmol glycerol/mol creatinine in the mutant subjects vs. < 1 mmol glycerol/mol creatinine in the normal subjects [65].

The studies above indicate that AQP7 functions as an entryway into the cells of the S3 segment, which is mainly involved in the reabsorption of glycerol. The same studies suggest that the deletion of AQP7 cannot be compensated by either AQP3 or other still unknown aquaglyceroporins.

7. Microvessels

A possible role of capillary aquaglyceroporins in the tissue handling of glycerol emerged from the study of Skowronski et al. [52]. The authors showed that AQP7 is localized in the endothelium of both brown and white adipose tissue capillaries, the heart, and skeletal muscles, but is surprisingly absent from the plasma membranes of either adipocytes or myocytes. Further, AQP7 expression in microvessels is doubled in streptozotocin-induced diabetes mellitus and in prolonged fasting [52]. For the first time, it has been proposed that insulin regulates the functioning of adipose tissue by controlling glycerol entry/exit through the vascular endothelium [52]. In humans, colocalization experiments of AQP7 and AQP10 with CD34, an endothelial cell marker, confirmed AQP7 expression in the endothelium of adipose tissue capillaries, and also demonstrated the presence of AQP7 in the plasma membranes of adipocytes; AQP10 was found to be localized in the adipocytes and not in the capillaries [45]. In the human small intestine, an AQP10 variant was found in the endothelial cells of the villus capillaries by Li et al. [116].

We are tempted to speculate that also in the small intestine aquaglyceroporin expression may be regulated by the hormones and drugs that regulate glycerol absorption.

Unfortunately, no evidence has yet been found of aquaglyceroporin expression in kidney capillaries, but their existence (and physiological importance) cannot be excluded.

8. Endocrine pancreas

Insulin secretion is physiologically turned on after a meal by the increased plasma concentration of glucose, free fatty acids and amino acids [117,118]. Glycerol does not seem to have a role in eliciting insulin secretion due to the low intracellular concentration of GK [119,120]. However, the artificial induction of GK in pancreatic β-cell experimental models makes the cells responsive to glycerol, which GK converts to lactate and glucose, for insulin production and secretion [119,120]. The possible role of glycerol content in the regulation of insulin secretion as well as in the aquaglyceroporins expression was successively investigated in pancreatic β-cells [62]. Of the four known mammalian aquaglyceroporins, only AQP7 was expressed in the β-cells of mouse pancreas. The role of AQP7 was further investigated by generating an AQP7-null mouse model. Compared to normal mice, Aqp7 −/− mice showed: 4–5-fold higher plasma insulin concentration without signs of insulin resistance, increased glycerol concentration in pancreatic islets, increased GK expression and activity, and increased triglyceride levels, reduced β-cell mass, higher abundance of intracellular insulin transcripts and lower intracellular insulin [62]. The authors concluded that AQP7 could be involved in insulin synthesis and secretion as well as in controlling β-cell mass.

Recently, a novel mechanism of insulin secretion has been proposed in addition to that of the metabolically-regulated ATP-sensitive K^+ channels. A rise in plasma glucose concentration determines its increased

entry into β-cells, which leads to β-cell swelling both directly and indirectly (through its metabolites). The swelling activates chloride efflux mediated by the volume-regulated anion channels (VRAC), membrane depolarization, the opening of voltage sensitive Ca^{2+} channels, and Ca^{2+} entry, which in turn promotes insulin secretion [118,121,122,123]. In this scenario, the role [of aquaglyceroporins in insulin secretion, and in particular of AQP7, has been investigated. Results obtained using pancreatic islets showed that the addition of isotonic and hypertonic glycerol caused β-cell swelling possibly through AQP7, VRAC activation, membrane depolarization and insulin release [123]. These data were also suggestive of an additional role of glycerol in insulin secretion related to glycerol metabolism, even though the GK expression in pancreatic β-cells is uncertain and the glycerol concentrations used were not in the physiological range [123]. The role of AQP7 and of glycerol in β-cell function was further confirmed by using pancreatic islets from AQP7 −/− mice [118,121]. Results showed a significantly lower release of insulin in AQP7 −/− mice after the iso-osmotic addition of glycerol or D-glucose, or in response to extracellular hypotonicity in comparison to wild-type mice [118,121].

These results appear to be consistent with a positive relation between AQP7 expression and insulin secretion, but are at evident conflict with previous ones, which reported a negative relation [62]. Future

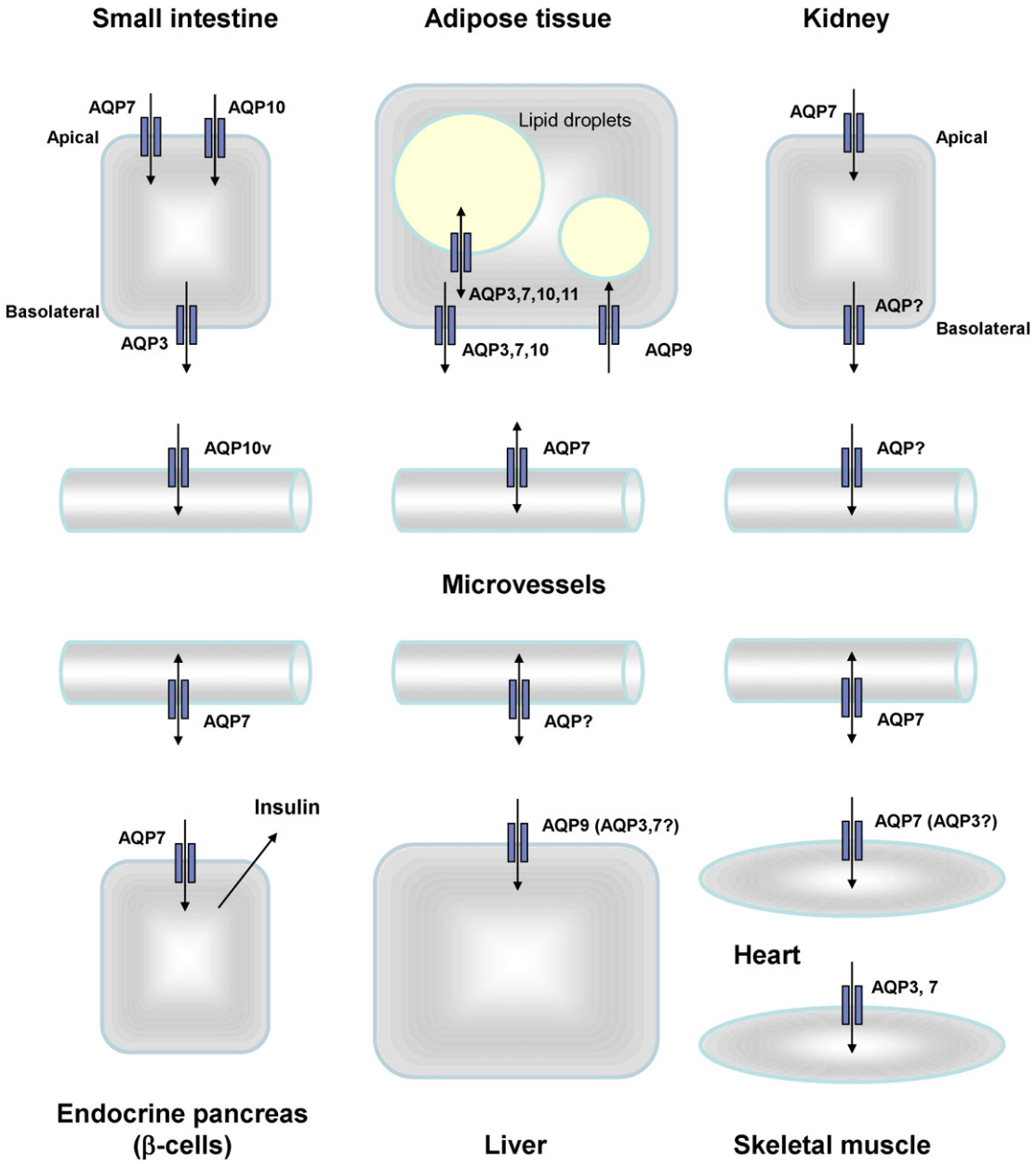

Fig. 1. Possible model of aquaglyceroporins involvement in the homeostatic regulation of plasma glycerol concentration. Glycerol absorption by the small intestinal epithelial cells occurs with mechanisms involving aquaporin-7 and -10 (AQP7 and 10) at the apical side and aquaporin-3 (AQP3) at the basolateral side. Glycerol accumulation in the interstitium is drained into the capillaries through an aquaporin-10 variant (AQP10v). In the adipocytes, glycerol movement into or out of the lipid droplets as well as the exit from the plasma membrane is mediated by AQP3, 7 and 10, while the entry of glycerol occurs through AQP9. AQP11 is involved in the transport of glycerol into/out of the lipid droplets. Depending on the metabolic state, glycerol is transported into or out of the microvessels through AQP7. In the kidney, glycerol is partially reabsorbed via the AQP7 localized in the brush border plasma membranes of the proximal tubule cells of S3 segment. The mechanism by which glycerol passes across the basolateral side and into the capillary endothelial cells is still unknown. Circulating glycerol (originating mainly from lipolysis, from intestinal absorption, and in part also from kidney reabsorption) enters hepatocytes through aquaporin-9 (AQP9), at the basolateral sinusoidal membrane, to be used for gluconeogenesis. Glycerol as such can also be used also as an energy substrate by the heart or skeletal muscle, entering through plasma membrane AQP3 and AQP7. Tissue aquaglyceroporin expression can be regulated by insulin, whose secretion is probably under the control (negative or positive is yet to be defined) of glycerol itself, that enters into pancreatic β-cells through AQP7.

studies may clarify the physiological function of AQP7 in the control of insulin release.

9. Heart and skeletal muscle

The cardiac tissue of different mammalian models (humans, mice and rats) has been analyzed for AQPs and their possible dual functional role: 1) water handling in both normal and pathological (myocardial edema) conditions, and 2) the uptake of glycerol, a substrate for energy production, into cardiomyocytes. Almost all AQP transcripts, AQP1, 3, 4, 5, 7, 8, 9, 10 and 11, were found in the heart of mammals, but only the AQP1, 3, 4, and 7 were confirmed at protein level [52,124,125,126,127]. While in normal conditions AQP1 and 4 are mainly involved in myocardial water balance, in edema and in ischemia reperfusion injury [124], AQP3 and especially AQP7 may play a role in cardiac energy production [124].].

Small amounts of AQP9 transcript were detected in both rat and human heart [43,125], but the protein was absent [35,126]. Interestingly, the increased expression of the AQP9 gene in infective endocarditis patients who experienced acute heart failure, suggests AQP9 is a potential prognostic factor for infective endocarditis [128]. The expression of the AQP3 protein in the heart is still uncertain: previously found in cardiac muscle by using human tissue microarrays [127], its presence in cardiac tissue was successively excluded [125,126].

At present, AQP7 is the only recognized glycerol channel expressed in the heart, localized in myocytes, fibroblasts and in capillaries [52,63,125,126,129]. Relevant insights were obtained with studies in AQP7-KO mice. Skowronski and coworkers revealed a specific signal in the capillaries but not in cardiomyocytes [52]. This localization in the capillaries is similar to what observed in the adipose tissue. Successively, the roles of cardiac AQP7 and of glycerol as an energy substrate of the heart were evidenced using a different AQP7-KO mouse model [126]. After confirming that AQP7 is the sole aquaglyceroporin in the mouse heart, Hibuse et al. [126] demonstrated that AQP7 silenced cardiomyocytes and cardiac tissue of AQP7-KO mice have lower glycerol and ATP contents than WT, consistent with a reduced glycerol entry into the cells. Further, cardiac overload by transverse aortic constriction and by continuous isoproterenol infusion for 1–2 weeks resulted in severe left ventricular hypertrophy in AQP7-KO mice and in a lower survival rate than in wild-type mice. Taken together, the results demonstrate that AQP7 is involved in the transport of glycerol in the cardiac muscle and that the glycerol taken up can be considered one of the energy sources of the heart, as it is converted to pyruvate by GK and glycerol-3-phosphate dehydrogenase −2.

Few data are available on the expression and function of aquaglyceroporins in the skeletal muscle of mammals. AQP3 transcript was first found by RNase protection assay in the skeletal muscle of rat [130] and was successively confirmed also at protein level in humans [127,131]. Immunofluorescence and immunohistochemistry showed that the protein is localized in the sarcolemma of slow-twitch human type 1 muscle fibers, and immunoelectron microscopy suggests its possible costameric distribution [131]. The localization in intramembranous particles was further confirmed by a fracture-label study, suggesting a role of AQP3 (together with AQP4) in maintaining osmotic homeostasis in the skeletal myofibers [132].

A second aquaglyceroporin was found at both mRNA and protein levels in the human and mouse skeletal muscle: the AQP7. The AQP7 protein was localized in the myofiber surface of type 1 and type 2 fibers in human muscles and of type 2 fibers in mouse muscles, but its functional role has not yet been analyzed [133]. Different results surprisingly showed a specific signal in the capillaries but not in the sarcolemma of skeletal muscle fibers [52], similarly to what found for the adipose tissue and the heart.

Finally, as regards AQP9, only a weak expression of its mRNA was observed in the skeletal muscle of mice [134] but, to date, no new results have emerged about the protein and its possible functional role.

As a whole, the aquaglyceroporins appear to be involved in supplying glycerol to the cardiac and skeletal muscle for energy production. This metabolic function of aquaglyceroporins is supported by the fact that insulin treatment decreases both AQP7 expression in the adipose tissue and triglyceride content in muscle tissue of mice [135].

Even though with some limitations (i.e. the extrapolation to humans of data obtained in rodents), the results are promising and beg further investigation.

10. Concluding remarks

As a whole, the involvement of AQPs in glycerol metabolism has been well documented. AQPs represent pathways for glycerol absorption by the gastrointestinal tract, for partial glycerol reabsorption or secretion by the kidney and for entry/exit processes in adipocytes, depending on the metabolic state of the organism. The glycerol thus made available can be used also as fuel by the heart and skeletal muscle and as a substrate for gluconeogenesis by the liver. Aquaglyceroporins in the capillaries may function as channels that modulate glycerol flow into and out of the bloodstream. A hypothetical working model for the involvement of aquaglyceroporins in the homeostatic regulation of plasma glycerol is summarized in Fig. 1.

Since AQPs can be controlled by hormones and drugs, they may represent potential therapeutic targets in the management of obesity and associated metabolic disorders. Thiazolidinediones, insulin sensitizer drugs for the treatment of type-2 diabetes and agonists of peroxisome proliferator-activated receptor γ, have revealed an upregulating effect on AQP7 in murine 3 T3-L1 cells and in OLETF rats [57,136]. Recently, Apelin-13 was demonstrated to reduce the lipid accumulation in hypertrophic adipocytes in vitro by increasing AQP7 expression, which indicates it could be used as an AQP modulator in the treatment of obesity [137]. A novel small molecule inhibitor of AQP9, HTS13286, has been identified and recognized to be effective in reducing glycerol gluconeogenesis in primary hepatocyte cultures [138]. However, at present, its therapeutic use cannot be recommended due to the high doses required and the low solubility in 1% DMSO aqueous solution.

We are only beginning to understand the importance and scope of the subject of AQPs, their function, and their promise as potential therapeutic targets, a promising field that that deserves further investigation.

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