Aspartoacylase (ASPA) inactivation leads to leakiness of the glomerular filtration barrier in zebrafish (Danio rerio)

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Chronic kidney disease (CKD) is a worldwide healthcare priority. Data for genes relevant to glomerular filtration barrier function or proteinuria as an early sign for CKD is continually increasing. Using our *in vivo* zebrafish model, we investigated the function of the enzyme Aspartoacylase for the integrity of the renal filtration barrier by morpholino knockdown. Preliminary data from our different proteinuria assays suggest damage of the glomerular filtration membrane by inhibition of the hydrolyzing enzyme ASPA (Aspartoacylase).

Aspartoacylase (ASPA) is primarily known to be involved in the integrity of the central nervous system (CNS). It is also expressed in liver and skeletal muscle tissue, but the highest expression levels are found in CNS and kidney. The gene ASPA encodes for the hydrolyzing enzyme that breaks N-Acetyl-L-Aspartic-Acid (NAA) down into aspartate and acetate. The metabolism of NAA is needed for the development of the white brain matter. Mutations of ASPA in the CNS result in Canavan Disease, a fatal disease[4,5]. NAA plays an important role in the biosynthesis of proteins and lipids. Aspartate, a derivative of aspartic acid, provides a nonessential liver-synthesized amino acid in the urea cycle. Acetate, a derivative of acetic acid, plays a substantial role in production of lipids, *e.g.* brain myelin.

Recent studies from our collaboration with the Jackson Lab suggested that a deregulation of ASPA also leads to renal damage. For further investigation we used our *in vivo* zebrafish-screening model to evaluate the effect of ASPA knockdown on the integrity of the glomerular filtration barrier[2]. To knockdown ASPA in zebrafish, an ASPA-morpholino and a scrambled control morpholino solution[6] were injected at different concentrations into one to four-cell stage fertilized embryos[1]. Morpholino sequences were designed and ordered from GeneTools (Pilomath, OR). We observed developing zebrafish embryos from 48 to 120 hours post fertilization and showed great differences between the phenotypes of control and ASPA knockdown group suggesting that the loss of the hydrolyzing enzyme leads to development of generalized edema (Fig 1)

To confirm that the observed zebrafish edema phenotype is associated with loss of high molecular weight plasma proteins due to damage of the renal filtration barrier after knocking down ASPA, we used a transgenic green fluorescent zebrafish model (Tg l-fabp:DBP:eGFP) and performed circulating fluorescence measurements on the retinal vessel plexus of the fish (eye assay[3]). This transgenic fish-line expresses a vitamin-D-binding protein fused with an enhanced GFP (MW 68kD) developing an increasing systemic fluorescence level from about 48 to 72 h post-fertilization on. Serial images of retinal vessel plexus showed significant differences with little development of systemic fluorescence in severely affected ASPA knockdown group compared to the control morpholino group (Fig 2, 3) indicating loss of systemic fluorescence levels.

To characterize the glomerular basement membrane we performed Transmission Electron Microscopy (TEM). Zebrafish larvae were sampled at 120 hours post-fertilization and fixed in epon plastic solution as per manufacturer´s protocol (Electron Microscopy Sciences, Hatfield, PA). Ultra thin sections (90 nm) of kidney were cut using a Leica Rotary Microtome. Stained sections were viewed on a JOEL-1230 Transmission Electron Microscope (Eching, Germany). Detailed analysis of the glomerular filtration barrier showed significant damage in the ASPA knockdown group to the podocytes, highly specialized epithelial cells of the visceral side of the glomerulus. This induction of "foot process effacement" of podocytes lead to leakiness of the renal filtration barrier and loss of high molecular plasma proteins into the urine (Fig 4).

These data indicate an important role for ASPA for the integrity of the glomerular filtration barrier and more studies in our lab are warranted to investigate the particular role of ASPA in podocytes.

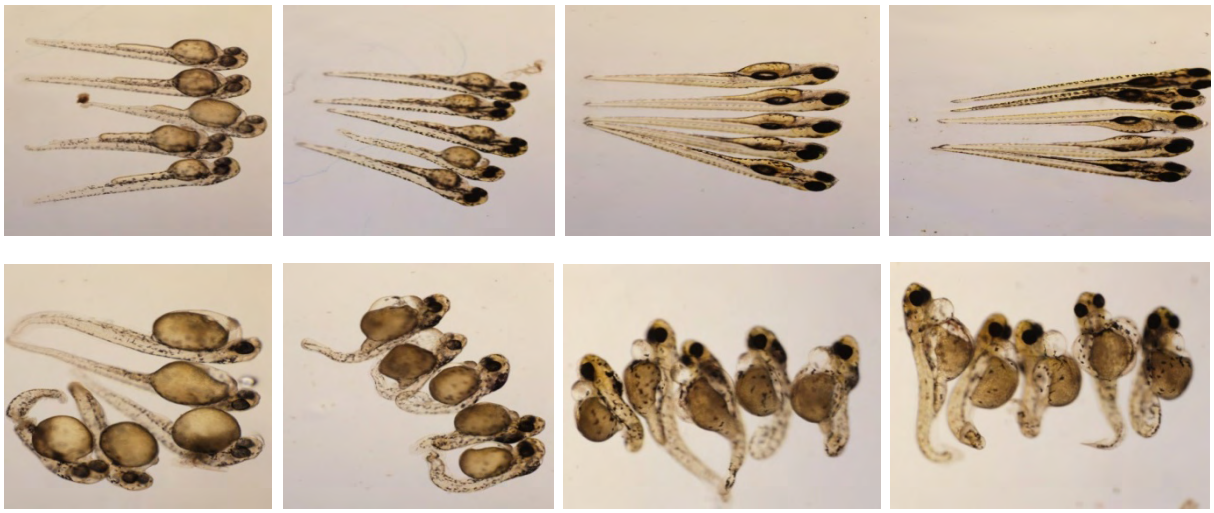

Figure 1. Knockdown of ASPA expression by morpholino silencing resulted in altered zebrafish phenotypes. The phenotypes of scrambled control morpholino zebrafish embryos was compared to ASPA knockdown morpholinos from 48 to 120 hours post fertilization. The ASPA knockdown group had several aberrant features including edema around the yolk and pericardial effusion.

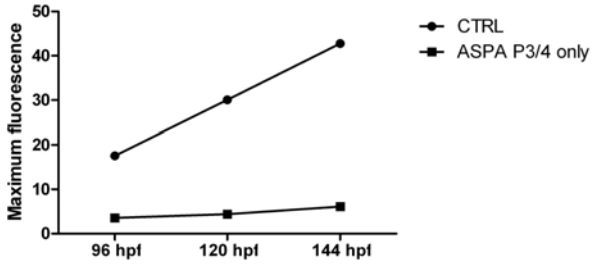

Figure 2. Results from the eye assay measurement using transgenic l-fabp:DBP-eGFP zebrafish. Compared to the control morpholino injected group there is hardly any development of systemic fluorescence in the ASPA knockdown group suggesting leakiness of the renal filtration barrier with loss of GFP into the urine.

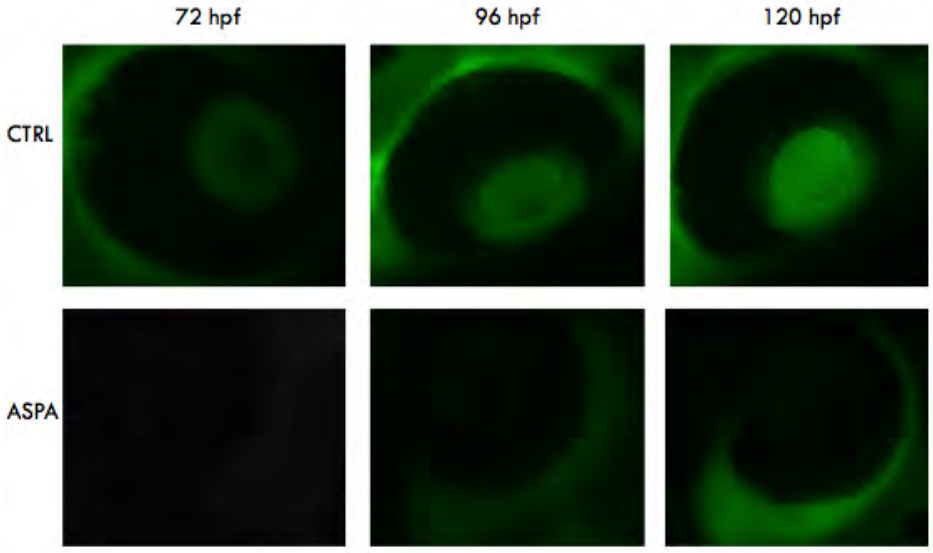

Figure 3. Eye assay for measuring renal filter integrity using transgenic l-fabp:DBP-eGFP zebrafish. The l-fabp:DBP-eGFP transgenic zebrafish produces a green fluorescent plasmaprotein. The transgene expression is driven by the FABP-liver promoter and leads to expression of a vitamin D binding protein fused with eGFP. The promoter becomes active at 48-72 h post-fertilization, leading to production and accumulation of fluorescent plasma protein that can be monitored over the retinal vessel plexus. In the ASPA knockdown group there was hardly any development of systemic fluorescence, suggesting produced GFP is lost *via* the kidney due to damage of the glomerular filtration barrier by ASPA knockdown.

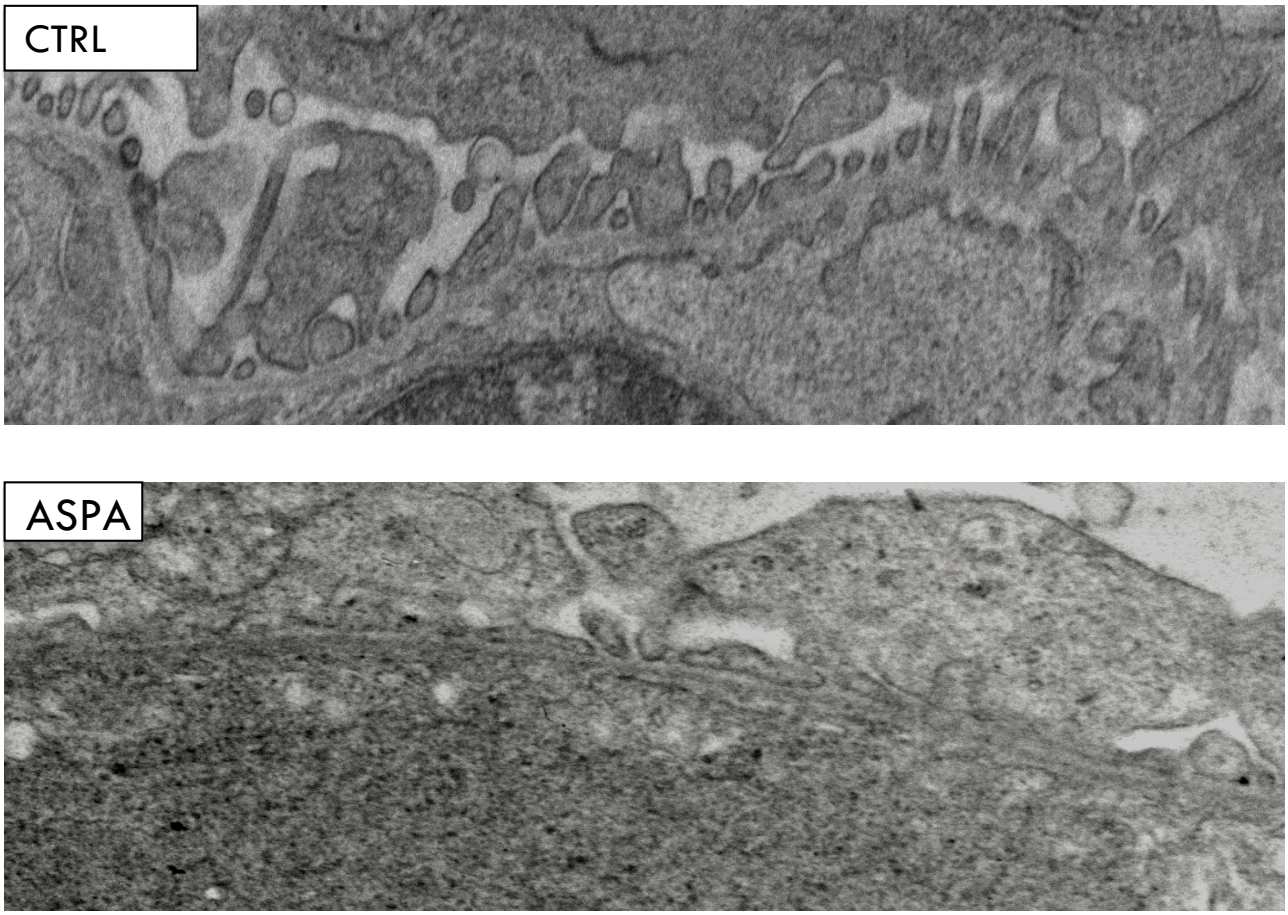

Figure 4. TEM-Analysis of zebrafish kidney (epon plastic embedding 120 hours post fertilization) to detect defects of the glomerular filtration barrier. It is essential to perform detailed structural analysis after proteinuria or leakage of the glomerular filtration barrier is detected, since any part of the filtration barrier (podocytes, glomerular basement membrane or endothelial cells) could be affected. Under normal conditions (Ctrl) the glomerular filtration barrier displays all features of a mammalian kidney with elaborate podocyte foot processes and a normal glomerular basement membrane. Analysis of the ASPA knockdown group (ASPA) shows loss of processes of the highly specialized podocyte cells with consecutive development of loss of high molecular plasma proteins into the urine.

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