Metabolic impact of the glycerol channels AQP7 and AQP9 in adipose tissue and liver

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Abstract

Obesity and secondary development of type 2 diabetes (T2D) are major health care problems throughout the developed world. Accumulating evidence suggest that glycerol metabolism contributes to the pathophysiology of obesity and T2D. Glycerol is a small molecule that serves as an important intermediate between carbohydrate and lipid metabolism. It is stored primarily in adipose tissue as the backbone of triglyceride (TG) and during states of metabolic stress, such as fasting and diabetes, it is released for metabolism in other tissues. In the liver, glycerol serves as a gluconeogenic precursor and it is used for the esterification of free fatty acid into TGs. Aquaporin 7 (AQP7) in adipose tissue and AQP9 in the liver are transmembrane proteins that belong to the subset of AQPs called aquaglyceroporins. AQP7 facilitates the efflux of glycerol from adipose tissue and AQP7 deficiency has been linked to TG accumulation in adipose tissue and adult onset obesity. On the other hand, AQP9 expressed in liver facilitates the hepatic uptake of glycerol and thereby the availability of glycerol for *de novo* synthesis of glucose and TG that both are involved in the pathophysiology of diabetes. The aim of this review was to summarize the current knowledge on the role of the two glycerol channels in controlling glycerol metabolism in adipose tissue and liver.

Key Words
- glycerol metabolism
- adipose tissue
- liver
- triglycerides
- obesity
- type 2 diabetes

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Glycerol metabolism

The metabolic switching between feeding and fasting is central to everyday life and involves tight hormonal control with effects on muscle, adipose tissue, and liver that maintains an adequate handling of metabolic precursors to support energy demands. One of the molecules affected by metabolic switching is glycerol, which is a small 3-carbon alcohol that in the fed state is stored as the backbone of triglyceride (TG) mainly in adipose tissue.

In the fed state, after ingestion of dietary fats (~90% TG), <30% of TG is fully hydrolyzed into free fatty acids (FFA) and glycerol. Glycerol rapidly enters the enterocytes of the small intestine and thereafter the circulation through the portal vein and is primarily metabolized by the liver (Lin 1977). In adipose tissue, the activity of glycerol kinase (GlyK), that catalyzes the initial phosphorylation of glycerol into glycerol-3-phosphate (G3P), is negligible and therefore glycerol is not normally utilized in adipose tissue. Instead G3P used for the synthesis of TG in the fed state is derived from glycolysis.

During states of increased energy demand, such as fasting and exercise, adipocyte lipolysis is increased by activation of adipose TG lipase and hormone-sensitive

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lipase, which initiates the breakdown of TG into FFA and monoacylglycerol (MAG), whereas the final step of converting MAG into FFA and glycerol is catalyzed by MAG lipase (Fig. 1). The major hormones involved in initiating lipolysis are low plasma levels of insulin, catecholamines acting through β-adrenergic receptors, and natriuretic peptides that in humans serve as potent stimulators of lipolysis (Lafontan & Langin 2009, Kolditz & Langin 2010). The generated FFA and glycerol are released to support energy demands in other tissues, and the efflux of glycerol from adipose tissue is facilitated by aquaporin 7 (AQP7). However, even in the fasted state, up to 65% of the generated FFA is re-esterified back into TG (Hanson & Reshef 2003). A substantial part of the re-esterification occurs already within the adipocytes (Hammond & Johnston 1987), and in contrast to the fed state, G3P is not derived from glucose, which is saved for metabolism by other tissues. Instead, the G3P used for re-synthesis of TG is primarily derived from glyceroneogenesis, which is a truncated form of gluconeogenesis that uses lactate, pyruvate, and alanine to generate G3P (reviewed in Hanson & Reshef (2003) and Reshef *et al.* (2003); Fig. 1).

As mentioned earlier, the activity of GlyK in adipose tissue is very low under normal circumstances, and therefore, generated glycerol is exported to other tissues for further metabolism. Even though it is not supported by all studies (Tan *et al.* 2003), the activity of GlyK in adipocytes has been shown to be increased to significant levels in response to peroxisome proliferator-activated receptor γ (PPARγ) activation. PPARs (PPARα, PPARβ/δ, and PPARγ) are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors. They are activated by the binding of ligands and regulate gene transcription by two pathways: i) heterodimerization with the retinoid X receptor (RXR) and binding to PPAR response elements and ii) interfering with other transcription factor pathways in a DNA-independent manner (Yki-Jarvinen 2004). PPARγ agonists (thiazolidinediones) are used in the treatment of type 2 diabetes (T2D) due to their insulin-sensitizing effects (Guan *et al.* 2002, Leroyer *et al.* 2006). In addition to its effects on GlyK activity, the activation of PPARγ also increases glyceroneogenesis in adipose tissue (Tordjman *et al.* 2003, Leroyer *et al.* 2006) and the increased generation of G3P from both sources results in an increased FFA re-esterification that lowers the efflux of FFA from adipose tissue, which contributes to the insulin-sensitizing effects of PPARγ agonists (Guan *et al.* 2002, Leroyer *et al.* 2006).

The FFA released in response to increased energy demands is metabolized mainly in liver and skeletal

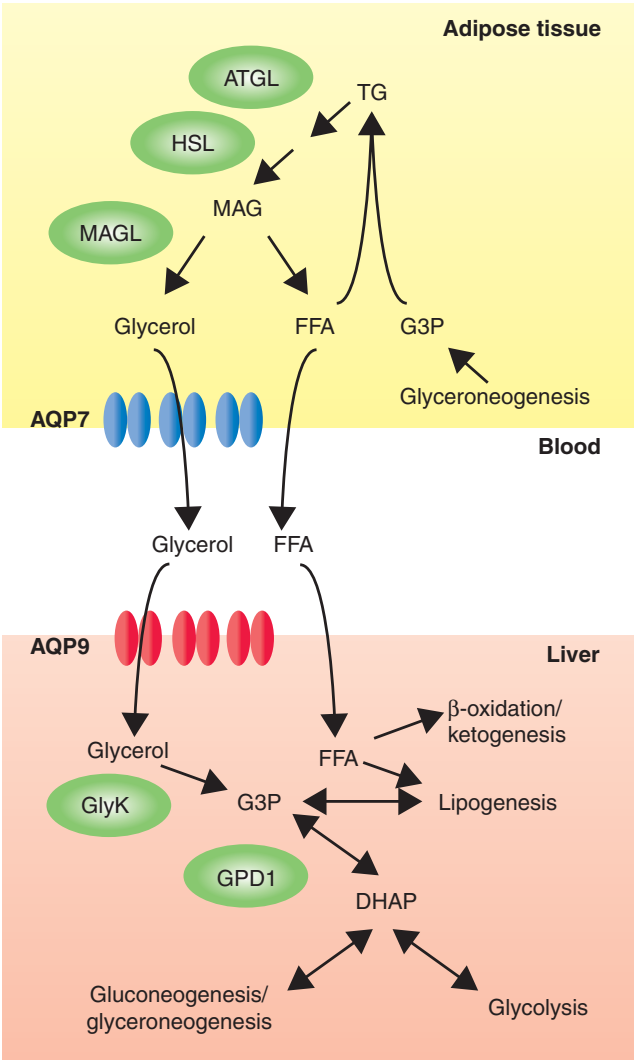

Figure 1
Cellular pathways involved in glycerol metabolism in adipose tissue and liver during fasting. In adipose tissue, triglyceride (TG) is hydrolyzed by adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) into monoacylglycerol (MAG) that is hydrolyzed into free fatty acids (FFA) and glycerol by MAG lipase (MAGL). A substantial part of the generated FFA is re-esterified into TG. Glycerol-3-phosphate (G3P) used for lipogenesis during fasting is mainly derived from lactate, pyruvate, and alanine through a truncated form of gluconeogenesis called glyceroneogenesis. The release of generated glycerol from adipose tissue is facilitated by the glycerol channel aquaporin 7 (AQP7). Both glycerol and FFA then reaches the liver, where AQP9 facilitates the uptake of glycerol. Once inside the hepatocyte, glycerol is phosphorylated by glycerol kinase (GlyK) into G3P. During fasting, glycerol serves as a substrate for hepatic glucose *de novo* synthesis through gluconeogenesis and in order to enter that pathway G3P is oxidized by cytoplasmatic glycerol-3-phosphate dehydrogenase (GPD1) into dihydroxyacetone phosphate (DHAP). GPD1 also catalyzes the reverse reaction generating G3P from DHAP as the final step of glyceroneogenesis. During fasting, FFA is in the liver used for energy synthesis through β-oxidation and ketogenesis, and a substantial part of FFA is re-esterified into glycerolipids. The G3P used for lipogenesis during fasting is derived either from glycerol or glyceroneogenesis.

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muscle (Jensen 2003). In the fasted liver, FFA is used for β-oxidation and synthesis of ketone bodies, and also a substantial part of FFA is re-esterified into TG (Wolfe *et al.* 1990, Hellerstein *et al.* 1993, Baba *et al.* 1995). In the liver, AQP9 facilitates the uptake of glycerol and with the liver being one of the main expression sites for GlyK (Lin 1977), G3P for TG synthesis can here be derived from glycerol in addition to being generated from glyceroneogenesis and glycolysis. A substantial part of the generated G3P is used for gluconeogenesis. To enter this pathway, G3P is reversibly oxidized by cytoplasmatic glycerol-3-phosphate dehydrogenase (GPD1) into dihydroxyacetone phosphate (Fig. 1). Measurements of how much glycerol ends up in TG synthesis vs gluconeogenesis have mainly been performed in humans. The results have been somewhat variable, with the reported proportion of glycerol used for TG synthesis ranging from only ∼6% in women after 16-h fasting (Kahn & Flier 2000) to > 60% in young men fasted for 14 h (Baba *et al.* 1995). Similarly, measurement of the amount of glycerol used for gluconeogenesis in the fasted state also demonstrates great variability and ranges from 38% in lean men after a short fast to ∼100% in obese women after prolonged starvation (Bortz *et al.* 1972, Nurjhan *et al.* 1986, Baba *et al.* 1995, Landau *et al.* 1996). However, in general, the proportion of glycerol used for gluconeogenesis increases as the fasting period prolongs. Even though a large fraction of glycerol goes into glucose *de novo* synthesis, it still only makes a minor contribution to the hepatic generation of glucose after overnight fasting but becomes increasingly important as a gluconeogenic precursor with prolonged fasting. Thus, the amount of *de novo* synthesized glucose ascribed to glycerol was 4.5 and 21.6% respectively in healthy men fasted for 14 and 62–86 h (Baba *et al.* 1995). In rats, glycerol has been found to account for ∼25% of the glucose derived from gluconeogenesis after both 6 and 48 h of fasting (Peroni *et al.* 1997).

AQP protein family

The AQPs are pore-forming transmembrane proteins that share a variable part of their amino acid sequences and have two highly conserved asparagine–proline–alanine (NPA) motifs, one in each half of the protein. They share the same basic architecture with a cytosolic N- and C-terminus and a hydrophobic stretch of six transmembrane domains, connected by three extracellular and two intracellular loops. The transmembrane domains form an hourglass structure with the NPA motifs at the center of the molecule (reviewed in Magni *et al.* (2006)). Several observations of AQP monomers forming a homotetrameric organization in the membrane have been made (Engel *et al.* 2000), and this feature is probably similar for all members of the AQP family. Transport through AQPs is not an active process and is dependent on the presence of a gradient across the membrane in question (Pastor-Soler *et al.* 2001).

At this time, 13 mammalian AQPs (AQP0–12) have been identified and the AQP family has been divided into three subgroups based on both their permeability characteristics and their amino acid sequence homology. The first group is the classical AQPs that are selective water channels. The second group is the aquaglyceroporins, which are capable of transporting glycerol and other small uncharged solutes in addition to water. Both AQP7 and AQP9 belong to this group together with AQP3 and AQP10. Atomic resolution analysis shows that GlpF, the aquaglyceroporin prototype in *Escherichia coli*, has a wider pore in the hourglass-like structure compared with the water-selective AQPs allowing the pore to accept larger molecules such as glycerol (Weissenborn *et al.* 1992). Finally, the third group is the unorthodox AQPs. As outlined above, AQP7 and AQP9 have emerged as important facilitators of glycerol transport across the cell membrane in adipose tissue and liver respectively and the focus of the present review will be the role of AQP7 in adipose tissue and AQP9 in liver in controlling the cellular availability and metabolism of glycerol.

Aquaporin 7

Structure and permeability profile

The AQP7 gene was first cloned from rat testis and encodes a protein of 269 amino acids with a predicted molecular mass of 28.9 kDa. The deduced amino acid sequences of human AQP7 and mouse AQP7 are 68 and 79% identical to the rat AQP7 respectively, and mouse AQP7 is 67% identical to human AQP7. Thus, in contrast to other AQPs, AQP7 demonstrates an unusual low conservation among species (Kondo *et al.* 2002). Hydropathy analysis predicts six putative transmembrane domains with the N- and C-terminal localized in the cytosol. No potential *N*-linked glycosylation sites or PKA phosphorylation sites have been identified in the predicted amino acid sequence; however, a potential PKC phosphorylation site has been found at residue Thr-174 (Ishibashi *et al.* 1997). In rat AQP7, the first NPA motif is present but the second NPA motif is NPS (asparagine-proline-serine) (Ishibashi *et al.* 1997). In human AQP7, it is the first NAA (asparagine-alanine-alanine) motif that is substituted by NAA, whereas the second NPA

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motif is preserved (Kuriyama *et al*. 1997). AQP7 shows permeability to water, glycerol, and urea when expressed in *Xenopus* oocytes, (Kuriyama *et al*. 1997, Ishibashi *et al*. 1997, Kishida *et al*. 2001*a*).

Localization of AQP7 in adipose tissue

An important part of establishing the function of a protein is to identify its cellular and subcellular localization within a given tissue. AQP7 has been localized to a wide range of different tissues in both rodents and humans. Here, we restrict our focus to AQP7 expressed in adipose tissue and only comment on the localization or function in other tissues when this could influence the interpretation of the role of AQP7 in adipose tissue glycerol metabolism. Hence, AQP7 expressed in kidney, pancreas, and muscle will also be mentioned below. The overall localization profile of AQP7 has been thoroughly revised by Rojek *et al*. (2008).

Controversy still exists on the cellular and subcellular localization of AQP7 in adipose tissue. The hypothesis on AQP7 as a facilitator of glycerol transport in adipose tissue has been founded on the assumption that AQP7 is expressed in the adipocyte plasma membrane (Fruhbeck 2005, Fruhbeck *et al*. 2006, Maeda *et al*. 2008, 2009). Supporting a localization in adipocytes, *AQP7* mRNA and protein is detected in adipocyte cell culture models such as differentiated 3T3-L1 (mouse embryonic fibroblasts – adipose-like cell line; Kishida *et al*. 2000, 2001*a*,*b*) and SGBS cells (human preadipocyte cell strain; Miranda *et al*. 2010). Furthermore, fractionation of human adipose tissue into adipocytes and stromovascular fraction cells resulted in the highest abundance of *AQP7* mRNA and protein in the adipocyte containing fraction (Miranda *et al*. 2010). Immunostaining for AQP7 in adipocyte cell culture models demonstrates a predominant localization in intracellular regions (Kishida *et al*. 2000, Miranda *et al*. 2010, Rodriguez *et al*. 2011), which does not apply to a role in transporting glycerol across the plasma membrane. Stimulation of the cells with epinephrine/isoproterenol have been reported to result in translocation of some of the AQP7 labeling to the plasma membrane domain (Kishida *et al*. 2000, Rodriguez *et al*. 2011, Laforenza *et al*. 2013), in turn suggesting that AQP7 traffics to the plasma membrane in response to lipolytic stimuli.

The above outlined observations are somewhat contrasted by the results obtained by immunohistochemical analysis of AQP7 expression in adipose tissue. Using paraffin sections from mouse and human adipose tissue, we have been unable to detect labeling for AQP7 in the adipocytes; instead strong labeling for AQP7 is found in the capillary endothelial cells (Skowronski *et al*. 2007, Lebeck *et al*. 2012*a*). Furthermore, in transgenic mice with eGFP expression driven by the AQP7 promoter, the expression of eGFP was also only found in the endothelial cells of the small capillaries within the adipose tissue (Lebeck *et al*. 2012*a*). The localization of AQP7 to the endothelial cells within human adipose tissue has been confirmed by others (Rodriguez *et al*. 2011, Laforenza *et al*. 2013). However, in their studies, they also detected labeling for AQP7 in the adipocytes. This discrepancy could be explained by differences in fixation methods or the specificity of the antibodies used. The unilocular nature of the adipocytes in white adipose tissue (WAT), with only a thin peripheral rim of cytoplasm, makes it difficult to interpret the subcellular localization of staining for AQP7 in adipocytes. Therefore, provided that AQP7 is also present in the adipocytes, studies using electron microscopy are needed to clarify whether trafficking of AQP7 from intracellular domains to the plasma membrane occurs *in vivo*. Thus, further studies are needed to sediment the cellular and subcellular localization of AQP7 in adipose tissue. However, whether AQP7 is found in endothelial cells alone or in both adipocytes and endothelial cells, it will either way play a role in facilitating glycerol transport between the adipocyte and the blood and thereby be able to influence glycerol metabolism.

Is AQP7 the sole aquaglyceroporin in adipose tissue?

The putative presence of other aquaglyceroporins in adipose tissue has been investigated with variable outcomes. Neither *Aqp3* nor *Aqp9* mRNA are detected by northern blotting of white or brown adipose tissue from mice (Kishida *et al*. 2000, Maeda *et al*. 2004), and although not supported by all studies (Rodriguez *et al*. 2011), similar results have been obtained for human WAT (Miranda *et al*. 2010, Lebeck *et al*. 2012*a*). A recent study suggests the presence of AQP10 in human WAT with localization only to the adipocytes (Laforenza *et al*. 2005). However, again this observation is disputed by others having been unable to detect *AQP10* mRNA in adipose tissue (Miranda *et al*. 2010). Thus, the cellular expression of aquaglyceroporins in adipose tissue needs clarification in order to validate their potential role and possible overlap in facilitating glycerol transport within adipose tissue.

Regulation of AQP7 expression in adipose tissue

In adipose tissue, the abundance of *AQP7* mRNA and protein is inversely regulated by insulin, with increased expression during fasting and decreased expression during

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refeeding (Kishida *et al*. 2001*a*). Increased abundance of *AQP7* mRNA (Kishida *et al*. 2001*a*) and protein (Skowronski *et al*. 2007) has also been found in response to streptozotocin (STZ)-induced diabetes mellitus as well as in insulin resistant db+/db+ mice (Kuriyama *et al*. 2002). These conditions are associated with an increased hydrolysis of TG into glycerol and FFA and release of these into the bloodstream, which agrees with AQP7 facilitating glycerol transport. A functional negative insulin response element (IRE) has been identified in the promoter region of the *AQP7* gene from both humans (−542 to −536 bp upstream of the translation initiation site) (Kondo *et al*. 2002) and mice (−443 to −437 bp) (Kishida *et al*. 2001*a*). The suppression of AQP7 transcription was abolished when an inhibitor of the PI3K pathway was administered, indicating that this pathway mediates the suppression of AQP7 expression by insulin (Kishida *et al*. 2001*a*).

In addition to the IRE, a peroxisome proliferator response element (PPRE) has also been identified in the promoter region of both the mouse (−93 to −77 bp) and human (−62 to −46 bp) *AQP7* gene (Kishida *et al*. 2001*b*, Kondo *et al*. 2002). Analysis of the mouse PPRE demonstrated that *AQP7* is a PPARγ target gene and binding of PPARγ after heterodimerization with the RXRα caused an increased *Aqp7* mRNA expression in 3T3-L1 adipocytes. As well as treatment of mouse and rats with PPARγ agonists increased the expression of AQP7 in adipose tissue (Kishida *et al*. 2001*b*, Lee *et al*. 2005). As mentioned earlier, PPARγ agonists have insulin-sensitizing effects and in addition to their effects on AQP7 in adipose tissue, they stimulate the generation of G3P and an increased availability of FFA subsequently resulting in an increased storage of TG (Lehrke & Lazar 2005). It does, however, seem contradictive that when AQP7 is already increased in insulin-resistant db+/db+ mice (Kuriyama *et al*. 2002), then a further increase due to stimulation by PPARγ agonists would be a means of improving insulin sensitivity. A possible explanation could be that a parallel increase in GlyK activity results in a shift in the gradient for glycerol flux and thus promotes the use of glycerol for TG synthesis in response to PPARγ-agonist treatment, whereas the increased AQP7 expression in db+/db+ mice facilitates an increased efflux of the glycerol generated from hydrolysis of TG.

Similar to the results obtained for PPARγ, activation of both PPARβ/δ (Patsouris *et al*. 2004) and PPARα (Walker *et al*. 2007) have been reported to increase the abundance of *AQP7* mRNA in adipocyte cell culture models. Finally, the effect of other hormones has been investigated in differentiated 3T3-L1 cells, and it has been shown that isoproterenol, tumor necrosis factor α, and dexamethazone decreases the expression of *AQP7* mRNA, while angiotensin 2, growth hormone, triiodothyronine, epinephrine, glucagon, and adrenocorticotropic hormone had no effect on the abundance of *AQP7* mRNA (Kishida *et al*. 2000, Fasshauer *et al*. 2003). The apparently diverging results obtained for isoproterenol and epinephrine could be due to differences in selectivity, with isoproterenol being a selective β-agonist. However, in differentiated 3T3-L1 adipocytes, the relative higher abundance of the β_3- vs α_2-adrenergic receptors (El Hadri *et al*. 1996, Monjo *et al*. 2005) predicts that the effect of epinephrine should also mainly be due to activation of β-adrenergic receptors and thus does not seem to explain the discrepancy. Furthermore, as outlined above, both isoproterenol and epinephrine have been reported to induce trafficking of AQP7 to the plasma membrane in *in vitro* systems. Should this trafficking also occur *in vivo*, then the effect on the total abundance of AQP7 protein in the plasma membrane needs to be determined.

Effects of AQP7 deficiency on glycerol metabolism

The metabolic impact of AQP7 in adipose tissue has been highlighted by the interesting phenotype observed in two different lines of AQP7 knockout (KO) mice (Maeda *et al*. 2004, Hara-Chikuma *et al*. 2005, Hibuse *et al*. 2005). The lack of AQP7 in both cases resulted in an increased accumulation of glycerol and TG within the adipocytes leading to adipocyte hypertrophy (Hara-Chikuma *et al*. 2005, Hibuse *et al*. 2005). The first AQP7 KO line had lower p-glycerol levels together with similar p-FFA levels in both the fed and fasted state compared with WT littermates. Furthermore, the young KO mice responded to fasting with lower p-glucose levels, suggesting an impaired *de novo* synthesis of glucose and no differences in p-insulin levels were observed (Maeda *et al*. 2004). When followed past the age of 12 weeks, these male AQP7 KO mice developed adult-onset obesity with adipocyte hypertrophy, and at 20 weeks of age, the KO mice responded to fasting with higher p-glucose, p-insulin, p-leptin, and p-FFA levels than WT littermates, whereas p-glycerol remained lower in the KO mice. The aged AQP7 KO mice demonstrated whole-body insulin resistance. The proposed mechanism behind the adipocyte hypertrophy is that an increased accumulation of glycerol within the adipocytes increases the activity of GlyK and, thus, results in an increased synthesis of TG within the adipose tissue (Fig. 2) which leads to obesity and secondary development of insulin resistance (Hibuse *et al*. 2005).

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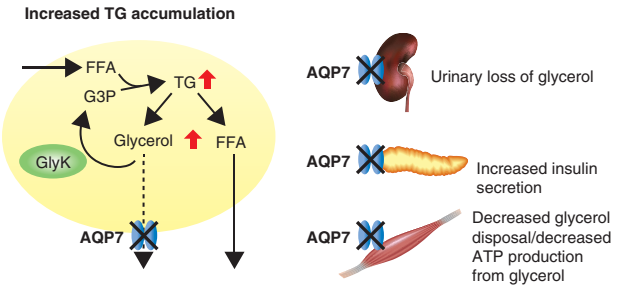

Figure 2
Tissues potentially contributing to the overall metabolic phenotype of AQP7 knockout mice. In adipose tissue, the lack of AQP7 is hypothesized to result in an increased accumulation of glycerol, which in turn stimulates the activity of glycerol kinase (GlyK). This leads to increased synthesis of glycerol-3-phosphate (G3P) and triglyceride (TG). The accumulation of TG eventually results in adipocyte hypertrophy and adiposity. In the kidney, lack of AQP7 in the proximal tubule brush border results in marked urinary loss of glycerol. In pancreatic β-cells, lack of AQP7 results in accumulation of glycerol and TG by a mechanism similar to that described for adipose tissue. This is associated with an increased secretion of insulin and, thus, increased plasma insulin levels, without changes in peripheral insulin sensitivity. Finally, in cardiac muscle, and potentially also in skeletal muscle, the lack of AQP7 results in a decreased uptake of glycerol and thereby a decreased generation of energy from glycerol.

In the second line of AQP7 KO mice, the age-dependent increase in fat mass with increased glycerol and TG content was confirmed (Hara-Chikuma *et al.* 2005). However, these KO mice had a similar weight gain as WT animals but had a reduced body length. Also no differences were found in serum levels of glycerol, FFA, and TG between KO and WT female mice, and the rate of lipolysis and lipogenesis in WAT seemed unaffected.

This intriguing phenotype suggests that lowering of adipocyte AQP7 expression or function is associated with an increased susceptibility for the development of obesity and insulin resistance. On the other hand, the studies performed in insulin-resistant db+/db+ mice demonstrated an increased expression of AQP7 in adipose tissue. Therefore, when investigating the expression of AQP7 in obese and insulin-resistant individuals, diverging results could be due to whether AQP7 is part of the development or the consequence of insulin resistance.

Nonetheless, it should be noted that two additional AQP7 KO mouse lines have been generated that do not support the findings presented above. In both these lines, no effect on body weight or fat pad masses were detected, and neither were there any differences between KO and WT animals in the plasma levels of glycerol, FFA, or TGs (Matsumura *et al.* 2007, Skowronski *et al.* 2007). Possible reasons for these variations could be due to different gene targeting strategies, different genetic backgrounds, or differences in collection and analysis of tissue samples.

Another important point to include when investigating the phenotype of AQP7 KO animals is the expression of AQP7 in other tissues with relevance for development of obesity and T2D as outlined in Fig. 2. In the kidney, AQP7 is localized to the apical plasma membrane of the proximal convoluted tubule (Ishibashi *et al.* 2000, Nejsum *et al.* 2000, Skowronski *et al.* 2007, Lebeck *et al.* 2012*a*). Investigations in AQP7 KO mice found that AQP7 only plays a minor role in water permeability of the proximal tubule (Sohara *et al.* 2005), whereas it plays a major role in urinary glycerol reabsorption. These mice display a marked urinary loss of glycerol and, thus, loss of energy, which *per se* would not predict susceptibility to development of obesity (Sohara *et al.* 2005, Skowronski *et al.* 2007). Like the lower efflux of glycerol from adipose tissue, then the urinary loss of glycerol would predict a reduction in p-glycerol levels in AQP7-deficient mice. AQP7 is also found in skeletal and cardiac muscle (Skowronski *et al.* 2007), and at least in cardiac muscle AQP7 is reported to play a significant role in supplying glycerol for energy production within the myocytes (Hibuse *et al.* 2009). The lack of AQP7 would here predict a reduced glycerol disposal that would increase the plasma glycerol levels. In parallel, it would also increase the need for other precursors than glycerol to support energy needs within muscle tissue. Finally, AQP7 has also been localized to pancreatic β-cells (Matsumura *et al.* 2007, Best *et al.* 2009). Despite the apparent intracellular localization of AQP7, the AQP7 KO mice were reported to have a higher intra-islet glycerol and TG content with an increased activity of GlyK similar to that observed in WAT from the first KO line. This was accompanied by an increased secretion of insulin, increased p-insulin levels with no effect on p-glucose, and with no evidence of insulin resistance (Matsumura *et al.* 2007). Owing to the wide expression profile of AQP7, the relative contribution of the different tissues to the phenotype of the total AQP7 KO mouse model seems difficult to define. Hence, there seems to be several candidate tissues affecting glycerol metabolism in parallel as well as the stated effect on pancreatic β-cells will influence energy metabolism at a more general level. In future studies, tissue-specific AQP7 KO mice would be a means to overcome this problem.

Human studies on the role of AQP7 in obesity and T2D

After the linkage between deficient AQP7 expression in adipose tissue and obesity with development of insulin resistance was proposed, several human studies have been

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undertaken to investigate whether dysregulation of adipose tissue AQP7 expression is involved in the pathophysiology of obesity and T2D in humans. In support of human relevance, the human *AQP7* gene is localized in a chromosomal region with reported linkage to T2D (Luo *et al*. 2001, Lindgren *et al*. 2002) and the metabolic syndrome (Loos *et al*. 2003).

An association study performed in Caucasian men and women has shown a connection between obesity and secondary development of T2D and a common single nucleotide polymorphism (SNP) (A-953G) in the promoter of AQP7. The SNP causes decreased transcriptional activity by impairing C/EBPβ binding to the promoter region and was coupled to decreased *AQP7* mRNA abundance in adipose tissue. Interestingly, this association was only observed among the female participants (Prudente *et al*. 2007). Thus, the role of AQP7 in human adipose tissue metabolism seems to be influenced by gender, and it indicates that in women a high AQP7 expression level in adipose tissue is desirable. This assumption is supported by the higher abundance of AQP7 in WAT found in women compared with men (Sjoholm *et al*. 2005, Lebeck *et al*. 2012*a*). Along the same line, we investigated the effect of a 10-week exercise program on AQP7 protein expression in abdominal subcutaneous adipose tissue (SAT) from healthy non-obese men and women and found that women responded by increasing the abundance of AQP7 protein, whereas in men, a reduced expression of AQP7 in SAT was observed (Lebeck *et al*. 2012*a*). Whether a similar gender-specific response to exercise also applies to visceral adipose tissue (VAT) remains to be investigated. However, the higher need for AQP7 to support glycerol efflux from adipose tissue in women seems well related to the higher plasma glycerol level found in women in response to metabolic stress such as fasting (Hales *et al*. 1965, Clore *et al*. 1989, Mittendorfer *et al*. 2001) and exercise (Davis *et al*. 2000, Galassetti *et al*. 2002, Mittendorfer *et al*. 2002).

The influence of gender might explain why an apparent metabolic phenotype was absent from a male subject homozygous for a loss-of-function mutation in the sixth transmembrane domain of AQP7 (G264V). A lack of increase in p-glycerol was observed in the G264V individual only when stressed by exercise (Kondo *et al*. 2002). Another group analyzed a mixed gender cohort of 178 Caucasians and found no association between the G264V mutation and either obesity or T2D. However, only one subject was homozygous for the mutation and that individual was obese, with T2D and p-glycerol levels below the 10th percentile (Ceperuelo-Mallafre *et al*. 2007). Recently, homozygosity for the G264V mutation in AQP7 was reported in three unrelated children with psychomotor retardation, hyperglyceroluria, normoglycerolemia, and a mild platelet secretion defect. No apparent metabolic phenotype was observed in either the three affected children or their normal homozygous siblings or heterozygous parents (Goubau *et al*. 2013).

Other human studies have evaluated the relative AQP7 abundance in adipose tissue from obese and T2D subjects with conflicting results. Several studies have found a reduced *AQP7* mRNA expression in SAT from obese men and women (Marrades *et al*. 2006, Ceperuelo-Mallafre *et al*. 2007, Rodriguez *et al*. 2011). However, one study reported unchanged *AQP7* mRNA abundance in SAT, whereas in VAT increased *AQP7* gene expression was observed when comparing obese with overweight individuals from a mixed gender cohort (Miranda *et al*. 2010). Most of the studies evaluating the *AQP7* mRNA expression in association with T2D did not find significant changes in the expression in SAT or VAT (Ceperuelo-Mallafre *et al*. 2007, Catalan *et al*. 2008, Miranda *et al*. 2009, 2010, Lebeck *et al*. 2012*a*), but an increased AQP7 abundance in VAT (Miranda *et al*. 2010, Rodriguez *et al*. 2011) has also been reported. These studies were performed in mixed gender cohorts, except for two studies (Catalan *et al*. 2008, Lebeck *et al*. 2012*a*).

In summary, the human data does point toward an association between a reduced expression of AQP7 and obesity, at least in women. The link to T2D, if any, seems to be secondary to obesity. Like the studies in the KO mice, no clear correlation between plasma glycerol and adipose tissue AQP7 expression was observed in the human studies. In order to further evaluate this, more studies with a focus on AQP7 expression in states prior to development of obesity or type diabetes are needed. Ideally, this would include a follow-up at a later time point, as the studies performed in obese or diabetics cannot provide much clarification as to whether a changed AQP7 expression is the cause or the consequence. In addition, gender-separated analysis would be preferable.

Aquaporin 9

Structure and permeability profile

The *AQP9* gene was first cloned from human liver cDNA and was found to encode a protein of 295 amino acids and, in contrast to AQP7, it contains the two highly conserved NPA motifs. It has a predicted molecular mass of 31.4 kDa (Ishibashi *et al*. 1998). The AQP9 protein homology is 76% between human and mouse, 75% between human and rat

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(Ko *et al*. 1999), and 90% between rat and mouse. Hydropathy analysis of human AQP9 predicts the characteristic six transmembrane domains with N- and C-terminal localized in the cytosol. A potential *N*-linked glycosylation site has been identified in the second extracellular loop at Asn-142 (Viadiu *et al*. 2007), and potential PKC and casein kinase II phosphorylation sites have been identified at the N-terminus (Ishibashi *et al*. 1998). The projection map at 7 Å resolution of two-dimensional AQP9 crystals indicates a tetrameric structure similar to other AQPs and that the pore size resembles the pore in GlpF (Viadiu *et al*. 2007). Immunoblotting for rat AQP9 protein results in a predominant band with a molecular size between 28 kDa (Elkjaer *et al*. 2000) to 33 kDa (Nihei *et al*. 2001), and additional bands at higher molecular weights that might represent glycosylated AQP9 products or oligomers (Elkjaer *et al*. 2000, Rodriguez *et al*. 2011). None of the additional bands were removed by deglycosylation with endoglycosidases (Nicchia *et al*. 2001); however, incubation with *N*-glycosidase F eliminated a ~35 kDa band (Viadiu *et al*. 2007), which is in agreement with the proposed *N*-linked glycosylation site in the second extracellular loop. In heterologous expression systems, AQP9 enhances the permeation of water and a wide range of small solutes, including polyols (glycerol, mannitol, and sorbitol), carbamids (urea and thiourea), monocarboxylates, purines (adenine), and pyrimidines (urasil and 5-FU) (Tsukaguchi *et al*. 1998, 1999, Carbrey *et al*. 2003, Jelen *et al*. 2011). Interestingly, AQP9 is also permeable to arsenite ($As(OH)_3$) (Liu *et al*. 2002, Carbrey *et al*. 2009), and one report also shows that AQP9 supports significant fluxes of NH_3 and NH_4^+ (Holm *et al*. 2005).

Localization of AQP9 in the liver

The localization of AQP9 has been investigated in humans as well as in rats and mice. The results are not fully unambiguous and some discrepancies between the various rodent and human expression sites exist (reviewed in Rojek *et al*. (2008)). In the liver, however, AQP9 protein demonstrates a similar localization in humans and rodents (Elkjaer *et al*. 2000, Rojek *et al*. 2007, Lebeck *et al*. 2012*a*). Here, AQP9 is localized to the plasma membrane domain of the hepatocytes, with the strongest labeling observed in the basolateral membrane domain facing the spaces of Disse (Nihei *et al*. 2001). Especially in females, AQP9 demonstrates a heterogeneous expression pattern within the liver. Thus, labeling for AQP9 is most abundant in the perivenous hepatocytes and gradually declines toward the portal triad. The gender-specific expression of AQP9 is also illustrated by female rats having ~20% lower AQP9 protein levels compared with males in the fed state (Nicchia *et al*. 2001).

Is AQP9 the sole aquaglyceroporin in the liver?

The presence of other aquaglyceroporins than AQP9 in the liver has been investigated with variable outcomes. Expression of AQP3 has been reported in murine liver (Patsouris *et al*. 2004). Contrasting results were, however, obtained in another study that was unable to detect either AQP3 or AQP7 expression at this site (Calamita *et al*. 2012). In the latter study, the authors also showed that the AQP inhibitor phloretin did not further reduce the glycerol permeability of the hepatocyte basolateral plasma membrane from AQP9 KO mice. This shows that AQP9 is the sole aquaglyceroporin contributing to the uptake of glycerol in murine liver. In human liver, the expression of both AQP3 and AQP7 protein has been reported (Rodriguez *et al*. 2011). Future studies are needed to clarify whether these aquaglyceroporins functionally overlap with AQP9 in human liver.

Regulation of AQP9 expression in the liver

Thus far, the regulation of hepatic AQP9 expression has only been observed at the transcriptional level. In male rodents, the hepatic expression of AQP9 is like AQP7 in adipose tissue inversely regulated by insulin. Thus, the hepatic AQP9 abundance is increased in response to fasting and decreased during refeeding (Kuriyama *et al*. 2002, Carbrey *et al*. 2003, Calamita *et al*. 2012), and STZ-induced diabetes mellitus results in an increased expression of AQP9 in the liver (Kuriyama *et al*. 2002, Carbrey *et al*. 2003). In accordance with the inverse relationship between insulin and hepatic AQP9 expression, a putative IRE (−502 to 496 bp) has been identified in the promoter region of AQP9 (Kuriyama *et al*. 2002; Fig. 3). This IRE is similar to the one found in the promoter of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in the gluconeogenic pathway. Insulin inhibits *PEPCK* gene transcription by inducing the phosphorylation of protein kinase B/Akt through the PI3K pathway. Akt then phosphorylates forkhead transcription factor 1 (FoxO1) and thereby causes its nuclear export and, thus, inhibits its binding to the IRE in the PEPCK promoter (Taniguchi *et al*. 2006). Whether the effect of insulin on *AQP9* gene transcription is regulated by this pathway remains to be experimentally confirmed.

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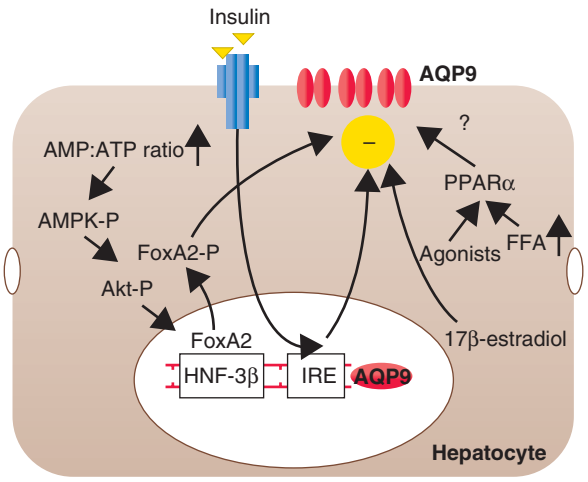

Figure 3
Signaling pathways reported thus far to be involved in regulating the hepatic expression of AQP9. In male rats, insulin inversely regulates the hepatic expression of AQP9 through an insulin response element (IRE) in the promoter region of the *AQP9* gene. In male rats, this results in an increased hepatic expression of AQP9 during states of low insulin levels, whereas in females 17β-estradiol inhibits this increase supposedly by interfering with other signaling pathways. Activation of AMPK by phosphorylation (AMPK-P) also reduces the transcription of AQP9 by phosphorylating protein kinase B/Akt (Akt-P), which then phosphorylates the forkhead transcription factor, forkhead box A2 (FoxA2)/(HNF-3β). This causes the nuclear extrusion of FoxA2 and thereby reduces its binding to the promoter region of AQP9. Under physiological conditions, AMPK activation occurs in response to increase in intracellular AMP:ATP ratios, such as seen during exercise and fasting. Finally, the peroxisome proliferator-activated receptor α (PPARα) is reported to be central for the increased expression of AQP9 in fasted male mice. Activation of PPARα is stimulated by increased cytosolic levels of FFA and by agonists such as fibrates used in the treatment of hypertriglyceridemia.

An important point regarding the inverse regulation of AQP9 by insulin is that it only occurs in male rodents. We recently reported that the increased expression of hepatic AQP9 in response to fasting and STZ-induced diabetes mellitus does not occur in female rats (Lebeck *et al*. 2012*b*). The lack of increase in hepatic AQP9 was paralleled by a lower glycerol permeability of the basolateral plasma membrane of hepatocytes and accumulation of glycerol in the blood in fasted females. Ovariectomy resulted in an increase in the hepatic abundance of AQP9 in fasted females similar to that observed in males, whereas ovariectomy did not influence the hepatic abundance of AQP9 in the fed state. This, together with results obtained by *in vitro* studies in hepatocytes, suggests that 17β-estradiol (E_2) prevents the increase in hepatic AQP9 abundance by influencing signaling pathways that are activated during metabolic stress (Lebeck *et al*. 2012*b*). In support for E_2 being involved in controlling hepatic AQP9 abundance, a separate study found that neonatal exposure to diethylstilbestrol was associated with lower hepatic AQP9 abundance in 20-day-old male rats (Wellejus *et al*. 2008).

Recently, an *in vitro* study in HepG2 cells showed that another forkhead transcription factor, forkhead box A2 (FoxA2 or HNF-3β), could be involved in regulating hepatic AQP9 expression (Yokoyama *et al*. 2011). As illustrated in Fig. 3, a decreased transcription of the *AQP9* gene was found in response to activation of AMP-activated protein kinase (AMPK), which increased the phosphorylation of Akt and thus the phosphorylation and nuclear extrusion of FoxA2 (Yokoyama *et al*. 2011). Several putative HNF-3β binding sites have been found in the promoter region of mouse AQP9 (Kuriyama *et al*. 2002), and the one affected by AMPK activation was reported to be between −1480 and −278 bp in the promoter of human AQP9 (Yokoyama *et al*. 2011). The physiological activator of AMPK is an increased AMP:ATP ratio such as seen during exercise and starvation and AMPK is known to promote energy generating pathways, whereas gluconeogenesis and lipogenesis are inhibited (Viollet *et al*. 2009). The nuclear extrusion of FoxA2 is also promoted by insulin, whereas during fasting and low insulin levels, FoxA2 increases the transcriptional programme of lipid oxidation and ketogenesis (Wolfrum *et al*. 2004). Hence, according to the *in vitro* studies on AMPK and AQP9, activation of AMPK during starvation in males would oppose the effects of the concurrent low insulin levels on AQP9 transcription. To this, it should, however, be noted that not all studies show increased activation of AMPK in response to fasting (Viollet *et al*. 2009).

PPARα has also been linked to regulation of hepatic AQP9 abundance (Fig. 3). In the liver, PPARα activation promotes fatty acid oxidation and ketogenesis and has also been linked to regulation of glucose metabolism (Kersten *et al*. 1999). In fasted WT male mice, increased levels of *Pparα* (*Ppara*) mRNA were paralleled by increased mRNA expression of *Aqp9*, *GlyK*, and *Gpd1*. This increase was abolished in male PPARα KO mice, suggesting that PPARα is central for the induction of AQP9 during starvation (Patsouris *et al*. 2004). In support, activation of PPARα using the agonist WY 14643 also increased the expression of AQP9 protein in HepG2 cells (Lee *et al*. 2005). However, in female mice, WY 14643 treatment failed to affect the expression of hepatic AQP9 (Patsouris *et al*. 2004). As no PPRE has been reported in the AQP9 promoter, it could be speculated that the effect of PPARα on hepatic AQP9 transcription is exerted through interfering with other transcription factor pathways. More studies are needed to elucidate the role of PPARα

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in the regulation of hepatic AQP9 expression. Finally, a single injection of glucagon in rats had no effect on the expression of AQP9 (Carbrey *et al*. 2003).

Role of AQP9 in the liver: insights from AQP9 KO mice

As outlined in Fig. 1, the glycerol released from adipose tissue through AQP7 during fasting reaches the liver where AQP9 facilitates its uptake. In the liver, the glycerol permeation step has been proposed to be rate-limiting for glycerol utilization within the hepatocyte (Li & Lin 1983). As mentioned earlier, a recent study demonstrated that AQP9 serves as the only glycerol channel in mouse liver and that changes in AQP9 abundance are paralleled by alterations in the glycerol permeability of the basolateral plasma membrane (Calamita *et al*. 2012). The significance of AQP9 in facilitating uptake of glycerol was reported to be limited to the fasted state, whereas in the fed state the glycerol permeability of the hepatocyte basolateral plasma membrane was similar in WT and AQP9 KO mice (Calamita *et al*. 2012). However, AQP9 KO mice demonstrate increased p-glycerol levels in both the fed and fasted state compared with WT mice (Rojek *et al*. 2007), thus indicating that also in the fed state, AQP9 plays a significant role in the hepatic uptake of glycerol.

Once inside the hepatocyte, glycerol can be utilized for lipogenesis, gluconeogenesis, and glycolysis (Fig. 1). AQP9 has mainly been considered in a role of supplying glycerol for gluconeogenesis, due to the increased expression during states of low insulin levels in male rodents (Maeda *et al*. 2008, 2009). Even though AQP9 KO mice do not suffer from hypoglycemia in response to fasting, AQP9-deficient $Lepr^{db}/Lepr^{db}$ mice that become obese and develop T2D have lower blood glucose levels than $Lepr^{db}/Lepr^{db}$ mice, suggesting that the obese AQP9 KO mice have a reduced capacity to generate glucose (Rojek *et al*. 2007). Furthermore, in obese insulin-resistant db+/db+ mice, a 1.3-fold increase in hepatic *AQP9* mRNA expression was paralleled by a higher hepatic glucose production from glycerol compared with lean control mice (Kuriyama *et al*. 2002). Finally, in contrast to WT mice, AQP9 KO mice do not increase the hepatic glucose output in response to perfusion with glycerol. Similar results were obtained in WT liver after treatment with an AQP9 inhibitor (Jelen *et al*. 2011). This shows that AQP9 plays a pivotal role in supplying glycerol for gluconeogenesis.

When considering the role of AQP9 in supplying glycerol for the different metabolic pathways, the distribution pattern of AQP9 within the liver should be taken into account. As outlined above, the expression of AQP9 is highest in the area surrounding the central vein with a gradual decline towards the portal triad. By contrast, according to the hepatic zonulation model, the expression of gluconeogenic enzymes is most abundant in the periportal hepatocytes with a gradual decline towards the central vein (Jungermann & Katz 1989, Krones *et al*. 1998, Postic & Girard 2008). Even though this, of course, gives a certain expression overlap, the physiological logic is not obvious. On the contrary, the hepatic zonulation model predicts relevance for AQP9 in supplying glycerol for glycolysis or TG synthesis.

As outlined above, a substantial part of the FFA that reaches the liver during fasting is re-esterified and either used for VLDL synthesis or stored in lipid droplets within the hepatocytes. The effect of AQP9 on hepatic TG generation remains largely unknown. Both fed and fasted AQP9 KO mice have higher plasma TG levels compared with AQP9 heterozygotes (Rojek *et al*. 2007), suggesting that AQP9 KO mice do not have a reduced capacity for generating TG. It should, however, be noted that plasma TG levels are often measured indirectly by detecting glycerol after hydrolysis of TG into glycerol and FFA, and therefore hyperglycerolemia can result in pseudohypertriglyceridemia. Two recent studies have linked AQP9 to hepatic TG accumulation and non-alcoholic fatty liver disease (NAFLD; Cai *et al*. 2013, Wang *et al*. 2013). Knockdown of AQP9 was reported to reduce the accumulation of TG in hepatocytes in both a rat and a cell culture model for NAFLD (Cai *et al*. 2013, Wang *et al*. 2013), thus suggesting a role for AQP9 in controlling hepatic TG synthesis.

Even though most studies have focused on the involvement of AQP9 in glycerol metabolism, the broad permeability profile of AQP9 suggests additional roles. As AQP9 is permeable to urea and as ureagenesis is increased in the catabolic states of fasting and diabetes, AQP9 could be hypothesized to play a role in facilitating the export of urea from the hepatocytes during these states. In support for this notion, the urea permeability of hepatocyte basolateral plasma membranes is reduced by 30% in AQP9 KO mice compared with WT mice. However, the AQP9 KO mice responded in a manner similar to WT mice when fed a high-protein diet, which increases the hepatic synthesis of urea, thus suggesting redundancy of AQP9 as a facilitator of urea transport (Jelen *et al*. 2012). This is also in agreement with the hepatic expression of AQP9 remaining unaffected by high-protein diets (Nicchia *et al*. 2001, Carbrey *et al*. 2003). Others have suggested a role for AQP9 in the hepatocellular hydration state and

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through this involvement in bile formation (Calamita *et al*. 2008). Finally, studies on AQP9 KO mice have also shown that AQP9 plays a role in hepatic arsenite excretion and thus provides partial protection of the whole animal from arsenic toxicity (Carbrey *et al*. 2009).

Human studies on AQP9 expressed in liver

Thus far, only a few studies have examined hepatic AQP9 in humans. The relative expression of AQP9 in the liver has been investigated in obese individuals with T2D and obese with no impairment of their glucose tolerance. This has given variable results with some reporting a reduced expression of AQP9 in T2D (Catalan *et al*. 2008, Rodriguez *et al*. 2011) and others finding no significant changes (Miranda *et al*. 2009). These findings contrast the increased expression of AQP9 found in male rodent models of diabetes (Kuriyama *et al*. 2002, Carbrey *et al*. 2003). Some of the human studies were performed in a mixed-gender cohort (Miranda *et al*. 2009, Rodriguez *et al*. 2011), which could affect the summarized outcome on hepatic AQP9 expression, if the gender-specific regulation of AQP9 in response to metabolic stress also applies to humans. On the other hand, studies on a human hepatocyte cell line (HepG2) showed an increased expression of AQP9 in response to insulin treatment, whereas leptin reduced the abundance of AQP9 (Rodriguez *et al*. 2011). This clearly contrasts the observations made in male rodents (Kuriyama *et al*. 2002, Carbrey *et al*. 2003), and even though the number of observations made in humans are still sparse, these results call for future studies to investigate whether the knowledge obtained for hepatic AQP9 in rodents has human relevance.

Conclusion and perspectives

Accumulating evidence suggest that AQP7 in adipose tissue and AQP9 in liver are glycerol channels with significant influence on glycerol metabolism within the two tissues. Even though not supported by all studies, results on mice and humans point towards a connection between low expression levels of AQP7 in adipose tissue and increased risk for development of obesity. Intriguingly, in humans, this association is found only in women. From a clinical point of view, a selective enhancement of AQP7 would seem like an interesting therapeutic target for treatment of obese women. However, before taking this step, the contribution of AQP7 expressed in other tissues to the metabolic phenotype needs further clarification.

In the liver, blocking of AQP9 could, at least in males, be a means to lower hepatic glucose production, especially in obese T2D, where glycerol availability and glycerol gluconeogenesis are increased. In addition, the novel association between AQP9 and NAFLD also suggests a beneficial effect of a reduced hepatic uptake of glycerol on hepatic TG accumulation. To clarify the potential for AQP9 as a pharmacological target, further studies on sexual dimorphism in hepatic glycerol metabolism and the role of AQP9 in TG synthesis seems to be of high importance. Finally, studies on whether results for AQP9 obtained in rodent models are of human relevance are of high priority.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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