

Universidade de Lisboa

Faculdade de Farmácia



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Dissertation Report supervised by Professor Janne Lebeck and co-supervised by
Professor Graça Soveral

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Abstract

Aquaglyceroporins are pore-forming transmembrane proteins that facilitate the transport of small solutes such as glycerol in addition to water. Some studies have reported expression of aquaglyceroporin 7 (AQP7) in both human and murine liver tissues. However, other studies have contradicted these findings showing very low or no expression of the glycerol channel AQP7 in liver. Taking into account these contradictory results, we re-evaluated the cellular and subcellular localization of AQP7 in human liver and determined whether a similar expression pattern was found in Human Hepatoma G2 (HepG2) cells by using immunofluorescence double labelling with well-validated antibodies. In addition, the role of AQP7 was also investigated. Results show marked labelling for AQP7 in hepatocytes, apical plasma membrane domain of the cholangiocytes lining the bile duct while few prominent labelling was observed in sinusoidal cells. The labelling for AQP7 in hepatocytes was intracellular predominantly confined to the perivenous region. Double immunofluorescence revealed the partial co-localization of AQP7 with lipid droplet associated protein perilipin-2 (Plin2). Almost a complete overlap was observed between the microtubule associated protein, dynactin and AQP7. Further, mRNA and protein expression of AQP7 in HepG2 was confirmed by PCR, immunoblotting and immunocytochemistry. However, in HepG2 cells, the localization of AQP7 was not similar to that found in hepatocytes in human liver tissue. Free fatty acid (FFA) treated HepG2 cells demonstrated increased expression of Plin2 in a concentration dependent manner. No significant change in the expression of AQP7 in FFA treated HepG2 cells was seen. In conclusion, evidence of AQP7 co-localizing with dynactin indicates that AQP7 is expressed in structures that are transported along the microtubule whereas co-localization of AQP7 with Plin2 points towards a role of AQP7 in lipid storage in liver. The involvement of AQP7 in lipid storage is also suggested by the abundant expression of AQP7 in the perivenous region which is the major site for triacylglycerol (TAG) synthesis.

Keywords: Aquaglyceroporin; AQP7; Perilipin 2; Human Liver; Triacylglycerol

Sumário

As aquagliceroporinas são proteínas canais transmembranares que facilitam o transporte de pequenos solutos, como o glicerol, para além da água. Alguns estudos reportaram a expressão de aquagliceroporina 7 (AQP7) em tecidos de fígado humano e murino. No entanto, outros estudos contradizem esses resultados mostrando muito baixa ou nenhuma expressão do canal de glicerol AQP7 no fígado. Para esclarecer esses resultados contraditórios, foi reavaliada a localização celular e subcelular de AQP7 no fígado humano e foi investigado se um padrão de expressão semelhante seria encontrado em células de Hepatoma G2 Humano (HepG2) usando marcação dupla de imunofluorescência com anticorpos bem validados. Além disso, o papel funcional da AQP7 também foi investigado. Os resultados mostram uma forte marcação da AQP7 em hepatócitos, no domínio da membrana plasmática apical dos colangiócitos que revestem o ducto biliar, enquanto que as células sinusoidais revelaram uma marcação fraca. A marcação para AQP7 em hepatócitos foi intracelular, predominantemente confinada à região perivenosa. A imunofluorescência dupla revelou a co-localização parcial de AQP7 com a proteína perilipina-2 associada a gotículas lipídicas (Plin2). Uma sobreposição quase completa foi observada entre a dinactina, uma proteína associada ao microtúbulo, e a AQP7. Além disso, a expressão de mRNA e proteína AQP7 foi confirmada em HepG2 por PCR, immunoblotting e imunocitoquímica. No entanto, em células HepG2, a localização de AQP7 não foi semelhante à encontrada em hepatócitos em tecido hepático humano. As células HepG2 tratadas com ácidos gordos livres (FFA) revelaram uma expressão aumentada de Plin2 dependente da concentração, mas não foi observada qualquer alteração significativa na expressão de AQP7 em células HepG2 tratadas com FFA. Em conclusão, a evidência de co-localização de AQP7 com dinactina indica que AQP7 é expressa em estruturas que são transportadas ao longo do microtúbulo, enquanto a co-localização de AQP7 com Plin2 aponta para um papel de AQP7 no armazenamento de lípidos no fígado. O envolvimento de AQP7 no armazenamento de lípidos também é sugerido pela expressão abundante de AQP7 na região perivenosa que é o principal local para a síntese de triacilglicerol (TAG).

Palavras-chave: Aquagliceroporina; AQP7; Perilipina 2; Fígado Humano; Triacilglicerol

Key Abbreviations

AQP7	Aquaglyceroporin 7
ATGL	Adipose Tissue Triacylglycerol Lipase
CD163	Cluster of Differentiation 163
CD68	Cluster of Differentiation 68
CO ₂	Carbon dioxide
CV	Central Vein
DAB	3,3-diaminobenzidine tetrahydrochloride
DHAP	Dihydroxyacetone Phosphate
dNTP	DeoxyriboNucleotide TriPhosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EGTA	Egtazic acid
FFA	Free Fatty Acid
G3P	Glycerol 3-Phosphate
GPD1	Glycerol-3-phosphate dehydrogenase 1
GlyK	Glycerol Kinase
H ₂ O ₂	Hydrogen Peroxide
HFD	High Fat Diet
Hsc70	Heat Shock Cognate 70
Hsp70	Heat Shock Protein 70
ICC	Immunocytochemistry
IF	Immunofluorescence
IHC	Immunohistochemistry
HepG2	Human Hepatoma G2
LD	Lipid Droplet
PBS	Phosphate Buffered Saline
Plin2	Perilipin-2
PFA	Paraformaldehyde
PV	Portal Vein
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase PCR
SEM	Standard Error of The Mean
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TAG	Triacylglycerol
T2D	Type 2 Diabetes
WAT	White Adipose Tissue

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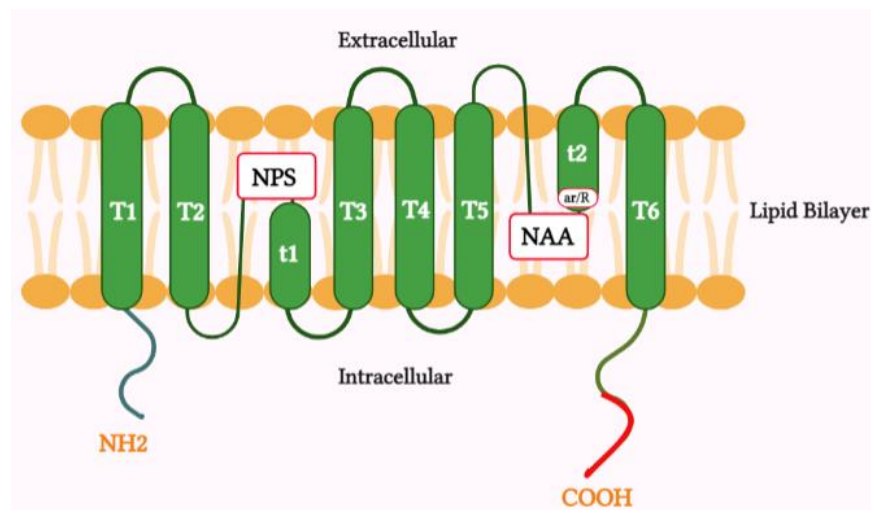
1. Introduction

Aquaporins

Aquaporins (AQPs), a family of small transmembrane proteins that include 13 known channel proteins (AQP0- AQP12), are further subdivided based on their solute selectivity and protein sequence similarity in humans (1). Generally, AQPs facilitate the transmembrane movement of water, however, some aquaporins also facilitate the transport of small solutes like glycerol and gases like CO₂ (2). The 13 isoforms of human AQPs are roughly assorted into 3 sub-families namely: a. Classical or Orthodox AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, AQP8) are mainly permeable to water, b. Aquaglyceroporins (AQP3, AQP7, AQP9, AQP10) that allow the movement of small solutes like urea and glycerol and gases such as ammonia beside water (2) c. Unorthodox AQPs (AQP11, AQP12) have low structural homology with other aquaporins and their permeability profile yet remain a point of debate (3).

Characteristics of AQP7

The AQP7 monomer comprises six transmembrane domains spanning the lipid bilayer, along with two half-helical segments plunging into the membrane (Figure 1.1). The AQP7 monomers form tetramers. This is essential for the stability and post-translational modification of proteins (4). Human AQP7 (hAQP7) has a narrow pore size of about 3 Å of diameter (5) with two constriction sites. One of the constrictions is called aromatic-arginine constriction site (ar/R) which excludes molecules above AQP7 pore size. The other constriction contains two motifs whose function is to prevent the passage of charged molecules such as protons. In hAQP7 the orthodox aquaporin NPA motifs are modified into NAA (asparagine–alanine–alanine) and NPS (asparagine–proline–serine) motifs (1,6). With a total of 342 amino acids, the cytoplasmic N and C terminals of AQP7 contain ~32 and ~63 amino acids, respectively (7). The cytoplasmic N and C domains are responsible for the regulation of AQP7 activity (7,8). It is found that AQP7 is less permeable to water in the presence of glycerol (5).



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Figure 1.1: Monomeric structure of human aquaglyceroporin (AQP7) in the plasma membrane.

AQP7 monomer possesses six transmembrane domains and two short half helices denoted by T (1-6), and t (1-2) respectively. The selectivity motifs are denoted by ar/R, NPS and NAA in proximity to half helices. The N and C terminals lie in the cytoplasm where the amino acid sequence of C terminal is longer than the N terminal. The antibody used in this study is directed

against the last 21 amino acids of the C terminal as indicated by red color. Figure adapted from Huang *et al.* (7)

Expression of AQP7 in liver

AQP7 was reported for the first time in rat testis with a molecular weight of 26 kDa (9). It was highly expressed in the plasma membrane of spermatids and maturing sperms and demonstrated permeability to water, glycerol and urea (9). So far, the expression of AQP7 has been reported in the adipose tissue, kidney, heart, skeletal muscle, endocrine pancreas, liver, gastrointestinal tract, eye, inner ear, male and female reproductive systems (1,10). However, the expression of AQP7 in liver has been a point of debate. While some studies have reported AQP7 expression in human and murine liver (11–17), other studies contradict these findings (18–22). One study demonstrated mRNA expression as well as protein expression of AQP7 in mouse liver. Immunohistochemistry (IHC) analysis of AQP7 expression in mouse liver showed immunoreactivity for AQP7 at the periphery of hepatocytes (11). The same study also revealed that the mRNA of AQP7 was expressed in humans. This was the first study to report the hepatic expression of AQP7 in both human and mouse. However, this study was limited to mRNA determination of AQP7 in humans. Another group backed up the presence of AQP7 in a healthy human and murine liver by reporting labelling for AQP7 in hepatocytes intracellularly (12). Besides hepatocytes, this group was indeed the first group to report the localization of AQP7 in cholangiocytes and endothelial cells in the human liver. Xiaohua *et al.* also demonstrated the presence of both AQP7 mRNA and protein in mouse liver using quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblotting (13). Apart from the expression of AQP7 in healthy human liver, other studies have confirmed the expression of AQP7 transcript and protein in liver from obese human subjects (14,15). The latter study showed the ratio between hepatic AQP7 and AQP9 mRNA expression to be 0.142/30, suggesting a relatively low AQP7 mRNA expression in liver. IHC performed in liver from obese individuals suggested an intracellular expression of AQP7 in hepatocyte which in some areas were associated with lipid droplet (LD) (14). Although their results seem to be appealing, they have not clearly specified about the AQP7 antibody they used in their study. Furthermore, other studies also confirmed the presence of AQP7 in human liver tissue (16,17). In the study by Chen and co-workers the labelling for human AQP7 was reported to have a cytoplasmic and nuclear distribution in hepatocellular carcinoma tissue. However, the staining for AQP7 was not convincing; it was difficult to differentiate actual labelling from background staining.

There are however several studies contrasting these findings by corroborating no expression of AQP7 in the human and murine liver (18–22). The study by Kondo *et al.* did not state the presence of AQP7 in human liver tissues, although a weak band seemed to appear in liver tissue lane from RT-PCR after 25 cycles (18). The absence of AQP7 in mouse liver is supported by both mRNA (20,22) and protein expression analyses (21). Moreover, rat liver also demonstrated a lack of expression of AQP7 (9).

Table 1.1: Overview of specification of AQP7 antibodies that were applied to the liver sections in the previous studies.

The table provides information about the species on which AQP7 was investigated, methods used to confirm the expression of AQP7 in liver, location of AQP7 in liver, specification of the epitope of AQP7 antibody and species (human, rat, mouse) that the antibody was generated against as well as the name of the company if given.

Ref.	Species Investigated	Methods to confirm AQP7 expression	Location of AQP7	Epitope of antibody and the species generated against
(11)	Human & Mouse liver	RT-PCR (Human & Mouse) Immunoblot (Mouse) IHC (Mouse)	Periphery of hepatocytes	Anti-human AQP7 antibody raised against the N-terminal peptide
(12)	Human & Murine liver	qRT-PCR IHC	Human: Hepatocytes (Intracellular); Cholangiocytes; Endothelial Cells Mouse: Hepatocyte (Intracellular)	Anti-rat AQP7 antibody raised against the N-terminal peptide. Also recognizes human and mouse sequences. (Alomone, Jerusalem, Israël)
(13)	Murine liver	qRT-PCR Immunoblot	–	Recombinant AQP7 antibody Abcam (ab32826)
(14)	Human obese liver	qRT-PCR Immunoblot IHC	Hepatocyte (Intracellular associated with LD)	Have not specified the epitope that the antibody was generated against and against which species the antibody was directed.
(15)	Human obese liver	qRT-PCR	–	–
(17)	Human hepatocellular carcinoma tissue	IHC	Cytoplasm and nuclear distribution	Epitope not specified, Directed against human sequence Abcam (ab85907)

Regulation of AQP7 expression in liver

In type 2 diabetic (T2D) human subjects, an increase in hepatic expression of AQP7 was seen where the liver would be expected to be insulin resistant (14). AQP7 was reported to be upregulated and downregulated by insulin and leptin respectively in human hepatoma G2 (HepG2) cells (14). The initial finding is in contrast to the effect of insulin on AQP7 expression in adipocytes (19). Insulin induces hepatic steatosis by inducing lipogenesis (23). The elevation of AQP7 expression in HepG2 by insulin has been suggested to increase hepatocyte triacylglycerol (TAG) concentration by increasing lipogenesis (14). The insulin mediated regulation of AQP7 was linked to the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and mammalian target of rapamycin (mTOR) signalling pathways where its pharmacological inhibitor wortmannin or rapamycin reversed the effect of insulin (14).

Similarly, the reduction of AQP7 by leptin stimulation was also reversed after treatment with wortmannin. Unlike in HepG2 cells, other studies did not find an association between the hepatic AQP7 gene and insulin in obese individuals (16). Rosiglitazone which improves T2D by improving insulin sensitivity transcriptionally increased AQP7 in HepG2 cells (16) similar to the finding in adipose tissue from the T2D model in rats (24). However, this result is not consistent with another hepatocyte cell line, HuH7, as well as primary murine hepatocytes wherein AQP7 mRNA level was unaffected by Rosiglitazone (12). Adiponectin, which also enhances insulin sensitivity in liver did not modulate HepG2 AQP7 gene expression (16). In all, the regulation of AQP7 by different regulators remains ambiguous and needs further investigation transcriptionally as well as at the protein level. Ovariectomy-induced hepatic steatosis in mice decreased the abundance of hepatic AQP7 (13) whereas the estrogen treatment in ovariectomized mice significantly raised AQP7 expression levels in liver. Estrogen alleviated hepatic steatosis by decreasing the lipogenic enzymes. Similarly, estradiol also declined the fat accumulation together with a reduction of TAG level in oleic acid induced steatosis in HepG2 cells. However, both of these outcomes were attenuated by inhibiting the gene expression of AQP7. Thus, the authors concluded that estrogen improves hepatic steatosis via both decreasing lipogenesis and enhancing AQP7 expression in hepatic tissue. These results indicate the involvement of AQP7 in TAG metabolism. Further research is necessary to fully understand the hormone-induced regulation of AQP7 in liver.

Glycerol metabolism in liver

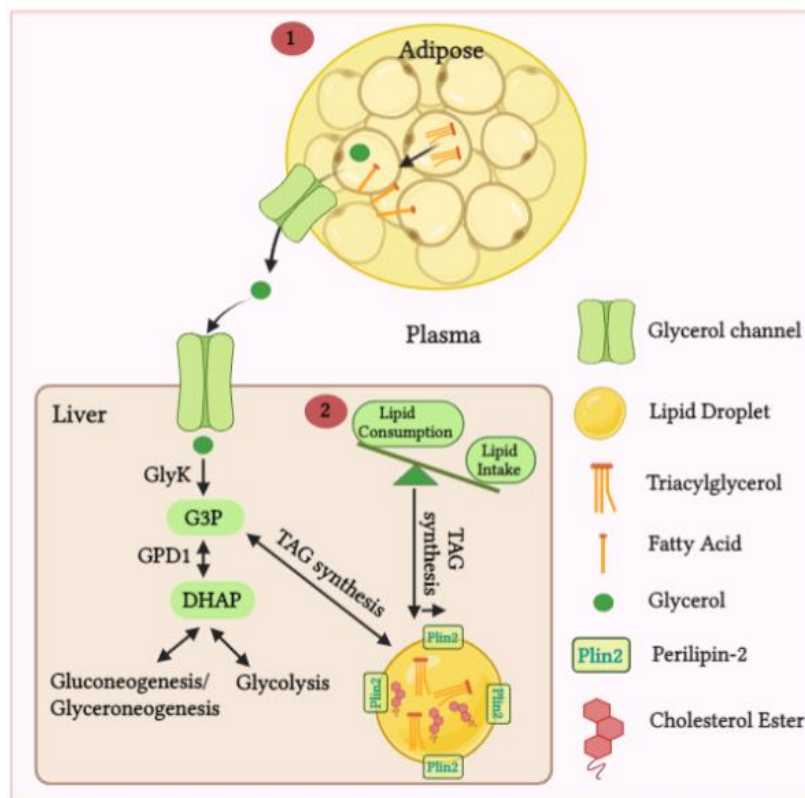
Glycerol is the backbone of TAG representing a key precursor for the synthesis of TAG and glucose. About 70-90% of glycerol metabolism occurs in liver (25). After the ingestion of dietary fat, TAG gets hydrolysed into free fatty acid (FFA) and glycerol by lipase enzymes (26). These fatty acids are resynthesized back into TAG in enterocytes and is packaged into chylomicrons. Chylomicrons from enterocytes, enter lymphatic capillaries and eventually pass into the bloodstream. Walls of blood capillaries have a surface enzyme called lipoprotein lipase which cleaves TAG components of chylomicrons into FFA and glycerol. These components are stored as TAG in fat droplet in adipocytes. Glycerol from the circulation is predominantly metabolized in liver. In a starvation or fasting state, adipocytes rapidly break down TAG via lipolysis and release the glycerol and FFA into blood circulation. Glycerol is primarily taken up by the liver where it is utilized for gluconeogenesis, glycolysis and TAG synthesis. During the fasted state, FFA entering the liver as non-esterified FFA is used for energy fuel through ketogenesis and beta-oxidation or re-esterified into TAG (27). Glycerol in hepatocytes is readily converted to glycerol-3-phosphate (G3P) catalysed by glycerol kinase (GlyK) whose activity in liver is significant (28). G3P is used either as a substrate for the formation of glucose through the hepatic gluconeogenesis pathway or used for the synthesis of TAG. In addition to G3P derived from plasma glycerol and glucose via glycolysis, G3P from glyceroneogenesis that uses alanine, pyruvate and lactate as substrates, accounts for 60% of hepatic triglycerides synthesis (27). Glycerol-3-phosphate dehydrogenase 1 (GPD1) catalyses the reversible conversion of G3P from dihydroxyacetone phosphate (DHAP), whereas conversion of G3P to DHAP directs G3P into the gluconeogenic/glycolytic pathways (26).

Under the feeding set, the input of glycerol to hepatic glucose production decreases (29) in parallel to the reduced rate of adipocyte lipolysis. FFA in hepatocytes is substantially re-esterified to TAG by combining with G3P. Consequently, the rate of TAG synthesis is dominant.

Lipid accumulation and lipid droplet protein in liver

The imbalance between lipid acquisition and lipid consumption can result in metabolic alterations like hepatic steatosis. The sources of hepatic TAG accumulation in liver are 1

Dietary fat from remnants of chylomicron (15%) as described above 2 *De novo* lipogenesis that synthesizes new fatty acid in the liver by metabolizing glucose to acetyl-CoA which is the main substrate for fatty acid (26%) 3 Non esterified FFA as a result of increased AT lipolysis (14%) (30). Additionally, the oxidation of fatty acid to energy and secretion of TAG into blood as VLDL particles (31) does not sufficiently compensate the excessive lipid intake. All these metabolic perturbations eventually lead to high lipid accumulation where esterified fatty acid in the form of TAG and cholesterol esters resides in lipid LD. The LD in liver is surrounded by a family of perilipin proteins, lipases and lipogenic enzymes. The perilipin family acts as a gatekeeper of LD promoting lipid storage. Among the perilipin protein family, perilipin-2 (Plin2) is the most abundant LD protein (32) in hepatocytes whose expression is elevated upon high fat intake (33). The fatty human liver had upregulation of Plin2 expression compared to control individuals (33). Plin2 ablation in mice resulted in a reduction of TAG content in hepatocytes, inhibiting the expression of lipogenic enzymes and eventually protecting against diet induced hepatic steatosis (34,35). Another mechanism via which suppression of Plin2 expression reduced hepatocyte TAG accumulation might be increase access of lipase enzymes like adipose triglyceride lipase (ATGL) to the surface of LD consequently enhancing the rate of lipolysis (36). Plin2 competes with ATGL's LD binding site, limiting ATGL's interaction with TAG. Collectively, the hepatic decline of Plin2 expression could be a viable strategy for preventing hepatic steatosis.



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Figure 1.2: Glycerol metabolism and fat accumulation in the liver.

1 During the fasting state triacylglycerol (TAG) gets hydrolysed into three molecules of FFA and glycerol in adipose tissue. Glycerol is uptake into the liver by aquaglyceroporin 9 (AQP9) glycerol channel (and possibly by others AQPs). Inside the liver, glycerol gets converted to glycerol-3-phosphate (G3P) catalysed by glycerol kinase (GlyK). G3P is dominantly used as a substrate for gluconeogenesis and partly for TAG synthesis and glycolysis during fasting. For G3P to enter the hepatic gluconeogenesis and glycolysis pathways is oxidized into

dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase 1 (GPD1). As the last stage in the process of glyceroneogenesis, GPD1 also catalyses the reverse reaction that produces G3P from DHAP which is also used as a substrate for TAG synthesis in fasting. The synthesis of G3P through glyceroneogenesis process uses alanine, pyruvate and lactate as substrates. When the fat intake exceeds the fat consumption, the process of TAG synthesis increases which results in lipid accumulation as well as the increment of lipid droplet (LD) protein perilipin-2 (Plin2) in the liver. The LD core encloses TAG and cholesterol esters as shown in the diagram. Figure adapted from Lebeck (26).

Liver structure and its cellular architecture in cargo transport

The liver is the second largest lipid storage organ in the body. The liver consists of four basic cell types, namely, hepatocytes accounting for 80% of cells in the liver, Kupffer cells within the hepatic sinusoid adhered to sinusoidal endothelial cells, endothelial cells, hepatic stellate (fat storing cells) also called Ito cells reside in space of Disse (perisinusoidal space) between hepatocytes and sinusoidal endothelial cells. The liver is made up of histological structures called lobules. Each lobule is hexagonal shaped surrounded by portal triads (hepatic artery, portal vein and bile duct) at each of the corners and with a central vein at the center. Both the portal vein and hepatic artery drain at the central vein. In human liver, approximately 25-30 hepatocytes make up the centro-portal axis in the liver lobule (37). The centro-portal axis is divided into three zones based on their metabolic activity.

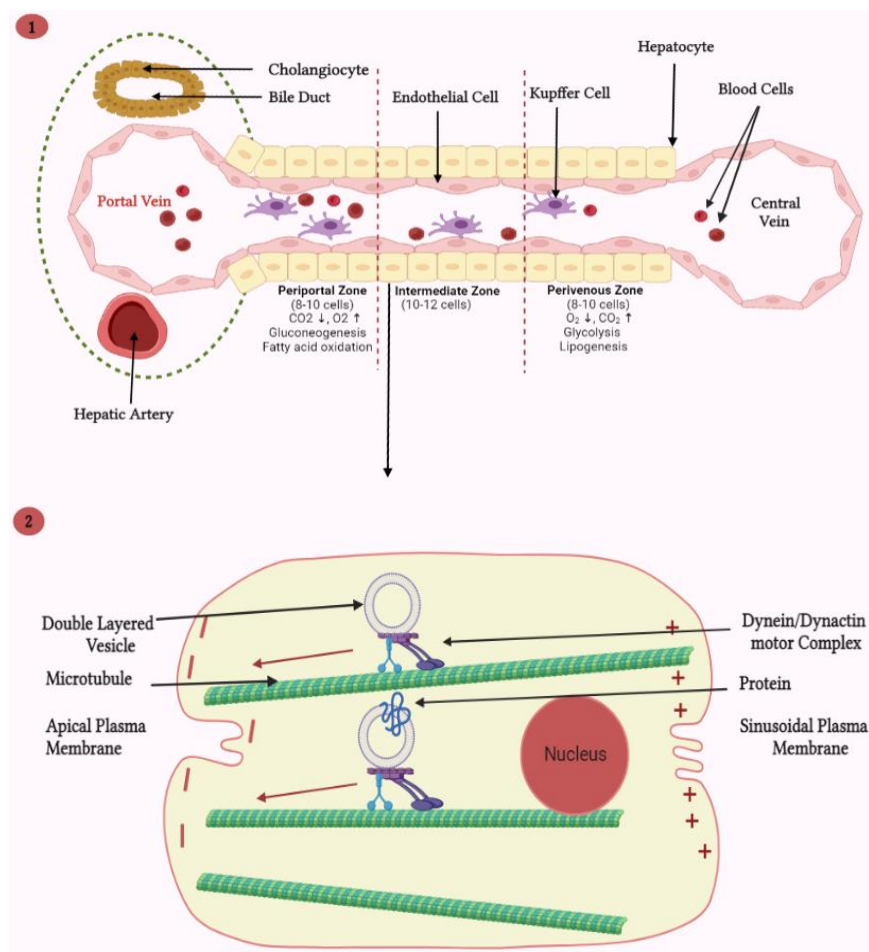
- Zone 1: Also called periportal is close to the portal canal and receives the most oxygen. It contains ~ 9 cells. Periportal has an oxygen supply of ~ 10–12% (38).
- Zone 2: Intermediate zone between periportal and perivenous. It is comprised of ~12 cells.
- Zone 3: Also known as pericentral/perivenous zone is nearest to the central vein and is least perfused. It comprises about 9 cells in its region. Perivenous hepatocytes have less exposure to the oxygenated blood level of about ~3–5% (38).

This heterogeneity of hepatocytes has previously been explored by several studies. One of the earlier studies showed that hepatocytes in the central part have more fat deposition than periportal hepatocytes in mouse liver (39). It was described as the “zonation within the lobule”. The regulation of liver zonation is ascribed to the differences in the amount of oxygen and nutrients received by hepatocytes lying in different regions.

Later another study by Jungermann *et al.* characterized the functional role of hepatocyte heterogeneity by introducing the idea of metabolic zonation (40). They pointed out that periportal hepatocytes undertake gluconeogenesis, and fatty acid oxidation whereas glycolysis, TAG synthesis primarily take place in pericentral hepatocytes. Besides the perivenous region exhibit stronger hypoxia-induced gene expression (41) and this gene has been reported to induce lipogenesis by modulating proteins involved in FFA uptake, and lipid storage (42,43). In short, the periportal region is an active site for high energy demanding metabolic processes. Apart from hepatocytes, metabolic heterogeneity is also applied to hepatic non-parenchymal cells like Kupffer cells and hepatic stellate cells.

Interestingly, the function of hepatocytes based on liver zonation changes with time (44). In the hours following feeding, glycogen is initially concentrated in the periportal area and later distribution in the pericentral region is reported (39). Successively, prolonged fasting caused the pericentral cells' glycogen reserves to be depleted first. The course of feeding and fasting was also reported to cause alterations in different gene expressions in hepatocytes (44). Therefore, the metabolic shift from feeding to fasting and vice versa is pivotal while analysing gene and protein expression that adheres to the notion of metabolic zonation.

Hepatocytes possess a canalicular membrane also called the apical domain and the blood facing domain called the sinusoidal (basolateral) membrane (45). The bidirectional movement (from and to the plasma membrane) of different molecules/proteins in the cells requires a microtubule network and motor proteins like dynein/ dynactin and kinesin. The proteins when newly synthesized are exported from the Golgi network in vesicles. For this, proteins use kinesin to shuttle towards the sinusoidal domain while dynein/dynactin is responsible for gaining access to the apical membrane or from the sinusoidal membrane to the interior of the cell. The microtubules in hepatocytes are organized with the negative end orientated towards the canalicular site and with the positive end towards the sinusoidal space. The dynein/dynactin complex drives the cargoes to the negative end of the microtubule (46). Some studies have shown that membrane-bound components reach their destined membrane sites via microtubule-based vesicle transport in liver cells (46,47).



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Figure 1.3: Metabolic zonation of centro-portal axis of liver lobule and a hepatocyte architecture.

1 Periportal, Intermediate and Perivenous regions are separated by red dotted line as indicated. Periportal region receives more oxygenated blood than the perivenous site shown by a greater number of blood cells in portal areas. Periportal region is an active site for gluconeogenesis and fatty acid oxidation whereas lipogenesis and glycolysis take place in perivenous region. **2** Hepatocyte microtubule cytoskeleton facilitates the trafficking of different organelles. Hepatocytes have the negative (-) end of the microtubule directed toward the apical plasma membrane whereas the positive (+) end is pointed toward the sinusoidal domain. The dynein/dynactin complex is the motor protein that drives different cargoes to the negative end

of the microtubule as indicated by long red arrows. Figure 1 adapted from Shani Ben-Moshe *et al.* (41). Figure 2 adapted from Hamm-Alvarez *et al.* (46)

What is the role of AQP7 in metabolic tissue?

The functional roles for AQP7 have been extensively studied in different tissues primarily in adipose tissue. The transport of glycerol and the effect on glycerol availability for the formation of TAG are the key physiological roles of AQP7 in adipose tissue and likely also in pancreas. However, the physiological role of AQP7 in liver and skeletal muscle is yet to be determined. AQP7 has been reported to be localized in adipocytes and capillary endothelium in human and murine adipose tissue (48–52). In response to different stimuli in adipose tissue, AQP7 has a dynamic localization pattern where it shuttles from the plasma membrane to intracellular space and vice versa. 3T3-L1 adipocytes display translocation of AQP7 from intracellular membrane to plasma membrane in lipolysis dependent manner (53). The relocation of AQP7 from LD to plasma membrane stimulated by isoproterenol is supposed to be the membrane trafficking mechanism for AQP7 (50). On the other hand, the binding of AQP7 with Plin1 associating with LDs in human adipose tissue has been suggested to enhance the intracellular localization of AQP7 (7,8,51). The interaction of AQP7 with Plin1 is increased during the feeding state whereas, in the fasted state, phosphorylation of the N-terminus of AQP7 reduced its binding with LD protein (7,8). Consequently, AQP7 moves to the plasma membrane where it is anticipated to exert efficient glycerol efflux which is a characteristic of lipolysis. In contrast, another study corroborated the localization of AQP7 from plasma membrane to intracellular domain around LD upon catecholamine induced lipolysis in the same cell type (49). Most studies have unveiled the role of AQP7 as a glycerol efflux channel during lipolysis (53–55) with AQP7 deficiency being associated with the onset of obesity (56,57) as a result of enhanced GlyK activity.

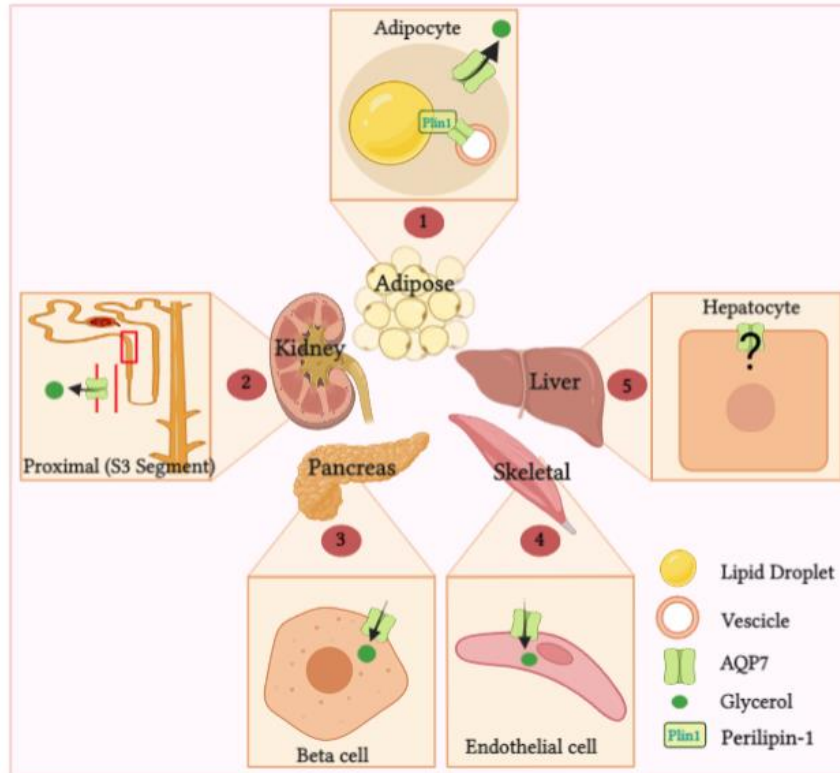
AQP7 in kidney is abundantly present in the apical plasma membrane of the S3 segment of the proximal tubule in mouse, human, rat (20,21,58,59). The physiological role of AQP7 in kidney is reported to be reabsorption of glycerol in the proximal tubule preventing the loss of glycerol in urine (60). Depletion of AQP7 in kidney could be expected to oppose the development of obesity by causing glycerouria (1).

AQP7 expression is reported in pancreatic islets of beta cells in mice (61) and rats (62,63). AQP7 was found to affect glycerol metabolism by increasing GlyK activity and eventually increasing TAG levels in the pancreas (61). AQP7 KO mice exhibited an accumulation of glycerol indicating that AQP7 facilitates glycerol release across the plasma membrane. However, it is yet unclear how this correlates with the reported intracellular localization of AQP7. AQP7 has also been shown to mediate a rapid uptake of glycerol and urea in the pancreatic beta cells. This resulted in cell swelling and triggered the release of insulin (63,64). Likewise, in pancreatic beta cells isolated from rats with T2D, metformin increased the AQP7 expression and promoted insulin secretion in parallel with an elevation of glycerol uptake by AQP7 (62). This implies that controlling AQP7 function in pancreatic cells may have significant effects on T2D management.

In skeletal muscle, AQP7 is localized in the capillary endothelium (21) and at the surface of myofiber (11). In obese T2D an increase in AQP7 expression in skeletal muscle has been reported (65). This increment of AQP7 leads to excessive lipid accumulation in skeletal muscle of obese T2D patients (65). This has led to the speculation that AQP7 provides the substrate for TAG synthesis by mediating the entry of glycerol. Further investigations are required to validate this hypothesis.

The physiological role of AQP7 in liver has not been determined so far as outlined above. One study has shown an association between AQP7 and lipid metabolism in liver (13). As stated earlier, the reduction of hepatic steatosis by estrogen via upregulating hepatic AQP7 expression

points AQP7 to influence TAG metabolism (13). Does this suggest that AQP7 could prevent hepatic steatosis by limiting glycerol availability as a substrate for TAG synthesis? In human liver from obese T2D patients, there is an increase in intrahepatic TAG content as well as AQP7 mRNA and protein expression when compared to obese normoglycemic and impaired glucose tolerance individuals (14). What is the role of increasing AQP7 expression in the liver of T2D obese subjects?



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Figure 1.4: Function of AQP7 in different metabolic tissues in terms of glycerol transport properties.

1 In adipocyte AQP7 facilitates the release of glycerol during lipolysis thereby reducing the availability of glycerol in the tissue. Intracellularly AQP7 binds to perilipin-1 (Plin1) during the feeding state. **2** AQP7 reabsorbs glycerol from the proximal tubule (S3 segment) and prevents urinary glycerol loss. **3** In pancreatic beta cells AQP7 has been shown to act as a glycerol influx channel. By exerting glycerol influx activity AQP7 causes beta cell swelling and eventually release of insulin. **4** AQP7 is speculated to mediate rapid entry of glycerol for providing substrate for TAG synthesis in skeletal muscle. **5** The role of AQP7 in hepatocytes is yet to be determined.

Expression and role of other AQPs in liver

AQP9 is the most significantly expressed glycerol channel among other glycerol channels in human liver (15). It is abundantly expressed in the basolateral plasma membrane of hepatocytes (66,67) and is also reported to be present in cholangiocytes (12). Intriguingly, marked AQP9 expression was found in the pericentral region of mice (68) and rat hepatocytes (69,70) compared to periportal hepatocytes. The plasma glycerol and TAG level were significantly increased in AQP9 deficient mice compared to wild type (WT) representing AQP9 as the glycerol import pathway in hepatocytes (22,68). Glycerol acts as a substrate for hepatic glucose production. Consistent with this fact, AQP9 KO mice displayed a reduction in hepatic glucose output in response to glycerol perfusion thereby suggesting the role of AQP9 to supply glycerol for gluconeogenesis (71). Interestingly, liver specific AQP9 was shown to be reduced in the

obese T2D subjects to improve hyperglycemic conditions by reducing the uptake of glycerol for gluconeogenesis substrate. Mouse and rat hepatic AQP9 expression was increased and decreased in response to fasting and feeding respectively (69,72). This implies that the supply of glycerol via AQP9 increases gluconeogenesis during the fasted state. In addition to controlling glycerol and glucose metabolism, AQP9 has also been reported to be involved in the water transport and formation of bile (73).

The expression of AQP10 in human hepatocytes is conflicting wherein one study detected mRNA expression (15) whereas another study did not find AQP10 expression in hepatocytes (12). AQP3 has been reported to be expressed in hepatocytes (15), hepatic stellate cells (16), and Kupffer cells (12). The subcellular localization and physiological meaning of the reported localization of AQP3 in hepatocytes are yet to be determined.

AQP8 in liver has a different location pattern. A previous study reported AQP8 to have a low plasma membrane and high intracellular vesicle distribution in rat hepatocytes under basal (unstimulated) conditions (74). Upon the stimulation, the intracellular vesicles bearing AQP8 were redistributed to the plasma membrane via the microtubule. Later another study identified the plasma membrane specifically to be the canalicular plasma membrane where it is ruled out to facilitate water transport for bile formation and regulation (75). Immunohistochemical studies in mouse hepatocytes showed AQP8 to have a periportal and mid-lobular expression pattern (76). On the contrary, rat hepatocytes demonstrated the distribution of AQP8 predominantly in the pericentral zone (75). Other studies have revealed that AQP8 also resides in the inner mitochondrial membrane (77) and smooth endoplasmic reticulum (76). However, the physiological meaning of its expression in these subcellular structures is not precisely known. This varied cytoplasmic localization of AQP8 has been linked with the presence of two different isoforms of AQP8 (78). It was found that both AQP8 and AQP9 were downregulated in extrahepatic cholestasis consequently decreasing canalicular and sinusoidal water permeability respectively during the bile formation (73,79). This conclusion reinforces the significance of coordinated regulation of hepatic AQP8 and AQP9 in bile formation and secretion.

2. Hypothesis and Objectives

So far, the observed results regarding the expression of AQP7 in murine liver tissue are contradictory and even though the studies in human liver agree with AQP7 being expressed in liver, then especially its subcellular localization needs further clarification. As outlined in the introduction, the limited investigations of the role of AQP7 expression in the liver do not fully agree with its reported localization in hepatocytes. Based on previous studies (12,14), we hypothesized 1) that AQP7 expression in human hepatocytes is found in intracellular domains and we expected HepG2 cells to have a similar expression pattern to that of human hepatocytes. In human primary adipocytes, AQP7 has been demonstrated to bind to Plin1 (8). In hepatocytes, the LD is surrounded mainly by Plin2 (32). Therefore, the present study hypothesized 2) that the intracellular expression of AQP7 would interact with Plin2. Finally, we hypothesized 3) that AQP7 plays a role in lipid storage in hepatocytes. Hence, the present thesis aimed to establish clarity about the presence of AQP7 in human liver and investigate the functional role of AQP7 in human hepatocytes.

The following aims were the objectives of this study.

1. To investigate the cellular and subcellular distribution of AQP7 in human liver by using well-validated immunohistochemistry antibodies.
2. To evaluate the expression of AQP7 in HepG2 and whether the subcellular expression of AQP7 in HepG2 cells is equivalent to the expression pattern of AQP7 in human hepatocytes.
3. To use a high fat diet model in HepG2 cells to explore the role of AQP7 in hepatocytes.

3. Materials and Methods

3.1 Subject

A liver biopsy was obtained from a human cadaver that was donated to the Department of Biomedicine, Aarhus University. Control human adipose tissue was obtained from the abdominal cavity during gastric bypass surgery as approved by the Danish Ethical stated in a previous study (51). Similarly, the tissue homogenate of a wild-type male mouse liver that was included as positive control was obtained from the samples obtained in a previous study (80).

3.2 Antibodies

AQP7 (8571) is an anti-human AQP7 rabbit polyclonal antibody raised against the peptide CANRSSVHPAPPLHESMALEHF. This antibody was previously validated against the C - terminus of human AQP7 for IHC and immunoblotting in human tissues (58). The following antibodies were used in paraffin-embedded liver tissue sections relative to positive control in referred to the earlier studies (51,81) before using them for immunofluorescence (IF) double labelling. These antibodies were chosen because they mark the different cellular and subcellular structures and were used previously (51,58,80,81) making them reliable markers.

Table 3.1: Overview of primary antibodies used in all the experimental procedures.

The table provides information about the name of the antibodies, the species they were produced in, what they were considered a marker of, source of purchase, application and dilution factor.

Antibody and its host	Marker of	Source	Application	Dilution
AQP7 Rabbit Polyclonal	AQP7	Lebeck, J., <i>et al.</i> (58)	IHC Peroxidase IHC fluorescence Immunoblot	1:25 1:25 1:400
Lysosomal-associated membrane protein 1 (Lamp1) Mouse Monoclonal	lysosome	Santa Cruz sc-17768	IHC Peroxidase IHC fluorescence	1:1000 1:1000
Plin2 Guinea pig Polyclonal	LD	Progen GP40	IHC Peroxidase IHC fluorescence Immunoblot	1:10 1:10 1:1000
Plin2 Mouse monoclonal	LD	Progen AP125	IHC Peroxidase IHC fluorescence	1:25 1:25
Dynactin p62 Mouse monoclonal	Dynactin	Santa Cruz sc-55603	IHC Peroxidase IHC Fluorescence Immunoblot	1:400 1:200 1:1000
Cluster of Differentiation 68 (CD68) Mouse monoclonal	Macrophage	Leica Biosystem (NCL-L- CD68)	IHC Peroxidase IHC Fluorescence	1:50 1:25
Cluster of Differentiation 163 (CD163) Mouse Monoclonal.	Macrophage	Abcam (10D6)	IHC Peroxidase IHC Fluorescence	1:50 1:25
Histone deacetylase 6 (HDAC6)	Aggresome	Santa Cruz sc-5258	IHC Peroxidase IHC Fluorescence	1:200 1:100

Goat Polyclonal				
Cell Death Inducing DFFA Like Effector C (CIDEK) Rabbit Monoclonal	LD	Novus Biologicals (NB100-430)	IHC Peroxidase IHC Fluorescence	1:100 1:50
Ras-related protein (Rab5) Rabbit Polyclonal	Early Endosome	Abcam ab109534	IHC Peroxidase IHC Fluorescence	1:100 1:50
GlyK Rabbit Monoclonal	Cytoplasm	Abcam 123ab126599	Immunoblot	1:1000
GPD1 Rabbit Polyclonal	Cytoplasm	Sigma Aldrich HPA044620	Immunoblot	1:1000

Secondary antibodies that were used for IHC peroxidase were Horseradish peroxidase-conjugated goat anti-rabbit IgG, Rabbit anti-goat IgG, Rabbit anti-guinea pig, Goat anti-mouse (Dako).

For fluorescence detection, donkey anti-rabbit – or mouse – or goat Alexa Fluor 488, 555, and Goat anti-Guinea pig 488 (Invitrogen) were used.

3.3 Immunohistochemistry

Immunoperoxidase and immunofluorescence double labelling were two main techniques in this study. Initially, immunoperoxidase was used to validate different cellular and subcellular markers with respect to positive control (see Figure S1 in Annex I) and to find the cellular localization of AQP7 in the human liver. Later immunofluorescence double labelling was applied to determine the subcellular localization of AQP7 in the human liver.

3.3.1 Tissue preparation and sectioning

The formalin-fixed paraffin-embedded tissue (FFPE) fixation method was used. Although formalin is a better fixative for retaining morphology, it interferes with IHC labelling by masking antigens and limiting antibody-target interaction. Liver biopsy was immersion fixed in 4% paraformaldehyde (PFA) in 0.01M Phosphate Buffered Saline (PBS) upon collection. The tissue section was dehydrated by full immersion in graded ethanol (70%, 96%, 99%) for 2 hours each followed by incubation in xylene overnight. The section was embedded in paraffin wax and subsequently, the paraffin-embedded tissue section was cut approximately into 2µm thick sections on a rotary microtome (Leica Microsystems GmbH, Wetzlar, Germany) and mounted on Super Frost slides. The covalent interactions between formalin-fixed tissue and the glass assist in attaching the tissue better with the usage of special adhesives. The section was then dried overnight at room temperature. These steps were performed by a lab technician.

3.3.2 Immunoperoxidase Staining

The prepared tissue sections were dewaxed by placing them in xylene overnight followed by rehydration in graded ethanol (99%, 96%). Endogenous peroxidase activity was blocked in 33% H₂O₂ in methanol to prevent false-positive staining. The sections were boiled in 1 mM Tris buffer, pH 9, 0.5 mM egtazic acid (EGTA) in the microwave to increase epitope availability and immunogenicity and then quenched in 50 mM ammonium chloride (NH₄Cl) in PBS. Successively, the sections were blocked with 1% Bovine Serum Albumin (BSA), 0.2% gel, 0.05% Saponin in PBS and were incubated with primary antibody diluted in 0.1% BSA, 0.3% Triton X-100 in PBS in the humidity chamber for 1 hr at room temperature and at 4°C overnight afterwards.

For light microscopy, sections were rinsed with 0.1% BSA, 0.2% gelatin, 0.05% Saponin in PBS prior to the incubation with horseradish peroxidase-conjugated secondary antibody in

0.1% BSA, 0.3% Triton X 100 in PBS. The sections were again rinsed with 0.1% BSA, 0.2% gel and 0.05% Saponin before being detected with 3,3-diaminobenzidine tetrahydrochloride (DAB) in PBS with 33% H₂O₂ for 10 minutes. The sections were counterstained with Mayer's Hematoxylin for 2-3 minutes, washed with running tap water then dehydrated with graded ethanol (70%, 96%, 99%) followed by xylene. The sections were finally mounted with coverslips using Eukitt (CellPath).

3.3.3 Immune cells blocking protocol

The protocol is the same as the immunoperoxidase staining above. Prior to incubating the sections with primary antibodies, the sections were incubated with 5% human serum in PBS. The sections were treated with the serum of the same species as the tissue host (Human) to block the binding of the antibody to Fcγ receptors present on the immune cells. Consequently, the primary antibody was diluted in 1% human serum to exclude unspecific binding of the primary antibody to the immune cells.

3.3.4 Immunofluorescence

For immunofluorescence double labelling, the endogenous peroxidase step was omitted. Two primary antibodies from different host species were mixed taking into account the individual desired concentration in the diluting buffer same as peroxidase staining above. The next day, after washing the sections, two fluorescence secondary antibodies corresponding to the host of primary antibodies were mixed along with Topro (Invitrogen) at the ratio of 1:1000. The sections were mounted with coverslips in hydrophilic mounting media called Glycergel Antifade Medium (Dako).

3.4 Imaging

For peroxidase and fluorescence imaging we used light and inverted confocal microscope respectively.

3.5 Light microscope

A Leica DM2500 light microscope with magnification: oil immersion objective lens, × 25, N.A.: 0.75, aberration correction: Fluotar, ×100, N.A.: 1.40, aberration correction: APO was used for brightfield imaging. Brightfield images were acquired using a Leica MC170 HD digital camera and the LAS V4.13 software.

3.6 Inverted Confocal Microscopy

A Leica DM IRE2 inverted confocal microscope with a Leica TCS SP2 laser module with magnification: ×63, N.A.: 1.32, aberration correction: APO, For image acquisition, Leica Confocal Software 2.61 was used. Fluorescence images were captured when the most intense signal reached to few saturated pixels with an image average of six frames and an 8-bit image depth, 1024x1024-pixel resolution. Fiji ImageJ was used for image processing and merging confocal images. The resolution of an optical system is one of the limiting factors to determine the co-localization or molecular interactions between two proteins. Practically, the average resolution of a confocal microscope is 2-500 nm.

3.7 Cell Culture

The HepG2 cells was chosen because some studies support the expression of AQP7 by HepG2 cells (13,16,82). The cryogenic vial of HepG2 cells was removed from the liquid nitrogen tank. The cells were thawed quickly for a short time by immersing the vial in the cold-water bath to avoid the ice shards capable of damaging the cells. The solutions were prewarmed in a water bath prior to the preparation of the culture medium. Culture medium was essentially prepared using 50 ml of 10% Fetal Bovine Serum (Invitrogen), 5 ml of 2 mM L-glutamine (Invitrogen), 5 ml of 1% Non-essential amino acid (Sigma) and 450 ml of Minimum Essential Medium Eagle (Sigma), 5 ml of 50 units/ml Penicillin (Sigma).

After thawing the cells from the vial were transferred into a sterile 15 ml conical tube containing 10 ml of prewarmed culture medium using a sterile pipette inside the hood. The vial containing cells was rinsed with 2 ml of the medium to recover any remaining cells. For this, the medium was gently pipetted up and down a couple of times and was transferred to the 15 ml conical tube. After that, the cell suspension was centrifuged for 2 minutes at 201 x g in order to remove the cryopreservation medium. The medium supernatant was discarded, and the cell pellets were resuspended in 5 ml of culture medium by gently agitating the suspension 2-3 times. The cell suspension was then transferred to a T25 cell culture flask and was gently rotated for an even dispersion of the cells over the surface. The flask was then placed in an incubator at 37°C and either 5% CO₂ or 7% CO₂, with 95% humidity. The medium was changed every 2 days by aspirating culture supernatant and adding fresh media.

The cells reached their confluence after 1 week of plating. After the cells reached confluence, it was split by trypsinization. Prior to trypsinization, the culture medium was aspirated. Initially, 2.5 ml of 0.25% trypsin was added. The flask was gently rotated to cover the cells' monolayer and was discarded right away. Subsequently, the cells were digested in 2.5 ml of trypsin and incubated in the incubator for approximately 3-4 minutes. The adhered cells got detached from the surface of the flask. The side of the flask was gently tapped to detach the cells completely. 3 ml of prewarmed culture medium was added to the flask to prevent the activity of the trypsin. For the determination of cell viability, 100 µl of cell suspension was pipetted in the centrifuge tube where 100 µl of lysis buffer (Chemometec) was added and vortexed followed by the addition of 100 µl stabilizing buffer (Chemometec) and mixed. Then the number of cells was counted using a hemocytometer. The cells were seeded at about 5×10^5 cells/cm² and 2.5×10^5 cells/cm² into T25 cell culture flasks and in the six-well plate respectively. The medium was replaced by a fresh medium until the cells reached their confluence.

3.7.1 Treatment of HepG2 cells with FFAs

The “high-fat diet (HFD)” model of HepG2 cells was prepared to analyse if AQP7 plays the role in TAG storage in hepatocytes.

Control, 0.5 mM and 1 mM of FFAs media was prepared. We had a stock solution of 30 mM concentration of mixtures of oleic acid and palmitic acid in the ratio of 2:1. The control medium was prepared by mixing the standard medium used to grow HepG2 cells as mentioned above and 0.02% BSA. 1 mM medium was prepared by mixing control medium and a certain volume of 30 mM stock solution of FFAs. And eventually, 0.5 mM medium was prepared by mixing a certain volume of control and 1mM media. All three media were filtered prior to their use.

The concentration of 0.5 mM and 1 mM FFA are regarded as the best ratio to induce lipid accumulation without causing lipotoxicity according to a previous study (83). The accumulation of LDs was monitored under the microscope. The 6 or 12 well plates containing cells were incubated with their respective medium for 24 hours. After that, the samples were prepared for either immunocytochemistry (ICC), qPCR or immunoblot.

3.8 Immunocytochemistry

To examine if AQP7 is present and has the same subcellular localization in HepG2 cells as that of human liver tissue, ICC was performed.

3.8.1 Sample preparation for ICC

The culture medium was aspirated. Cells were rinsed twice with 0.01 mM PBS. The cells were then fixed with 4% PFA in a 12-well plate for 15 minutes. After that, cells were shortly rinsed again with 0.01 mM PBS 3 times prior to permeabilization. The wells containing cells were incubated with 0.2% saponin for 10 minutes. Thereafter it was washed with 0.01 mM PBS. The cells were then incubated with a mixture of 10% fetal calf serum, 0.1% BSA, 0.05% saponin in 0.01 mM PBS for 30 minutes followed by 1% BSA, 0.2% fish gelatin, 0.05%

saponin, 0.05M glycine in 0.01 mM PBS for 30 minutes again. This step was to block the cells toward the non-specific binding of antibodies. The cells were then incubated with primary antibodies in 0.5% BSA, 0.05% saponin in 0.01 mM PBS in a humidity chamber at room temperature for 30 minutes followed by 4°C overnight afterward. For double labelling, two primary antibodies were mixed together taking the individual dilutions in account.

For immunofluorescence, the cells were washed with 0.5% BSA, 0.05% saponin in PBS at room temperature thrice for 5 minutes each. Then it was incubated with Alexa secondary antibody (which is the same as the description in IHC above) diluted in 0.5% BSA, 0.05% saponin in PBS for 1 hour at room temperature in a humidity chamber. In the case of double labelling, two secondary antibodies were mixed in diluting buffer at the ratio of 1:1000. Cells were washed again with 0.01 mM PBS 3 times and counterstained with Topro for the visualization of the nucleus. Cells were washed with 0.01 mM PBS again and were mounted in DAKO's medium for fluorescent staining.

3.9 Quantitative Real-Time PCR

To confirm if the AQP7 gene is expressed in HepG2 cells, agarose gel electrophoresis was performed. qRT-PCR was also applied to see the effect of FFA on the gene expression of AQP7. It is to note that qRT-PCR experiment was performed by a lab technician, however, the samples were prepared by me and I followed most of the experiment steps. Also, the primers used here had previously been designed.

3.9.1 Sample preparation for PCR

The medium was removed from the wells. 250 µl of trizol reagent (Invitrogen) were added and the cells were scraped. The cell suspension was then transferred to the Eppendorf tube.

3.9.2 Extraction of RNA, Reverse Transcription and Quantitative PCR

300 µl of chloroform was added to the homogenate. It was used to aid phase separation allowing RNA to be separated from DNA and proteins. The mixture was vortexed for approximately 15 seconds prior to incubating at room temperature for 5 minutes. The mixture was then centrifuged at 14,000g for 15 minutes at 4°C. This step separates the upper aqueous phase containing RNA from the lower organic and the interphase containing proteins and DNA. 100 µl of the aqueous phase was transferred to a new centrifuge tube and 50 µl of 100% ethanol was added. The mixture was immediately vortexed to avoid any kind of RNA precipitation. The samples were transferred to a new tube with filter cartridges and were centrifuged at 12,000g for approximately 1 minute. The flow-through was discarded whereas the filter cartridge where RNA was bound was returned to the same collection tube. 500 µl wash solution (Invitrogen) was applied to the filter cartridge-collection tube assembly and was centrifuged again for 1 minute. The flow-through was discarded again. The steps with wash solution were repeated twice. The collection tubes with filter cartridges were centrifuged for 1 minute without wash solution to remove residual wash solution. The filter cartridge was then transferred to a new collection tube and any contact between the filter cartridge and flow-through was avoided to prevent carryover of ethanol to the eluted RNA and potential inhibition of downstream enzymatic reactions. 50 µl of elution buffer (Invitrogen) was added to the filter cartridge and was incubated at room temperature for 2 minutes. It was then centrifuged for 1 minute to elute the RNA from the filter. The recovered RNA was placed on the ice and stored at -80 °C until use.

The concentration of RNA was determined by using the NanoPhotometer (IMPLEN) by measuring its absorbance at 260 nm. All the required kits were used according to the manufacturer's instructions. The RNA was treated with DNase I (Invitrogen) in DNase reaction buffer (Invitrogen) and, Dimethyl Dicarboxylate (DMDC) water for 15 minutes at room temperature. 2µl of EDTA (Invitrogen) was added to the mixture to deactivate DNase activity in order to prevent RNA degradation and the mixture was heated for 10 minutes at 65°C on a

Thermal Cycler Block (Thermo Fisher Scientific). The treatment with DNase was required to digest single and double stranded DNA resulting in purified RNA.

224 ng RNA was then reverse transcribed to cDNA using the SuperScript™ II Reverse Transcriptase kit (Invitrogen). 2µl of first random hexamer primers (Invitrogen) and 2µl of DeoxyriboNucleotide TriPhosphate (dNTP) (GenScript) were mixed with 224 ng extracted RNA. Random hexamer primers unbiasedly bind to the whole RNA sequence to generate cDNA and thereby increase the likelihood of sample amplification during PCR. dNTP helps to extend the strand of DNA by supplying individual DNA bases. The mixtures were heated at 65°C on a Thermal Cycler Block for 5 minutes and afterward, the tubes were put on ice where first strand buffer (Invitrogen) and dithiothreitol (DTT) were added along with diethylpyrocarbonate (DEPC) water. The samples were then heated at 42°C for 2 minutes and were then split into positive and negative control PCR tubes. SuperScript II reverse transcriptase (Invitrogen) was added in the positive PCR tube whereas the reverse transcriptase enzyme was excluded in the negative control to exclude genomic DNA contamination. The samples were then heated at 42°C for 50 minutes followed by 70°C for 10 minutes.

PCR

cDNA was mixed with DEPC water and Hot FIREPol Blend Master Mix (Solis Biodyne) that contains a modified DNA polymerase, and 2 sets of forward and reverse primers designed to amplify human AQP7 transcript were used separately (5'- ACTGCAGAGGAAGATGGTGC -3', 5'- TCCGAAGCCAAAACCCAAGT-3') and (5'-CAAAATGGTCTCCTGGTCCG-3' and 5'-GACTCCGAAGCCAAAACCC-3') with expected product sizes mentioned in Table 2 below. Thermal Cycler Block performed 30 cycles. Elongation was carried out at 72°C; annealing to primers was done at 60°C, and denaturation was done at 95°C. The PCR products were separated by 1% agarose gel electrophoresis with ethidium bromide and were run for 30 minutes at 120V. The PCR products were detected with UV light using an ultraviolet transilluminator.

qRT-PCR

Primer pairs utilized are shown in the table below. 18s RNA was used as the housekeeping gene. Amplification was performed using 4 µl of cDNA, 10 pmol of 0.5µl of each primer, and 5µl of the LightCycler 480 SYBR Green I Master (Roche Applied Science). Cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 62°C for 20 s, and 72°C for 30 s. The RT-qPCR reactions were run on a LightCycler 480 (Roche), with fluorescence measured at the end of each elongation step to determine Ct values. Relative quantitation of gene expression was determined using the delta-delta Ct method.

Table 3.2: Overview of primer pairs utilized in RT-PCR and RT-qPCR.

The table provides information about the name of the gene, primer pairs utilized and the expected product sizes of those that were used in RT-PCR.

Name of Gene	Primer Pairs	Expected Product Size
Human AQP7	(5'-CAAAATGGTCTCCTGGTCCG-3', 5'-GACTCCGAAGCCAAAACCC-3')	193 bp
	(5'-ACTGCAGAGGAAGATGGTGC-3', 5'- TCCGAAGCCAAAACCCAAGT-3')	148 bp
GPD1	(5'-CACGGGTGACCATGTGGGTAT-3' 5'- GGGGCACCACAAAGATCAGGA-3')	
18s RNA	(5'-GGATCCATTGGAGGGCAAGT-3, 5- ACGAGCTTTTAACTGCAGCAA-3)	

3.10 Immunoblot

This technique was used to analyse if the AQP7 protein was detectable in HepG2 cells and also to analyse the effect of FFA treatment on the protein expression of AQP7 and other markers.

3.10.1 Sample preparation for immunoblotting

After the cells reach 80%-100% confluency in 12 well plates, the medium was removed, and the cells were rinsed with 0.01 M PBS twice. The cells were then scraped in 200 µl sample buffer. Sample buffer was prepared by mixing 4x sample buffer (62 mM Tris (hydroxymethyl)-aminomethane, 0.1 M Sodium dodecyl Sulphate (SDS), 8.7% glycerol, Bromophenol blue, 0.04 M DTT in milliQ water, pH 6.8) with dissection buffer (0.3M sucrose, 25mM Imidazol, 1mM EDTA, pH 7.2) at a ratio of 1:3. The cells suspension was collected in an Eppendorf tube. The samples were then sonicated 3 times with 5 pulses each time and heated at 65°C for 15 minutes afterward to facilitate denaturation and linearization of proteins.

3.10.2 Gel Electrophoresis

The samples containing proteins from HepG2 cells were prewarmed in a heat block at 37°C for 5 minutes. Electrophoresis buffer was prepared and filled in the electrophoresis chamber. The samples were loaded into AnykD Criterion TGX Precast Midi Protein gel (Bio-Rad) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) consecutively at 200V. This allowed linearized proteins in the samples to migrate through the gel in a size-dependent manner.

Polyvinylidene fluoride (PVDF) (Bio-Rad) membranes were soaked in 100% methanol for 30 seconds prior to equilibration in the transfer buffer. PVDF membranes are very hydrophobic, which means they are difficult to combine with standard aqueous transfer buffers. This would hinder buffer flow and protein binding in the membrane during membrane transfer. Therefore, to activate and promote the transfer of proteins, it was preincubated with 100% methanol.

The membrane and filter pads were then transferred to transfer buffer and incubated for 3 minutes. The membrane was not allowed to dry after it was hydrated with the buffer otherwise dry spots would form and proteins would not bind to the dry membrane. For the transfer of protein, a Trans-Blot Turbo transfer system (Bio-Rad) was used. At first, one of the wetted filter pads was placed on the bottom of the cassette. Any wrinkles on the stack were avoided by rolling the stack with the blotting roller. The membrane was placed on top, and the gel was placed on the membrane. The blot roller was used to avoid air bubbles between the membrane and the gel. Finally, the second wetted filter pad was placed on top of the gel and the sandwich was rolled again to expel trapped air bubbles. The Trans-Blot Turbo transfer system (Bio-Rad) was run to transfer the proteins from the gel to the PVDF membrane.

After the transfer of proteins, the membrane was blocked with 5% skimmed milk to avoid any non-specific binding of antibody. After a washing step, primary antibodies of interest were then incubated overnight followed by incubation with horseradish peroxidase enzyme-conjugated secondary antibody. The protein of interest was then determined by using Enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences). The ECL consists of two types of substrates namely peroxide and luminol enhancer solution. Upon the reaction of these substrates with horseradish peroxidase enzyme bound via the secondary antibody to the membrane, resulting in the emission of fluorescent light facilitating the detection of antigen when exposed to x-ray film on Image Quant LAS 4000 Luminescent Image Analyser (GE Healthcare).

In my case, I did not perform the Coomassie staining of my samples. We assumed that each sample consist of an equal amount of proteins. Nevertheless, it would be better to quantify the protein in the future. The band was quantified within the linear range by ImageJ software after subtracting the background. The value of each band was normalized to the housekeeping protein, beta-actin.

3.11 Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). GraphPad Prism 8.0.2 is used for graphical representation and statistical analysis. One Way Anova was used in most of the result analysis as there were more than 2 experimental groups. It is the statistical analysis that determines the significant differences in the mean between more than 2 groups and uses one independent variable. For instance, in this study, the effect of FFA treatment was analysed (Control, 0.5 mM and 1 mM FFA) on the expression of the protein.

4. Results

4.1 Cellular and subcellular localization of AQP7 in liver

In order to evaluate the cellular and subcellular expression of AQP7 in human liver I performed IHC using a previously characterized antibody designed against the c-terminal of human AQP7. Immunoperoxidase labelling for AQP7 in human liver shows labelling for AQP7 in hepatocytes surrounding the central vein (CV) (Figure 4.1, **a**). At higher magnification, the labelling for AQP7 is found in the cytoplasmic domain of the hepatocytes (Figure 4.1, **b**). This pattern was not observed in the hepatocytes around the portal vein (PV) (Figure 4.1, **a, c**). The immunolabelling for AQP7 was also found in the apical plasma membrane domain of the cholangiocytes lining the bile duct (BD) (Figure 4.1, **c**) as indicated by arrows. In addition, labelling for AQP7 was found in sinusoidal cells (Figure 4.1, **b, c**) as indicated by arrowheads. Labelling for AQP7 in human adipose tissue was included as a positive control and as previously reported labelling for AQP7 was found predominantly in the capillary endothelial cells (Figure 4.1, **d**) (51).

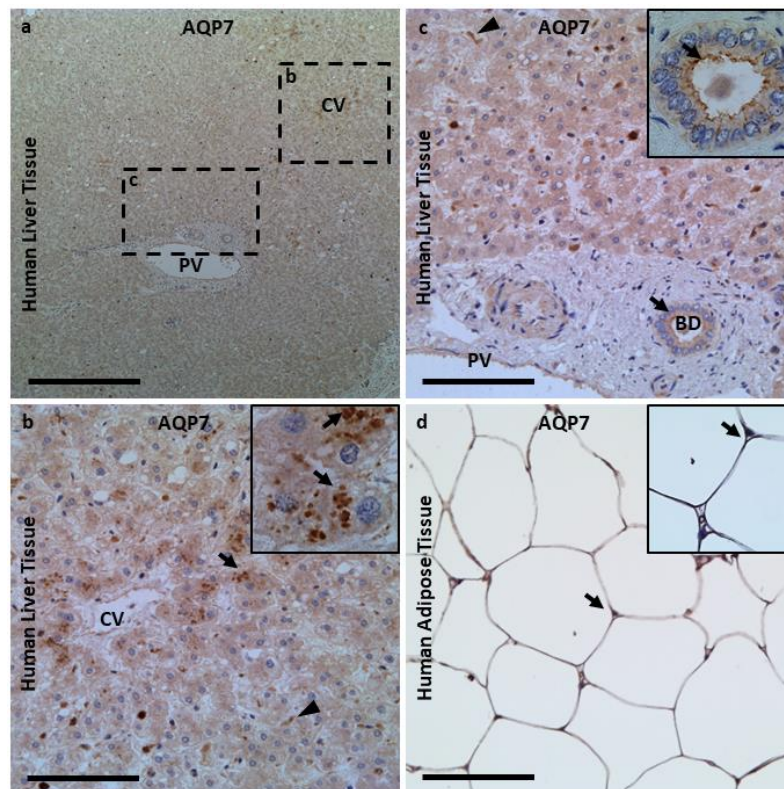


Figure 4.1: Immunoperoxidase imaging of AQP7 expression in human liver tissue.

a labelling for AQP7 in human liver tissue at low magnification (5x). Scale bar indicates 500 μm. **b** labelling for AQP7 in hepatocytes surrounding the central vein at 25x magnification. **c** labelling for AQP7 in the apical plasma membrane domain of cholangiocytes indicated by arrow at 25x magnification. **b, c** labelling for AQP7 in sinusoidal cells indicated by arrowheads. **d** labelling for AQP7 in the capillary endothelial cells as well as in the adipocytes in human adipose tissue as a positive control. Arrows indicate labelling for AQP7. Insets in **b** and **c** represent higher magnification (100x). Scale bars indicate 100 μm.

Immunofluorescence imaging was used to investigate the subcellular localization of AQP7 in human liver tissue. Double labelling with the lysosomal marker Lamp1 and AQP7 demonstrated no overlap between the labelling for AQP7 and Lamp1 as illustrated in Figure

4.2, **a and b**. Instead, an almost complete overlap was seen between labelling for AQP7 and dynactin with the yellow color indicating co-localization between the two proteins at the pixel level (Figure 4.2, **c**). Dynactin is a protein complex that plays a role in linking the microtubule motor protein dynein with membrane vesicles and assists dynein in the trafficking of the cargo along the microtubule (84). Due to the resolution of fluorescence imaging the overlap of fluorescence signals does not necessarily imply an interaction between two proteins, however it does indicate that they are associated with the same structures (85). Nonetheless, the frequent overlap between AQP7 and dynactin in several regions within a cell augments the probability that they are associated with the same subcellular structures.

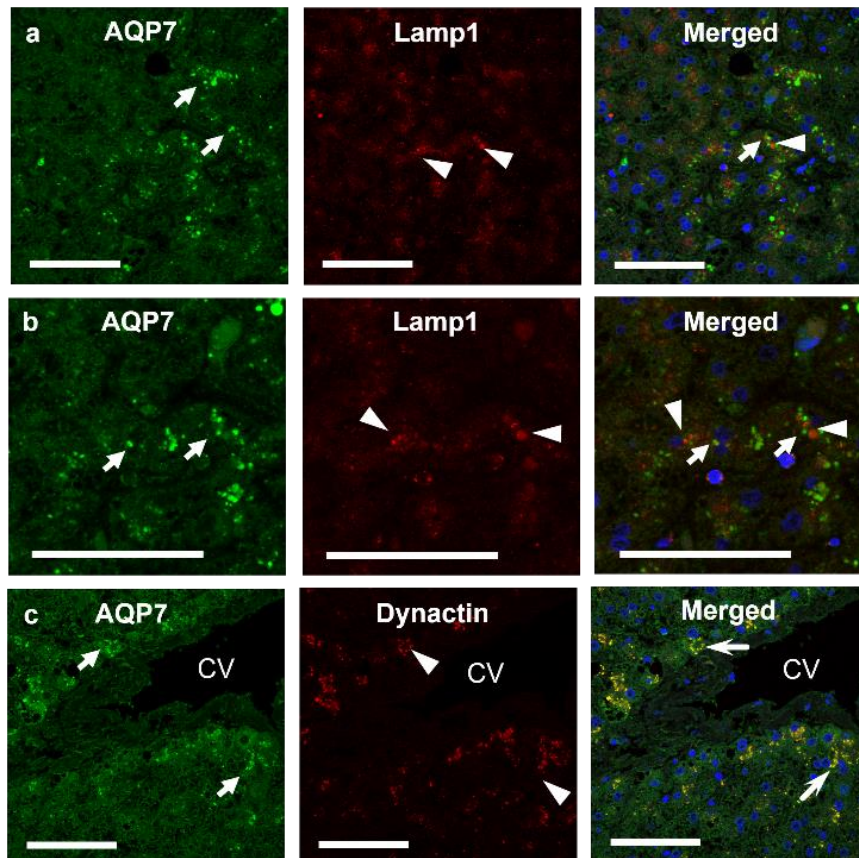


Figure 4.2: Immunofluorescence imaging of AQP7 in human liver tissue.

a Immunofluorescence labelling for AQP7 (Green) and Lamp1 (Red) and the merged image in human liver tissue (63x). Arrows indicate labelling for AQP7. Arrowheads indicate labelling for Lamp1 in lysosomes. **b** Immunofluorescence labelling for AQP7 and Lamp1 and merged image in human liver tissue at high magnification (63x2). **c** Immunofluorescence labelling for AQP7 (Green) and Dynactin (Red) and merged image in human liver tissue (63x). Arrows indicate labelling for AQP7 in the liver. Arrowheads indicate labelling for dynactin. Long arrows indicate overlap between AQP7 and dynactin labelling in the merged image. Scale bars indicate 50 μm .

Labelling for Plin2, a marker of LDs, is as illustrated in Figure 4.3, **a, b, c, d and e** identified in the liver. We used two different Plin2 markers namely Plin2 (GP40) (Figure 4.3, **a, b, d, e**) produced in guinea pig and Plin2 (AP125) (Figure 4.3, **c**) produced in mouse. The distribution of labelling for AQP7 was heterogeneous, with some regions exhibiting greater overlap (greater density of yellow pixels) with labelling for Plin2 (GP40) than others (Figure 4.3, **a, b**). This is most obvious at higher magnification (Figure. 4.3, **b**). The co-localization had a dot-like pattern in clusters as indicated by the yellow color in some regions of hepatocytes (Figure 4.3, **a, b**).

However, some of the labelling for AQP7 did not overlap with Plin2 labelling (Figure 4.3, **b**). Interestingly, labelling for Plin2 (AP125) showed no overlapping with AQP7 at all.

Furthermore, to investigate which of these LD markers is the legit one, we performed co-labelling of AQP7 with another LD marker; CIDEA. CIDEA is known to be present on LD surface (86,87) and also to be expressed in hepatocytes (87). CIDEA has been demonstrated to co-localize with perilipins and promote lipid accumulation (86). With this in mind, we expected that if AQP7 co-localizes with Plin2, then we would expect AQP7 to also co-localize with CIDEA. In the event of a positive result, we would assume Plin2 (GP40) to be a true marker rather than Plin2 (AP125). The merged image showed almost complete co-localization between AQP7 and CIDEA. However, the overlap between labelling for AQP7 and CIDEA was also found in immune cells. We did not expect labelling for CIDEA in immune cells and with both the AQP7 and CIDEA antibody being produced in rabbit, we cannot rule out that the observed co-localization is not due to the secondary antibody used against CIDEA also binding to the antibody against AQP7. Thus, the complete overlapping was interpreted as being a false positive staining. However, for further experiments, we proceeded with Plin2 (GP40).

See Figure S2a in Annex I.

The labelling for Plin2 (GP40) and dynactin both showed overlap with the labelling for AQP7 above. Since dynactin is binding a lot of different vesicles to the microtubule, then it was expected that a lot of the staining for dynactin would not co-localize with Plin2. However, the merged images (Figure 4.3, **d, e**) both at lower and higher magnification seem to be a complete overlap between dynactin and Plin2.

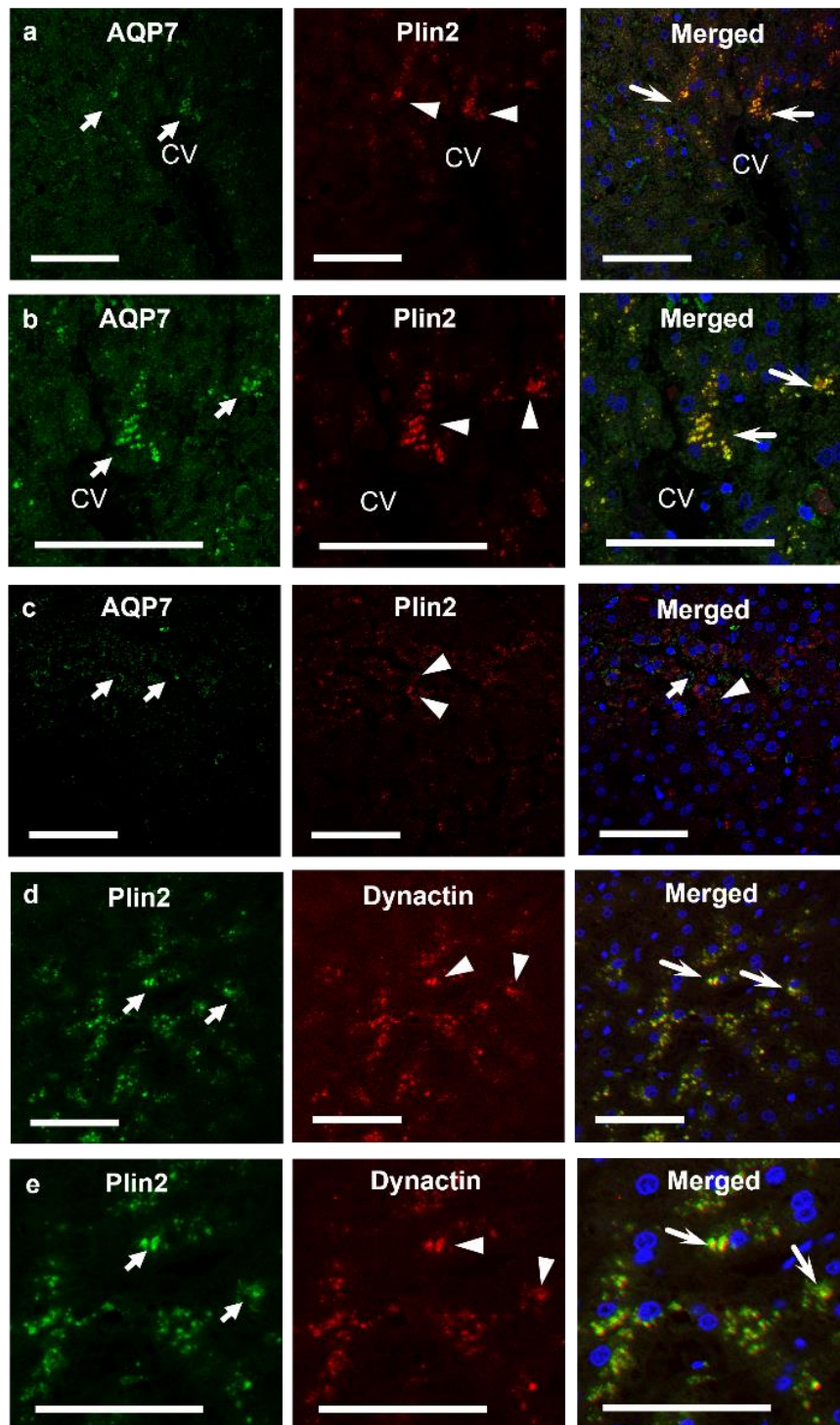


Figure 4.3: Immunofluorescence imaging of AQP7 & Plin2 in human liver tissue. **a** Immunofluorescence labelling for AQP7 (Green) and Plin2 (GP40) (Red) and the merged image in human liver tissue (63x). **b** Immunofluorescence labelling for AQP7 and Plin2 GP40 and merged image in human liver tissue at high magnification (63x2). Arrows indicate labelling for AQP7. Arrowheads indicate labelling for Plin2 (GP40). Long arrows indicate co-localization between labelling for AQP7 and Plin2 (GP40) in the merged images. **c** Immunofluorescence labelling for AQP7 (Green) and Plin2 (AP125) (Red) and merged image of human liver tissue (63x). **d** Immunofluorescence labelling for Plin2 (Green) and Dynactin (Red) and the merged image in human liver tissue (63x). **e** Immunofluorescence labelling for Plin2 and dynactin and merged image in human liver tissue at high magnification (63x2). Arrows indicate labelling for Plin2. Arrowheads indicate labelling for Dynactin. Long arrows

indicate co-localization of Plin2 and dynactin at pixel level in the merged images. Scale bars indicate 50 μm .

Initially, dynactin was used as an aggresomal marker (81). Since the labelling for dynactin demonstrates a high degree of overlap with the labelling for AQP7 in perivenous hepatocytes, we further investigated if labelling for AQP7 would be associated with other aggresomal markers. HDAC6 is an enzyme that is predominantly distributed in the cytoplasm (88). HDAC6 is reported as an aggresomal marker in some studies (81,89). For this, double labelling for HDAC6 and AQP7 was performed. An overlap in the labelling for the two proteins was observed especially in perivenous hepatocytes (Figure 4.4, **a, b**).

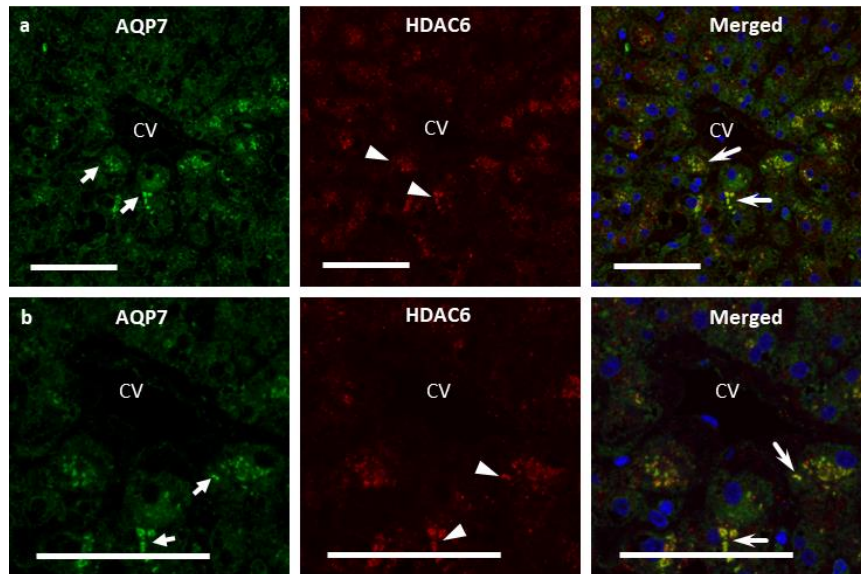


Figure 4.4: Immunofluorescence imaging of AQP7 & HDAC6 in human liver tissue.

a Immunofluorescence labelling for AQP7 (Green) and HDAC6 (Red) and the merged image in human liver tissue (63x). **b** Immunofluorescence labelling for AQP7 and HDAC6 and merged image in human liver tissue at high magnification (63x2). Arrows indicate labelling for AQP7. Arrowheads indicate labelling for HDAC6 in the cytoplasm. Long arrows indicate an overlay of fluorescence signal between AQP7 and HDAC6 in the merged images. Scale bars indicate 50 μm .

Moreover, immunofluorescence double staining of AQP7 was also performed with Rab5 which marks endosomes. The signalling pattern of both the antibodies was utterly identical. Also, both antibodies were from the same host species. Hence due to the same reasons as for CIDEC I am concerned that the overlap is not true.

See Figure S3a in Annex I.

We next evaluated the expression of AQP7 in Kupffer cells. It appeared that labelling for AQP7 in the sinusoidal space was confined to certain cells (Figure 4.5, **a, b**). CD163, a membrane marker of macrophages visualized several elongated cells (Figure 4.5, **a**). Strong AQP7 labelling was found in only some of the CD163 positive cells (Figure 4.5, **a**).

To further examine the cellular localization of AQP7 in Kupffer cells, double labelling for AQP7 was also performed with CD68. CD68 is a marker associated with lysosomes in macrophages. AQP7 and CD68 displayed a similar pattern like that observed with labelling for CD163 as illustrated in Figure 4.5, **b**.

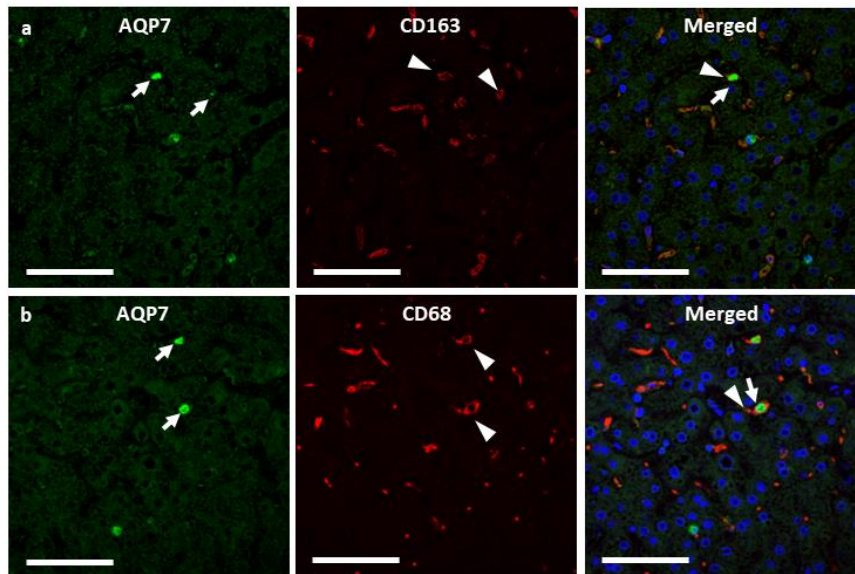


Figure 4.5: Immunofluorescence imaging of AQP7 in human liver tissue.

a Immunofluorescence labelling for AQP7 (Green) and CD163 (Red) and the merged image in human liver tissue (63x). Arrows indicate labelling for AQP7. Arrowheads indicate labelling for CD163 in kupffer cells. **b** Immunofluorescence labelling for AQP7 (Green) and CD68 (Red) and merged image in human liver tissue (63x). Arrows indicate labelling for AQP7 in liver. Arrowheads indicate labelling for CD68 in Kupffer cells. Scale bars indicate 50 μ m.

A monocyte is an immune cell that expresses CD163 and CD68. Immune cells like monocytes exhibit the Fc receptor at the cell surface that binds to other antibodies via its Fc domain. A human serum blocks the Fc domain of the immune cells, ensuring that only antigen-specific binding takes place and prevents false-positive results. We performed, an immune cell blocking experiment by using human serum as previously described (90) in human liver tissue to further confirm if AQP7 expression is present in Kupffer cells. The labelling for AQP7 in Kupffer cells was seen in control (Figure 4.6, **a**) and serum treated sections (Figure 4.6, **b**) of the human liver as indicated by arrows. The staining of immune cells did not fully disappear even after treating the section with human serum as illustrated in Figure 4.6, **b** indicated by arrowheads. AQP7 in human adipose tissues with or without human serum (Figure 4.6, **c, d**) and dynactin in human liver tissues with or without human serum (Figure 4.6, **e, f**) act as positive controls. While the labelling for AQP7 in hepatocytes seemed to be reduced, no change in AQP7 labelling was observed in Kupffer cells in serum treated liver section (Figure 4.6, **b**) relative to control human liver section (Figure 4.6, **a**). On the contrary, the labelling for AQP7 appeared to be enhanced in serum treated human adipose tissue (Figure 4.6, **d**) compared to control adipose tissue (Figure 4.6, **c**). However, the labelling for dynactin remained the same in both control (Figure 4.6, **e**) and serum treated (Figure 4.6, **f**) liver sections.

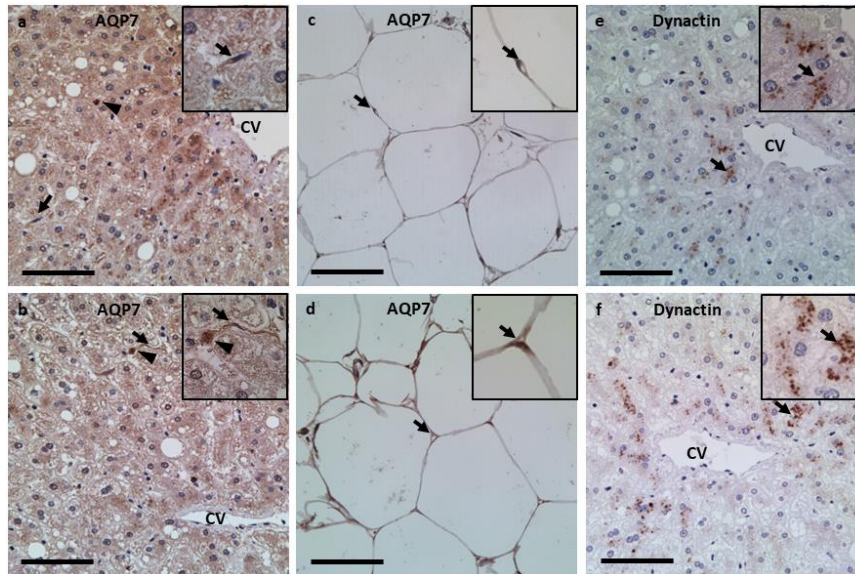


Figure 4.6: Immunoperoxidase imaging of AQP7 & dynactin in human liver tissue and human adipose tissue.

a labelling for AQP7 with the standard immunohistochemical protocol (control) in the human liver tissue. **b** labelling for AQP7 after an additional step of blocking human Fc-receptor using 1% human serum (blocking) in the human liver tissue. Arrows indicate labelling for AQP7 in Kupffer cells. Arrowheads indicate labelling for AQP7 in immune cells. **c, d** labelling for AQP7 in control and serum treated human adipose tissues. **e, f** labelling for dynactin in control and serum treated human liver tissues. Arrows indicate labelling for dynactin. Insets represent higher magnification (100x). Scale bars indicate 100 µm. CV- central vein.

4.2 Gene and protein expression of AQP7 in HepG2 cells

In present study, AQP7 was reported to be expressed abundantly in the perivenous hepatocytes where the hepatocytes are exposed to higher concentration of CO₂ (Figure 4.1, **a, b**). Therefore, we analysed the presence of the AQP7 gene in HepG2 cells grown with 5% and 7% CO₂. The presence of AQP7 mRNA was confirmed by PCR using 2 different AQP7 primer pairs. Human adipose tissue was included as a positive control. Both sets of AQP7 primers produced a strong band with the expected band size (148bp and 193bp respectively) (Figure 4.7, **a, b**). However, additional weak bands with a size close to 300 bp were also seen with both sets of primers in both samples from HepG2 cells and adipose tissue samples (Figure 4.7, **a, b**) probably indicating unspecific products. No PCR product was seen in negative control samples suggesting that the samples were free from nuclear DNA contamination.

Immunoblot analysis showed a lot of unspecific bands in HepG2 cells with the AQP7 antibody (Figure 4.7, **c**). It was difficult to pick a band corresponding to AQP7. However, the human adipose tissue homogenate which was included as positive control showed one specific band at ~25kDa. Therefore, the band for AQP7 in HepG2 cells was chosen as the band found in parallel to the band observed in human adipose tissue.

In addition, the expression for AQP7 protein in HepG2 cells grown at 5% CO₂ compared to 7% CO₂ (Figure 4.7, **d, e, f, g**) was evaluated by ICC. The staining with the anti-AQP7 antibody was heterogeneous in HepG2 cells under both conditions. No distinct change in signalling intensity was observed between 5% and 7% CO₂.

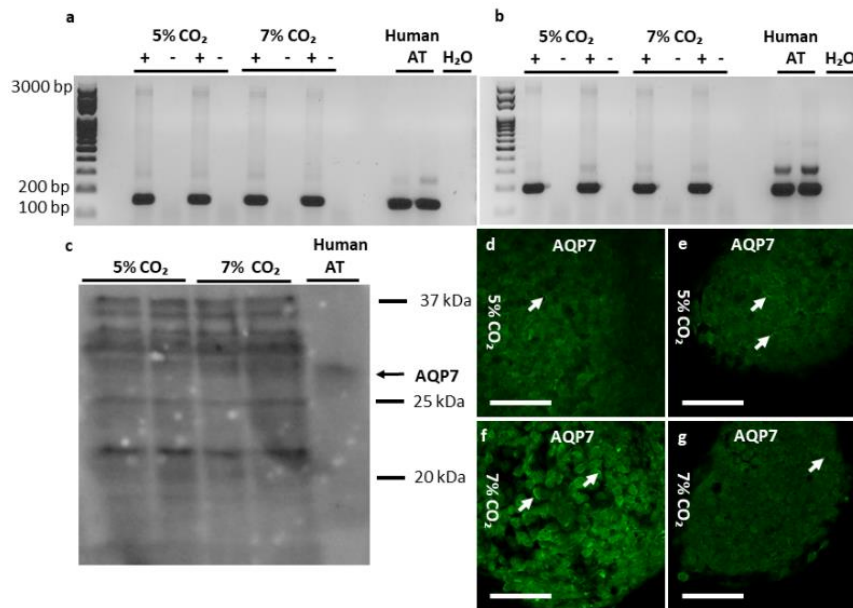


Figure 4.7: Gene & protein expression of AQP7 in HepG2 cells.

a, b PCR for mRNA encoding AQP7 in HepG2 cells using 2 different primer sets for AQP7. 1% gel electrophoresis of AQP7 mRNA in HepG2 cells grown with 5% and 7% CO₂. + and – denote the presence and absence of reverse transcriptase in the RT reaction, respectively. Human adipose tissue (AT) was included as a positive control as indicated. **c** Immunoblot for AQP7 in HepG2 cells grown with 5% or 7% CO₂. Human adipose tissue (AT) was included as a positive control as indicated. Arrows indicate the band for AQP7. **d, e, f, g** Immunofluorescence imaging of AQP7 labelling in HepG2 cells grown with 5% and 7% CO₂. **d, e** labelling for AQP7 in HepG2 cells grown with 5% CO₂ (63x). **f, g** labelling for AQP7 in HepG2 cells grown with 7% CO₂ (63x). Scale bars indicate 50 μm.

4.3 Subcellular localization of AQP7 in HepG2 cells

The subcellular localization of AQP7 in HepG2 was anticipated to resemble the localization to that of human hepatocytes. The initial labelling for AQP7 in HepG2 cells demonstrated a rim-like structure in some of the cells, indicating that it is localized within the plasma membrane domain of the hepatocytes (Figure S4, **a, b** in Annex I). This is unlike the expression pattern observed in human liver tissue (Figure 4.1, **a, b**). However, after adjusting the concentration of AQP7 antibody, labelling was present in intracellular domains in the HepG2 hepatocytes (Figure S4, **c, d** in Annex I). To analyse if AQP7 labelling would co-localize with labelling for Plin2 (GP40) and dynactin like that observed in human liver tissue, double labelling of AQP7 along with these two markers was performed. Unfortunately, the signalling for dynactin was not strong enough to make conclusions. However, with Plin2, there seemed to be no overlap with AQP7. See Figure S5, **a, b** in Annex I.

4.4 Effect of FFA in the expression of Plin2 and dynactin

The LD in the liver is primarily coated with Plin2 (32), where its overexpression is reported to promote TAGs accumulation in hepatocytes (91). Here, to investigate if the FFA exposure in HepG2 cells upregulates the expression of Plin2, immunoblot analysis and ICC were performed. A distinct band corresponding to Plin2 at ~50kDa was detected in both HepG2 cells and mouse liver homogenate which was included as positive control (Figure 4.8, **a**). The band intensity increased with increasing concentrations of FFAs when compared to control ($p < 0.01$) (Figure 4.8, **a, b**).

The immunoblotting for dynactin was also analysed. A ~55 kDa band representing dynactin protein (Figure 4.8, **c, d**) was present in control and treated HepG2 cells, although no differences in the protein expression were observed in FFA treated cells relative to control. Additionally, ICC also supported an increment in Plin2 abundance in 0.5 mM and 1 mM FFA treated HepG2 cells as compared to control conditions (Figure 4.8, **e, f, g**). The quantification of Plin2 expression was not done here, as there was only one sample from each group. Also, the images were not taken with the same gain and offset values. Both results uphold the anticipation that Plin2 is a marker for the FFA model and that FFA treatment upregulates the expression of Plin2.

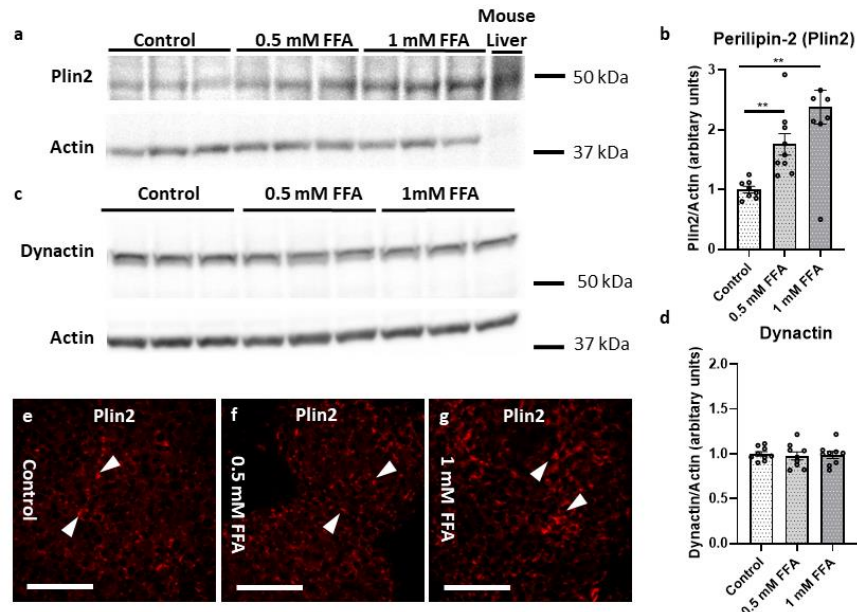


Figure 4.8: Expression of Plin2 & dynactin in FFA treated HepG2 cells.

a Representative immunoblots for Plin2 and Actin. Mouse liver tissue was included as a positive control as indicated in **a**. **b** Densitometric analysis of the immunoblots for Plin2. Band densities were normalized to internal control Actin. Mean band densities ($n=9$ in each group) were normalized relative to the Controls. Asterisks indicate significant differences between groups: $**p<0.01$. **c** Representative immunoblot for Dynactin and Actin. **d** Densitometric analysis of the immunoblots for dynactin. Band densities were normalized to internal control Actin. Mean band densities ($n=9$ in each group) were normalized relative to Controls. **e** Immunofluorescence labelling for Plin2 in control HepG2 cells. **f** Immunofluorescence labelling for Plin2 in 0.5 mM FFA treated HepG2 cells. **g** Immunofluorescence labelling for Plin2 in 1 mM FFA treated HepG2 cells. Arrowheads indicate labelling for Plin2.

4.5 Gene and protein expression of AQP7 in FFA treated HepG2 cells

To evaluate whether the increment in fat accumulation in HepG2 cells would modulate the expression of AQP7 I performed immunoblotting and qPCR. A tendency towards an increased expression of AQP7 protein abundance was observed in cells treated with 1 mM FFA ($p=0.09$) when compared to controls (Figure 4.9, **a, b**). Whereas qPCR analysis revealed no changes in AQP7 gene expression between control and FFA treated cells (Figure 4.9, **c**).

The results obtained using immunoblotting and qPCR do not follow the same pattern. The possible reason could be the samples prepared for RT-PCR and immunoblot were from the different well plates, although they were set up on the same day under the same conditions. As a consequence, a lesser number of cells might have reached confluence in the well plates for qPCR than immunoblot resulting in lesser AQP7 mRNA. Nonetheless, this finding is common as only around 40% of the time the two measurements coincide (92).

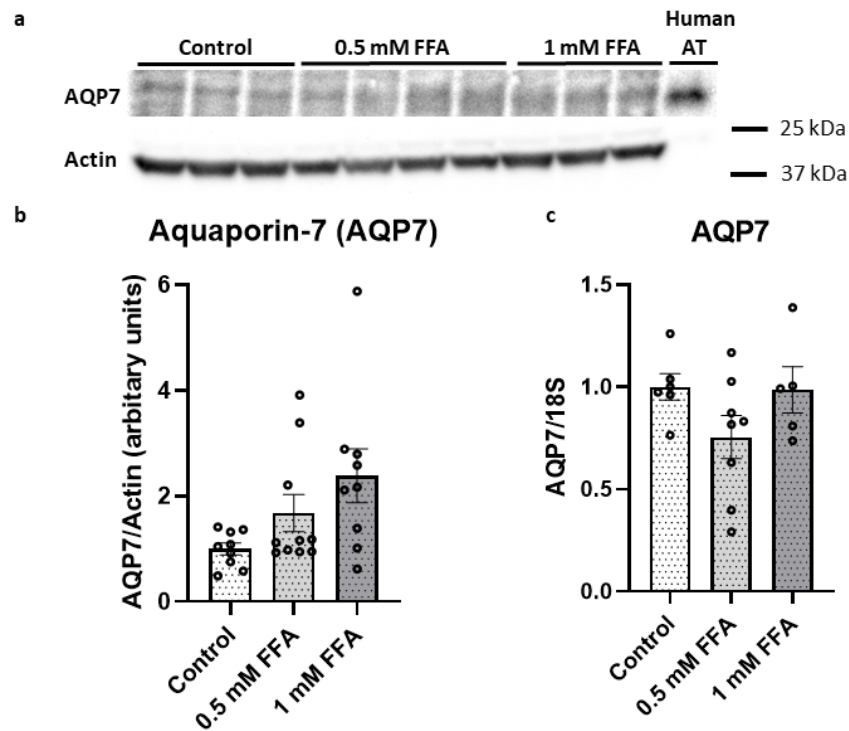


Figure 4.9: Expression of AQP7 in FFA treated HepG2 cells.

a Representative immunoblots for AQP7 and Actin. Each lane represents a sample treated with either Control media or Control media including 0.5 mM or 1 mM of free fatty acid mixtures (FFA). Human adipose tissue (AT) was included as a positive control as indicated. **b** Densitometric analysis of the immunoblots for AQP7. Band densities were normalized to internal control Actin. Mean band densities ($n = 9$ in each group) were normalized relative to Controls. **c** Analysis of AQP7 mRNA expression in HepG2 cells treated with either control media or control media including 0.5 mM or 1 mM concentrations of FFA.

4.6 Gene and protein expression of hepatic glycerol metabolizing enzymes in FFA treated HepG2 cells

My present results suggest that AQP7 co-localizes with Plin2 in perivenous hepatocytes, which is associated with LD. In adipose tissue, AQP7 has been shown to co-localize with Plin1 and in adipose tissue AQP7 plays a role in glycerol and triglyceride metabolism. Therefore, we here wanted to investigate whether FFA exposure would influence the expression of enzymes involved in glycerol metabolism. As in white adipose tissue (WAT), an increased or decreased abundance of AQP7 is associated with significant changes in GlyK expression while partly in GPD1 (80). Immunoblotting results displayed no alteration in GlyK protein expression between FFA treated HepG2 groups and controls (Figure 4.10, **a, d**). Similarly, the gene and protein levels of GPD1 also demonstrated no differences between control and treated cells (Figure 4.10, **b, e, f**).

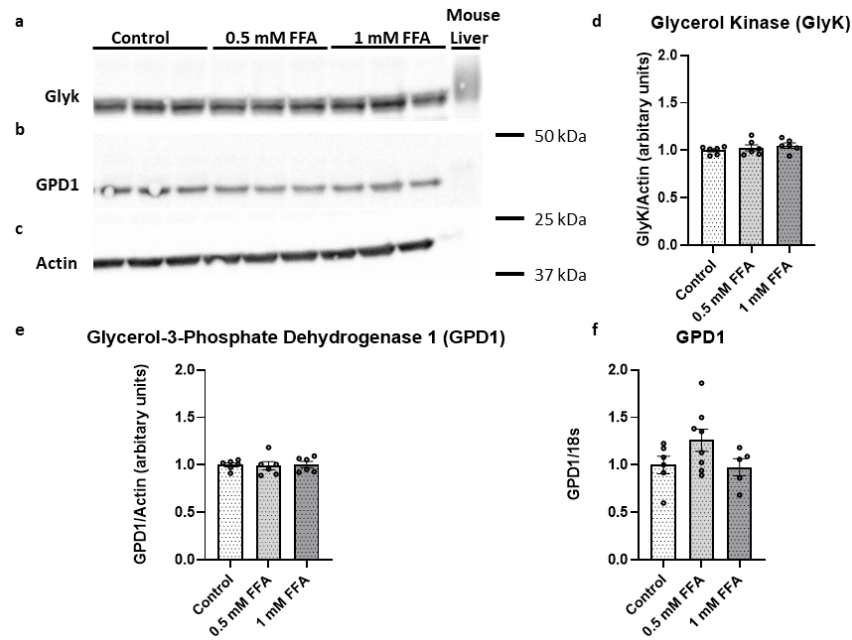


Figure 4.10: Expression of glycerol metabolizing enzymes in FFA treated HepG2 cells. Representative immunoblots for **a** GlyK **b** GPD1 and **c** Actin in HepG2 cells exposed to either control media or control media including 0.5 mM or 1 mM of free fatty acid mixtures (FFA). Mouse liver tissue was included as a positive control as indicated. **d**, **e** Densitometric analysis of the immunoblots for GlyK, GPD1 respectively. Band densities were normalized to internal control Actin. Mean band densities ($n = 6$ in each group) are normalized relative to Control. **f** Analysis of GPD1 mRNA in HepG2 cells treated with either control media or control media including 0.5 mM or 1 mM concentrations of FFA.

5. Discussion

AQP7 gene is documented to be the third most expressed glycerol channel in the human liver where the ratio between hepatic AQP7/AQP9 and AQP7/AQP3 are 0.142/30 and 0.142/1 respectively (15). There are few previously published studies supporting the presence of AQP7 in the liver and no study to date investigating the subcellular localization in the liver. The present research investigated the cellular and subcellular location site of AQP7 in human liver using IHC. In addition, we also examined the functional role of AQP7 in hepatocytes. By analyzing gene and protein expression in HepG2 cells, we provided additional evidence for the presence of AQP7 in the human hepatocytes. The expression of AQP7 was found intracellularly within the hepatocytes and in the apical plasma membrane of cholangiocytes as well as in sinusoidal cells. The cellular localization of AQP7 in hepatocytes and cholangiocytes is consistent with the findings by Gregoire *et al.* (12). The cytoplasmic presence of AQP7 in hepatocytes also agrees with the study by Chen *et al.* (17). However, the later group also reported the nuclear distribution of AQP7 in hepatocyte which is inconsistent to our findings. The present study reports the localization of AQP7 in the sinusoidal cells of human liver for the first time. Additionally, intracellular labelling of AQP7 in our study is in contrast with the finding in the liver section of a lean mouse, wherein immunostaining of AQP7 was present at the plasma membrane domain of hepatocytes (11). In this study, however, they used an AQP7 antibody that was directed against the human N-terminal cytoplasmic domain for the mouse study. Mouse and human share the homology sequence of about 67% (93). According to the Uniprot database sequence alignment tool (<https://www.uniprot.org/>), the amino acid sequences of human that are homologous to mouse are mostly present in the transmembrane domain. In all, the antibody used has a very low affinity to the epitope of mouse antigen. It is striking to note that the immunostaining of AQP7 in my study was mostly confined to the perivenous hepatocytes while no labeling was observed in the periportal hepatocytes. The previous studies that reported the localization of AQP7 in human hepatocytes have not specified the zones where the AQP7 was expressed (12,17). The difference in expression between perivenous and periportal hepatocytes has previously been observed for other aquaporins. The expression of AQP9 is mainly found in the perivenous hepatocytes and the expression of AQP8 occurred in all three regions of hepatocytes based on mouse and rat liver data (68–70,76). It is also interesting to note the intracellular location of AQP7 in the perivenous region in our study does not suggest a functional overlap with AQP9, which is exclusively found in the basolateral plasma membrane domain. The intracellular localization of AQP8, on the other hand, shows that its expression might overlap with AQP7 in hepatocytes. As described in the introduction earlier, the perivenous region is an active site for TAG synthesis. This gives an impression of the relevance of the expression of AQP7 involved in TAG synthesis.

While identifying markers for different subcellular organelles, dynactin was originally taken as a marker for aggresomes as previously reported (81). It is involved in the transfer of aggregates (misfolded protein) to the aggresome. However, after exploring into more literature, I found out that dynactin not only facilitates the transfer of misfolded protein but is also implicated in the transport of different other cargoes. In association with dynein, it assists in the retrograde transport of different cargoes intracellularly as illustrated in Figure 1.3 in the introduction. The co-localization of AQP7 and dynactin in our study could potentially suggest that dynactin is involved in the transport of AQP7 inside the hepatocyte. There is evidence of the dynein-dynactin complex being associated with membrane vesicles that transport AQP2 along the microtubule to the apical plasma membrane of kidney cells upon stimulation with vasopressin (94). As summarized in the introduction about the architecture of hepatocytes it

seems possible that dynactin recruits membrane vesicles carrying AQP7 to the motor protein dynein and helps in the transport of the vesicles bearing AQP7 to the negative end of microtubule in hepatocytes. Dynactin might assist the movement of cargoes bearing AQP7 either to the apical plasma membrane or from the basolateral plasma membrane to intracellular domains. Additional investigations are required to ascertain if the movement of AQP7 is occurring towards the interior of the hepatocytes or to the apical plasma membrane by dynactin.

We herein detected the partial overlapping of labeling for AQP7 with Plin2 (GP40) in the human liver. The expression of AQP7 was not even throughout the tissue section. Apparently, some hepatocyte cells had a high level of AQP7 expression formed in the clusters while others had a little or barely any expression. In previous studies, the interaction between the N-terminal of AQP7 and Plin1 has been corroborated in human adipocytes under lipogenic conditions (8). There is other literature supporting the labelling of AQP7 around the LD in human adipocytes (50) and human liver (14). However, in the later study (14) AQP7 staining upon the LD surface was not very convincing potentially due to strong background staining. Moreover, they only exhibited negative control staining omitting the primary antibody in their study which does not say anything about the specificity of the primary antibody. Apart from AQP7, other aquaporin like AQP3 have also been reported to bind with Plin1 protein at the surface of LDs (7). AQP7 being a transmembrane protein, we assumed that it is not integrated within the lipid monolayer of the LD. But we rather expect AQP7 to interact with LD in a peripheral manner while being localized in a vesicle with a lipid bilayer by binding to Plin2 to some extent as illustrated in Figure 5.1 below. In contrary to Plin2 (GP40), we did not see any co-localization between AQP7 and Plin2 (AP125). Although the immunolabelling of Plin2 (AP125) seems very convincing (Figure S1, **d** in Annex I) the labelling was not seen around the CV like that of Plin2 (GP40). This could be the reason why AQP7 did not show co-localization with Plin2 (AP125), as the distribution of AQP7 was primarily seen in the perivenous zones.

I speculate that some of the AQP7 proteins which did not overlap with Plin2 (GP40) may not be in their functional state and somewhat be misfolded. This speculation is derived from our data where the overlapping of AQP7 with HDAC6 was observed. It has been well documented that one-third of newly synthesized proteins are degraded by the proteasomal system under physiological conditions (95). Also, most of the time, the misfolded proteins originate from the proteins of a long-life span. One of the key functions of HDAC6 is to link misfolded protein to the dynein/dynactin complex and facilitate its transport to aggresomes, so called an aggresomal marker (89). Thus, HDAC6 may mediate in transporting some of the old non-functional AQP7 proteins that have essentially performed their function, to the aggresome for degradation. However, HDAC6 is also responsible for the transport of stable protein to its final destination based on the observations that HDAC6 facilitated the shift of the glucocorticoid receptor (GR) protein to the nucleus from the cytoplasm by forming GR and Hsp90 (chaperone) complex (96). Inhibition of HDAC6 results in hyperacetylation of Hsp90 and impairs the complex assembly of Hsp90-GR reducing its translocation. Interestingly, the trafficking of aquaporin requires its interaction with the chaperone proteins. As shown in the rat kidney tissues, Hsc70 and Hsp70 regulate AQP2 movement (97). Intriguingly, Hsc70 has been identified as a substrate for HDAC6 activity in mouse liver (98). It can thus also be speculated that AQP7 which shows the co-localization with Plin2 is functional and that HDAC6 is involved in the movement of functional AQP7.

The immunohistochemical data demonstrate that very few CD68 and CD163 positive Kupffer cells express a high amount of AQP7. The immunolabelling of AQP7 in macrophages is also seen after the blocking of immune cells. However, there seems to be some background staining

with the AQP7 antibody in the liver. The single cell transcriptomic data from the liver cell atlas (<https://www.livercellatlas.org/>) carried out on the liver biopsies of 19 human subjects shows very few Kupffer cells expressing AQP7 gene. It was recently revealed that macrophages in AT express AQP7 (51). This gives the impression that very few sinusoidal cells might express AQP7.

To investigate our thoughts regarding the differences in AQP7 expression between the portal and venous hepatocytes, HepG2 cells were grown with 5% CO₂ and 7% CO₂ mimicking the periportal and perivenous conditions respectively. The labeling for AQP7 in HepG2 cells was, however, very heterogenous in the different regions of the section. Hence, no differences in expression were observed. The reasons behind this might be that CO₂ incubators may not be the best to simulate hypoxic condition of perivenous site as it doesn't have an O₂ sensor. Hence it perhaps either requires further elevation of CO₂ level or an incubator with an O₂ cylinder thereby maintaining the O₂ level to 3-5% mimicking the perivenous site. Nevertheless, it would be very intriguing to examine the expression of AQP7 under hypoxia conditions in the future.

Some studies provide evidence of HepG2 cells expressing both the AQP7 gene (13,16) as well as protein (13,14,82). However, the localization of AQP7 in HepG2 has not been reported to date. We aimed at determining the localization of AQP7 in HepG2. We anticipated that AQP7 has the same localization pattern as that of human hepatocytes where I here found evidence that it co-localizes with Plin2. RT-PCR analysis showed mRNA expression for AQP7 in HepG2 which is in support of the prior findings (13,16,82). Surprisingly, I found that the localization pattern of AQP7 in HepG2 cells as evaluated by ICC differed with different dilutions of antibodies. The possible explanations for this discrepancy could be **1** highly concentrated AQP7 antibody may result in overstaining as staining was also seen in the nucleus region as illustrated in Figure S4a, S4b in Annex I. **2** due to handling errors. In contrast to the observations in human liver, we did not observe a co-localization between AQP7 and Plin2 in HepG2 cells. It can be argued that the expression pattern of AQP7 in human differs from that of a corresponding cell line. A multitude of factors is involved in this differential localization pattern for instance the drastically different cell environment, and culture system (99).

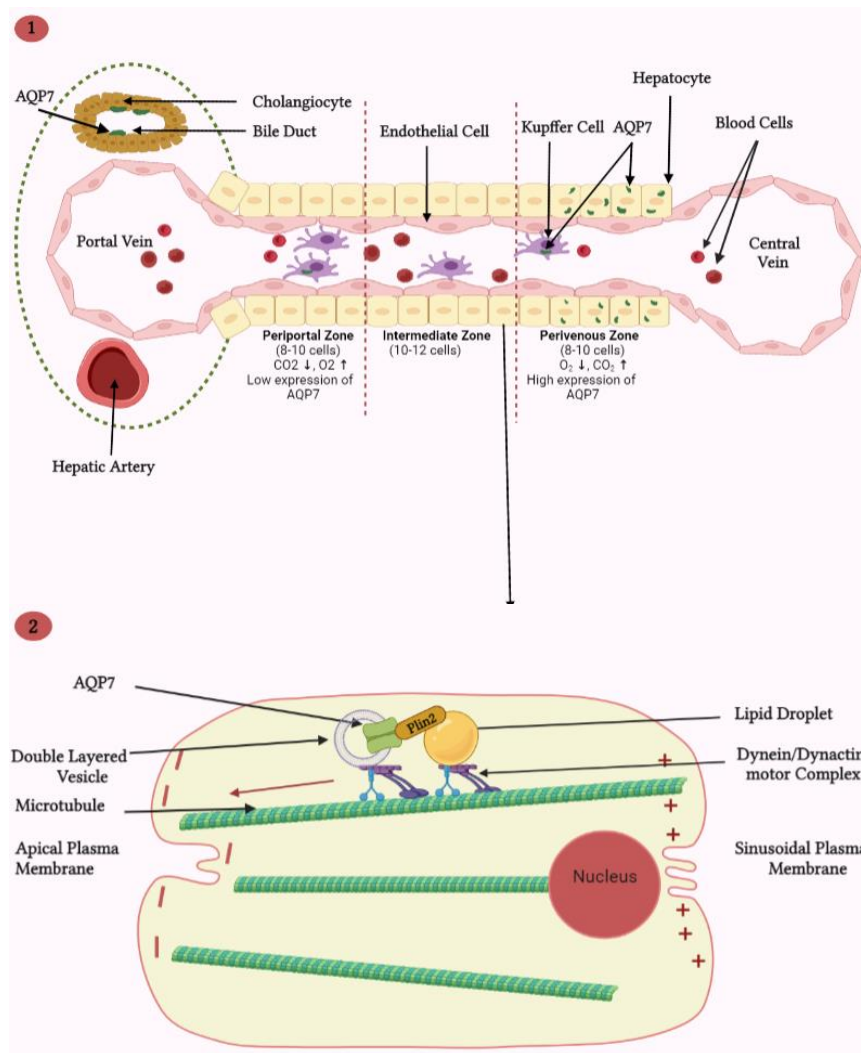
One of the contributing factors that stimulate lipogenesis in the liver is HFD. Plin2 is abundant in the liver. There was a marked increase in the Plin2 protein level in response to FFA treatment of HepG2 cells as anticipated. This result is in parallel with the previous observations made in mice and human (33,80). Both male and female mice showed substantial hepatic elevation of Plin2 after 24 weeks of feeding HFD. Overall, it advocates that Plin2 is a marker for FFA induced lipid accumulation. Moreover, we set out to investigate the *in vitro* functional role of AQP7 in hepatocytes using HepG2 cells. For that, we proposed that AQP7 would co-localize with Plin2 like that of human while the co-localization would further rise under FFA treatment conditions. Unfortunately, co-localization was not observed between AQP7 and Plin2 as stated above (Figure S5a, S6a, S6b in Annex I). However, the results showing overlapping between AQP7 and Plin2 in human liver might implicate a role of AQP7 in lipid storage. Nonetheless, further studies are needed to elucidate how AQP7 helps in lipid storage upon co-localizing with Plin2.

HFD induces hepatic steatosis as a result of obesity (100). Some metabolic tissues respond to obesity by having an increased expression of AQP7 (65,80,101), a reduced expression in others (102) while some remain unaffected (65,80). Thus, it follows that HFD has a different effect on the expression of AQP7 in different tissues. In the present study, the HepG2 AQP7 protein expression exhibited a tendency towards a slight elevation whereas no difference in AQP7 gene expression was observed with the increasing concentration of FFAs. This finding is in contrast

to the study made on ovariectomy induced hepatic steatosis in mouse liver (13) and in line with the study in human liver where stronger expression of AQP7 was found in obese subjects with hepatic steatosis (14). Also, the abundance of hepatic AQP7 protein was shown to be higher in obesity-associated T2D rather than in obese human subjects linking this consequence with elevated hepatic steatosis (14). Thus, the increased expression of AQP7 in FFA treated HepG2 might be correlated with steatosis as indicated by increasing Plin2. It is to note that the abundance of hepatic AQP9 decreases during the feeding state (69,72) implying that uptake of glycerol for TAG synthesis also decreases. This could suggest that the physiologic mechanism of AQP9 is not responsible for increased lipid storage in hepatocyte during feeding condition. Hence the correspondence of elevation of AQP7 and lipid accumulation in FFA treated HepG2 support the role of AQP7 in lipid storage. Having said that, the AQP7 mRNA and protein level in control as well as in FFA treated HepG2 cells seem to be low as indicated by high Ct values (>30) and the faint bands found in immunoblotting respectively. A previous study also reported the low mRNA expression level of AQP7 compared to AQP9 and AQP3 in human hepatocytes (15). Also, HepG2 cells might not be a good model with regard to AQP7 expression as HepG2 might mimic periportal hepatocytes based on our observations that AQP7 expression did not increase in 7% CO₂. So precise elucidation of the role of AQP7 in the process of lipid storage in hepatocytes by using other hepatocyte cell lines is a matter for further study.

FFA exposure did not affect the abundance of GlyK in HepG2 cells in our study. This result contradicts the earlier finding (80). However, this can be somewhat expected. Although lipid accumulation occurred in HepG2 after 24hrs of incubation with FFA, it does not necessarily result in a high level of hepatic steatosis like that of HFD fed mice after 12/24 weeks (80). Also, there is evidence that Plin2 primarily coats the surface of small LDs (103). It is conceivable that smaller LD accumulates in hepatocytes inducing mild hepatic steatosis in response to FFA. Keeping this in mind, it could be expected that GlyK is involved in generating G3P for TAG synthesis after prolonged exposure to FFA and therefore no change in its expression was seen. Moreover, the slight elevation in AQP7 could impact the expression of GlyK as the abundance of AQP7 has been reported to be inversely correlated with GlyK abundance in adipose tissue (57,80). Similar to GlyK, the expression of GPD1 also did not exhibit any change in FFA treated HepG2. This finding agrees with the findings earlier where both male and female mice exhibited no hepatic GPD1 changes after 24 weeks of HFD treatment (80). Likewise, we observed no change in the level of dynactin protein in response to FFA treatment indicating that the activity of dynactin is independent of diet induced fat accumulation.

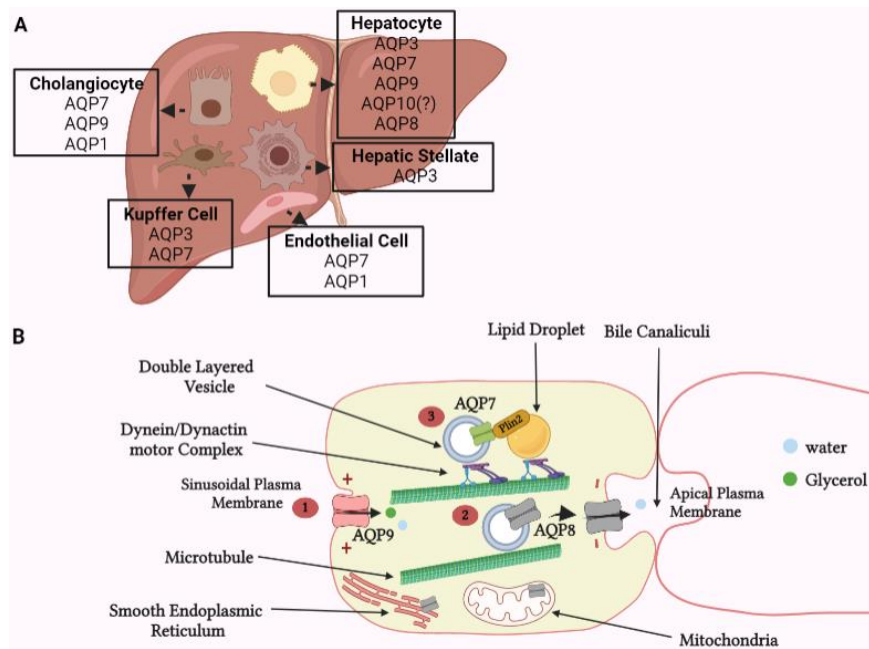
Our study has some limitations. The study was only made using tissue sections from one human subject whose characteristics were unknown. It would indeed be important to confirm the results obtained here regarding the subcellular localization of AQP7 in liver tissue from more individuals. IHC fluorescence double labelling is not the appropriate method to confirm the molecular interactions between two proteins. Thus, high resolution techniques like Proximity ligation assay or electron microscopy are required to further confirm the observations made in these studies.



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Figure 5.1: Proposed localization of AQP7 in liver.

1 AQP7 is localized intracellularly within hepatocytes mostly around the perivenous regions. The perivenous region is exposed to lower levels of oxygen than the periportal region. AQP7 is reported to be localized around the apical plasma membrane of cholangiocytes and Kupffer cells. **2** Schematic of a single hepatocyte with microtubule organizing center. The negative (-) end of the microtubule is directed toward the apical plasma membrane whereas the positive (+) end is pointed toward the sinusoidal domain. AQP7 is hypothesized to be localized in vesicles with a lipid bilayer where it interacts with lipid droplets (LDs) in a peripheral fashion by binding to LD protein, perilipin-2 (Plin2). Dynactin is the protein complex that is suggested to govern the transport of the vesicle bearing AQP7. The LD is also hypothesized to be associated with dynactin as seen by the overlapping between dynactin and Plin2. Since dynactin is associated with a motor protein called dynein which is a negative end-oriented microtubule motor protein, then transport of vesicles containing AQP7 is assumed to be either oriented towards the cell interior or to the apical plasma membrane indicated by the long red arrow.



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Figure 5.2: Expression of Aquaporins (AQPs) in liver.

A Reported expression of AQPs in different cells in human liver. **B** Subcellular expression of **1** AQP9 **2** AQP8 (based on rat and mouse data) **3** AQP7 in hepatocytes. **1** AQP9 as indicated by red color is expressed as the sinusoidal basolateral plasma membrane where it mediates the uptake of glycerol and water. **2** AQP8 as indicated by grey color is located mainly in the vesicle during the basal state. The vesicle containing AQP8 translocates to the apical plasma membrane along microtubules (via dynactin/dynein complex?) after stimulation where it facilitates the passage of water for bile formation. AQP8 has also been reported to locate at the inner mitochondrial membrane and smooth endoplasmic reticulum. **3** AQP7 as indicated by green color is hypothesized to be located in vesicle bilayer where it interacts with LDs in a peripheral fashion by binding to LD protein, Plin2. The subcellular expression of AQP3 in hepatocyte is yet to be determined.

6. Future Work

As outlined above, further studies should be carried out on more individuals to confirm the results obtained here regarding the subcellular localization of AQP7 in liver tissue. Furthermore, the study using additional subcellular organelles markers is required to find out which of the hypothesis that are suggested above is the right one (AQP7 associating with LD protein or aggresomal protein for degradation). Also, identification of which vesicles with a lipid bilayer are involved in the transport of AQP7 would be important. Some observations in this study point AQP7 to being involved in the process of lipid storage. However, the precise mechanism through which AQP7 does so remains to be elucidated. Concerning the hepatocyte architecture and data obtained from this study, it can be speculated that the movement of AQP7 probably occurs in the cell interior or to the apical plasma membrane via the dynactin complex. However, more investigations are required to verify this hypothesis. The localization of protein varies as per the metabolic demand. It would be interesting to look at the expression of AQP7 in liver under different metabolic as well as hypoxic conditions as described above.

7. Conclusion

The present study sheds light on the controversies about the cellular and subcellular localization of AQP7 in human liver. This study confirmed the cellular localization of AQP7 in hepatocytes and cholangiocytes. Very few kupffer cells showed strong labelling for AQP7. Interestingly, the expression of AQP7 was only found in the perivenous hepatocytes suggesting the involvement of AQP7 in lipid storage. The subcellular location of AQP7 in hepatocytes was shown to overlap with the labelling for dynactin, HDAC6, LD associated protein Plin2, whereas no overlap with labelling for the lysosomal marker, Lamp1, was observed. This suggests that AQP7 is intracellularly expressed in structures that are transported along the microtubule by dynactin. The co-localization of AQP7 with Plin2 also points towards a role of AQP7 in lipid storage in liver. In HepG2 cells, the expression of AQP7 was confirmed using RT-PCR and immunoblotting, however no overlap between labelling for AQP7 and Plin2 was observed in HepG2 cells. Treatment of HepG2 cells with FFA was associated with a substantial elevation in the expression of Plin2 and a non-significant tendency towards an increased AQP7 protein expression. Further investigations are required to understand the intracellular function and trafficking mechanism of AQP7 in detail.

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Annex I- Supplementary Figures

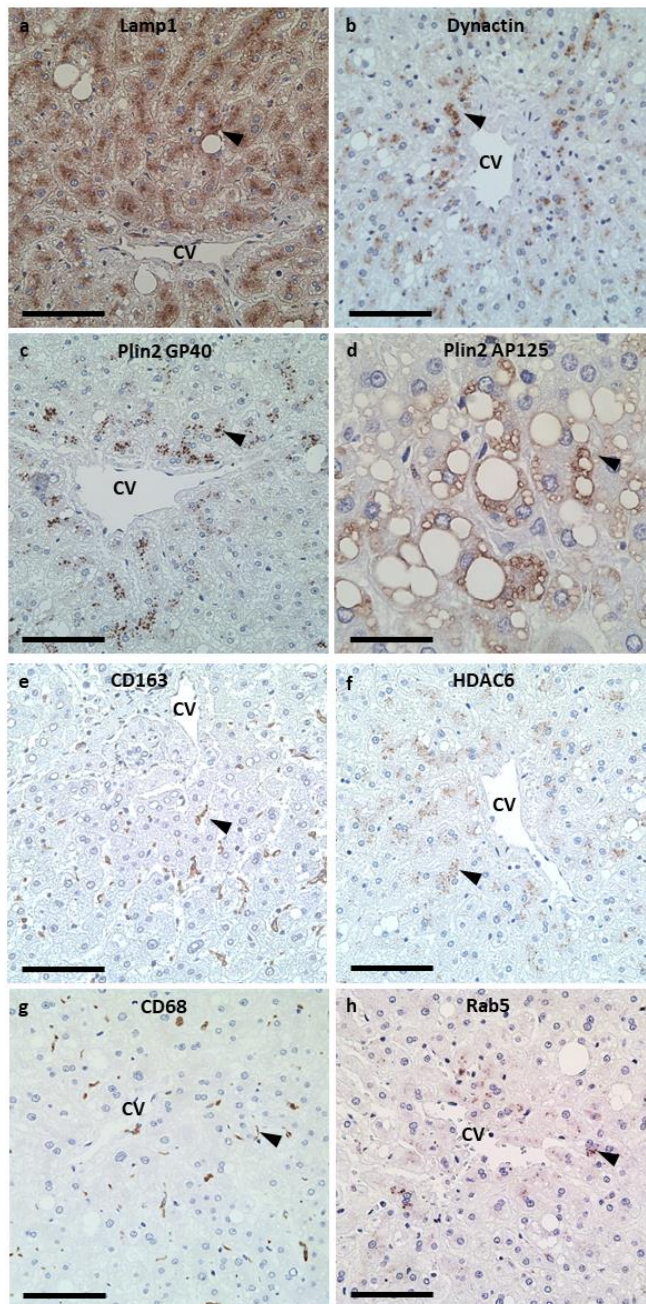


Figure S1

Immunoperoxidase labelling of different markers in human liver tissue. **a** Lamp1 **b** Dynactin **c** Plin2 (GP40) **d** Plin2 (AP125) **e** CD163 **f** HDAC6 **g** CD68 **h** Rab5. Arrowheads indicate labelling of markers. Scale bars indicate 50 μ m.

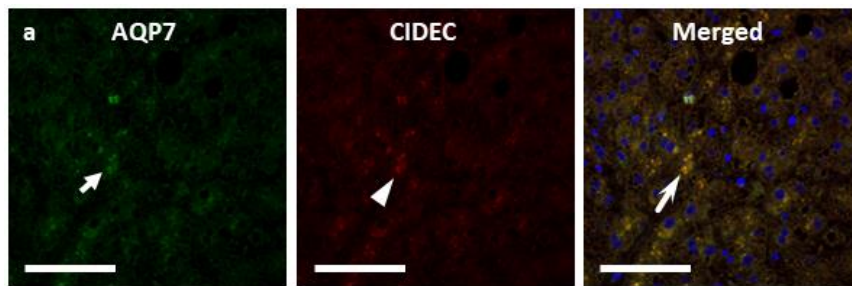


Figure S2

Immunofluorescence imaging of AQP7 and CIDEA in human liver tissue. **a** Immunofluorescence labelling for AQP7 (Green) and CIDEA (Red) and the merged image in human liver tissue. Arrow indicates labelling for AQP7. Arrowhead indicates labelling for CIDEA. Long arrow indicates co-localization between AQP7 and CIDEA. Scale bars indicate 50 μ m.

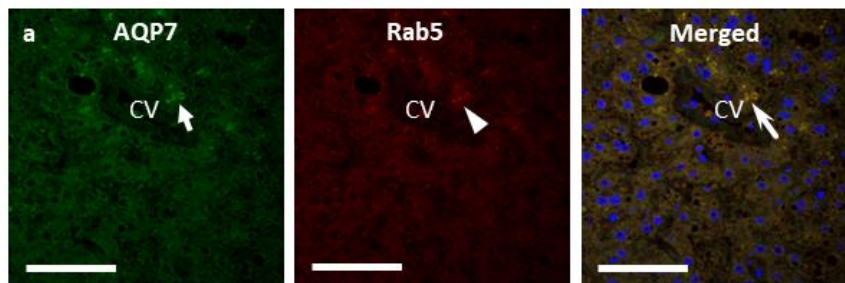


Figure S3

Immunofluorescence imaging of AQP7 and Rab5 in human liver tissue. **a** Immunofluorescence labelling for AQP7 (Green) and Rab5 (Red) and the merged image in human liver tissue. Arrow indicates labelling for AQP7. Arrowhead indicates labelling for Rab5. Long arrow indicates co-localization between AQP7 and Rab5 in the merged image. Scale bars indicate 50 μ m.

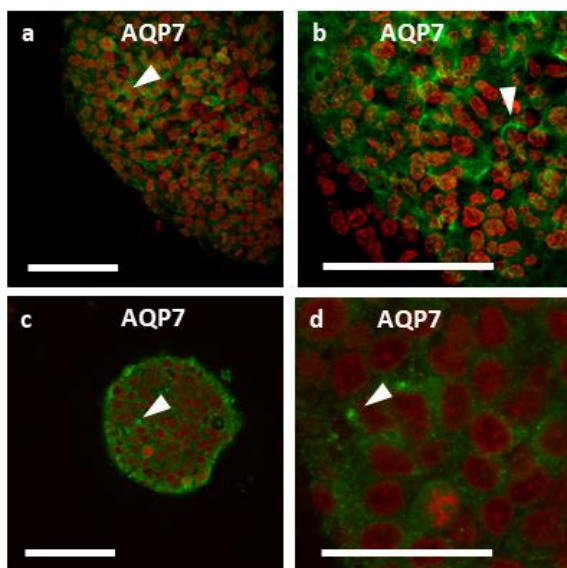


Figure S4

Immunofluorescence imaging of AQP7 in HepG2 cells. **a, c** Immunofluorescence labelling for AQP7 (Green) at 1:50 & 1:150 dilution respectively at 63x magnification. **b, d** labelling for

AQP7 at 63x2 magnification at 1:50 & 1:150 dilution respectively. Arrowhead indicates labelling for AQP7. Scale bars indicate 50 μ m.

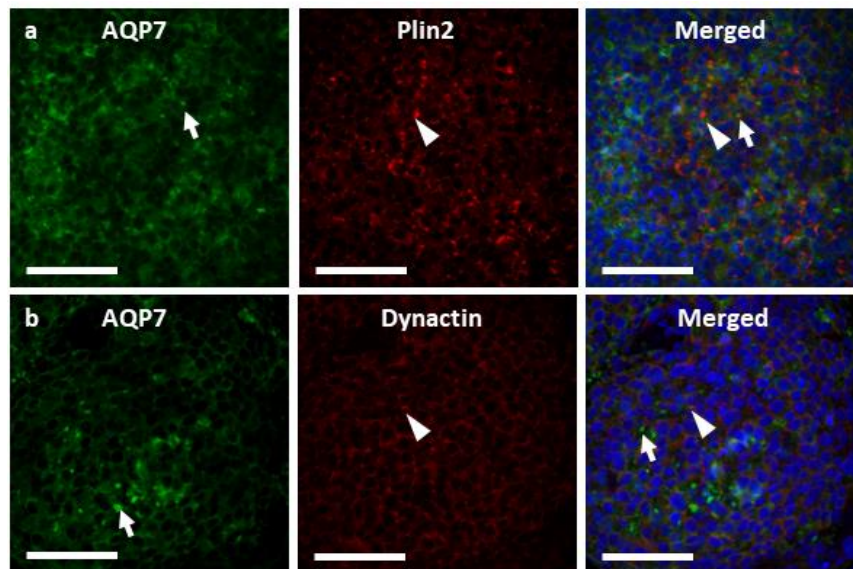


Figure S5

Immunofluorescence imaging of AQP7 in HepG2 cells. **a** Immunofluorescence labelling for AQP7 (Green) and Plin2 (GP40) (Red) and the merged image in HepG2 cells. Arrow indicates labelling for AQP7. Arrowhead indicates labelling for Plin2. **b** Immunofluorescence labelling for AQP7 (Green) and Dynactin (Red) and the merged image in HepG2 cells. Arrow indicates labelling for AQP7. Arrowhead indicates labelling for Dynactin. Scale bars indicate 50 μ m.

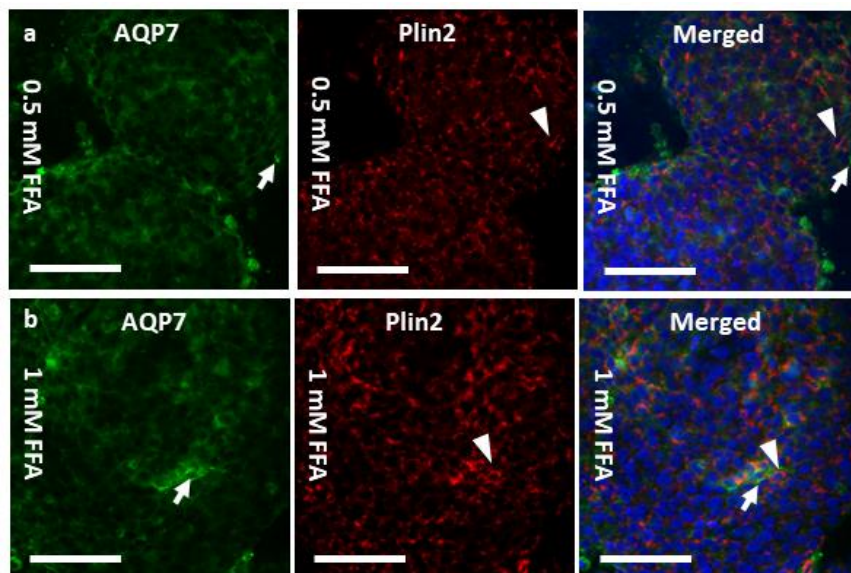


Figure S6

Immunofluorescence imaging of AQP7 and Plin2 in FFA treated HepG2 cells. **a** Immunofluorescence labelling for AQP7 (Green) and Plin2 (GP40) (Red) and the merged image in HepG2 cells in 0.5 mM treated HepG2 cells. **b** Immunofluorescence labelling for AQP7 (Green) and Dynactin (Red) and merged image in HepG2 cells in 1 mM treated HepG2 cells. Arrows indicate labelling for AQP7. Arrowheads indicate labelling for Plin2. Scale bars indicate 50 μ m.