Localization of the water transporting protein aquaporine-1 in the heart: a transmission immuno electron microscopy study

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2009

Acknowledgements

This thesis is based on experimental work done at the department of Anatomy of the Institute of Basic Medical Sciensec and Centre for Molecular Biology and Neuroscience (CMBN), University of Oslo, and at the Institute of Experimental Medical Research (IEMR), Univeristy of Oslo, Oslo Univeristy Hospital, Ullevål.

This project started off as a threesome cooperation of Jarle Vaages group at IEMR, Ole Petter Ottersens group at CMBN and Guro Valens group at the Department of Physiology, Institute of Basic Medical Sciensec, Faculty of Medicine, University of Oslo. I am grateful not only for my two supervisors Kåre-Olav Stensløkken and Jarle Vaage, but also to Mahmood Amiry-Moghaddam and Guro Valen.

I am grateful for the competent technical assistance provided by "the ladies of the lab", which includes Bjørg Riber and Karen-Marie Gujord for training me in the process of cutting tissue, Jorunn Knutsen for helping with blinding of the studies. I am also indebted to Bashir Hakim for learning me to operate an electron microscope, Anna Elisabeth Toren for helping with knock out mice, Martine Eilert-Olsen for providing brain tissue.

Furthermore I would like to thank me colleagues Thomas Misje Mathiisen, Abrar Quazi and Suleman Hussain for good advice during this prosess, wich has thought me many lessons, some tougher than others.

Contents

Abbreviations and Glossary

AQP = aquaporin

LV = left ventricle

mRNA = messenger RNA (ribonucleic acid)

SR = sarcoplasmatic reticulum

PC = preconditioning

BL6 = Black 6 (rat strain)

AR = area at risk

KO = knock out mice

WT = Wild type

IMGAP = ImmunoGold-Analysis-Programme

Paper Included

LOCALIZATION OF AQUAPORIN-1 IN THE HEART: AN ELECTRON MICROSCOPY STUDY

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(unpublished manuscript)

Mubashar Amin has performed in the paper:

1. Most of the fixations
2. Most of the preparations and cutting of the tisssues
3. Most of incubation and immunolabelling
4. Most of the image analysis
5. Prepared histograms
6. Made the basics on the figures
7. Made initial text version

INTRODUCTION

Cardiovascular diseases and especially myocardial infarction is today a major cause of death in the western world (Yusuf et al., 2001). World Health Organization is warning that already by 2020, cardiac diseases will become the major world wide cause of disability and death. The fact that third world countries are undergoing a more rapid increase prevalence of cardiovascular disease than the western world, is alarming.

It is known that myocardial edema occurs during ischemia and reperfusion, and also in heart failure (Schrier and Fassett, 1998). Ischemia of the myocardium is characterized by swelling of both interstitial and cellular compartments of the myocytes causing cardiac dysfunction. However, data are limited concerning the molecular mechanisms of fast water fluxes across cell membranes in ischemic hearts. Little attention has been given to water transport of the heart, and its possible role in physiology and pathophysiology.

In 1988 Peter Agre and his colleagues isolated a membrane protein that belongs to a large family of water channels: the aquaporins (AQPs). He was in 2003 awarded the Nobel Prize for this finding. Prior to this revolutionary finding it was hypothesized that specialized water channels that control rapid transmembrane water fluxes probably existed, because water flux through the lipid bilayer of the plasma membrane is rather slow and not subjected to regulation. In mammals, 12 members of the AQP family have been identified (Agre et al., 2002; Agre and Kozono, 2003). Different aquaporins exist in different organs and have been shown to play an important role in physiology and several disease states (Agre and Kozono, 2003; Amiry-Moghaddam and Ottersen, 2003). Aquaporins were traditionally regarded as merely water channels, but appear to have many additional functions including transport of ions and small molecules, gases and solutes such as glycerol (Verkman, 2005). Furthermore, AQPs may be involved in signal transduction, cell volume regulation and organellar physiology (Amiry-Moghaddam and Ottersen, 2003; Verkman, 2005). There are strong evidence that AQP4 may be of great importance in cerebral water balance and brain edema (Amiry-Moghaddam and Ottersen, 2003). There is information that ischemia and reperfusion influence the expression of AQP4 in the brain (Ribeiro et al., 2006). Furthermore modulation of AQP4 expression attenuated ischemia-induced brain edema (Kleindienst et al., 2005).

The myocardial AQPs are poorly characterized, and their localization and possible functions are still almost unknown. There is evidence of AQP-expression in the human heart (Egan et al., 2005), including AQP1 expression in endothelium and myocytes of cardiac tissue. Human, mouse and rat hearts all contain AQP7 transcript. Low levels of AQP11 expression were seen by Western blot analysis in the mouse heart. For other AQPs, reports differ, depending on the species examined and techniques used. AQP4 is present in the brain, muscle, kidney, lung, stomach and small intestine (King et al., 2004). For many years it was thought that no AQP4 exist in the heart, mostly based on RNA-studies (King et al., 2004; Ma et al., 1997). However, the RNA quality of these early papers were probably poor, partially due to a unstable "housekeeping gene" used in the analysis of realtime-PCR. Later publications have suggested that AQP4 mRNA is present in the human heart, along with AQPs 1, 3, 4, 5, 7, 9 and 11. (Butler et al., 2006) However, appreciable protein expression was demonstrated only for AQP1 and AQP4.

In the mouse hearts AQP 1, 4, 6, 7, 8 and 11 is described. The expression of AQP4 mRNA at different time points of ischemia in a murine model of myocardial infarction have been studied, and a correlation between the upregulation of AQP4 mRNA and the size of the infarction was found (Warth et al., 2007). In situ hybridization experiments showed comparably higher expressions levels of AQP4 mRNA in ischemic myocytes (Warth et al., 2007).

In summary, little is known about the role of AQPs in the heart physiology. Our research group have focused on AQP4 and AQP1, because of available knock-out mice. From pilot electron microscopy analysis we see that AQP4 in the mice heart is diffusely spread in the heart tissue with no clear pattern detected, and further investigation is planned. With immunohistochemistry it was shown that AQP1 in the mice heart is concentrated along capillaries. It has been suggested that AQP1 is also present in the t-tubules (Egan et al., 2005) and that it co-localizes with t-tubular and caveolar proteins (Au et al., 2004). Figure 1 is showing a schematic overview of the suggested distribution, but due to technical consideration with the immunohistoligical method the exact location in the heart has not been confirmed.
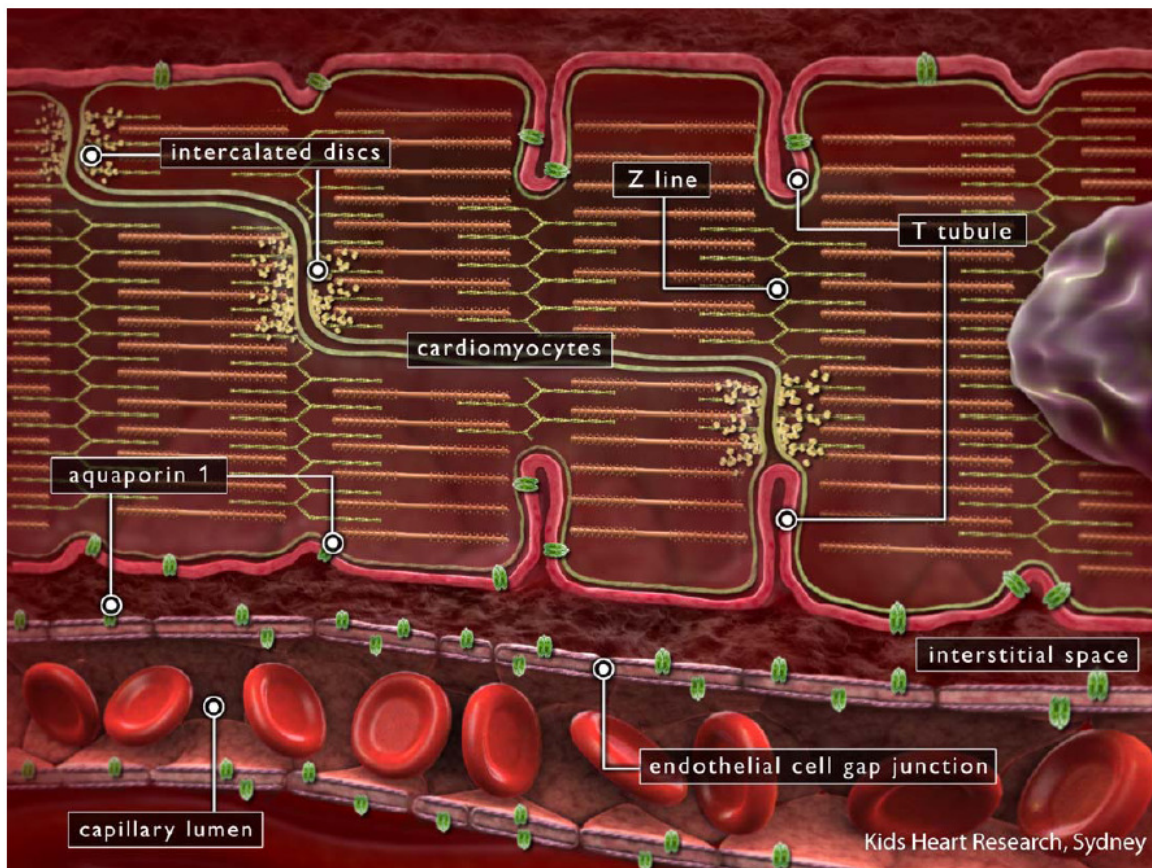
Fig. 1. *This is how expression of AQP1 in the heart is thought to be. AQP1 is distributed on the vascular endothelium. Note the AQP1 is also situated in the t-tubili and the myocyte membrane At the right we can se a part of a mitochondria. Modified from Egan, J. R., T. L. Butler, et al. (2006). Myocardial water handling and the role of aquaporins. 4th International Conference on Aquaporins, Genval, BELGIUM, Elsevier Science Bv.*

One of the overall hypotheses prior to this project has been that AQPs may play an important role in heart physiology and in ischemic injury. In human hearts during cardiac surgery mRNA of AQP1decreased during ischemia and reperfusion (Rutkovskiy unpublished observations). mRNA of both AQP1 and AQP4 decreased during perfusion of the isolated, perfused mouse heart, and ischemia-reperfusion had no additional effect (Rutkovskiy, unpublished data).

Our research group also investigated the effect of ischemia-reperfusion in isolated perfused hearts of AQP4-knock out mice (genotype AQP4 -/-). AQP4 -/- mice had increased resistance to ischemia-reperfusion injury, as shown by reduced infarct size and improved postischemic

function compared with the control group of AQP-WT (genotype AQP4 +/+) (Rutkovskiy, unpublished data).

An interesting finding was that AQP1 mRNA was significantly increased in AQP4 -/- mice, whereas Western Blot showed no difference at protein-level of AQP1 in AQP4 -/- compared to AQP4 +/+ (Rutkovskiy, unpublished data) . Increased AQP1 might be a compensatory mechanism in AQP4 -/- mice.

The purpose of the present study was by using immunoelectronmicroscopy: 1. To investigate the distribution and localization of AQP1 in mouse cardiac tissue, and 2. To study if deletion of AQP4 leads to an upregulation of AQP1.

HYPOTHESES

1) AQP1 in the heart is concentrated along the endothelial cellular membrane.

2) Is there a difference in the expression of AQP1 between the luminar/apical surface of the endothelial cell membranes compared to the basal membrane?

3) AQP1 is up-regulated in AQP4-KO, as a compensatory mechanism.

4) There is more AQP1 in t-tubuli compared to myocyte-membrane.

2. MATERIAL AND METHODS

2.1 Experimental animals

All experiments conform to the guidelines for use and care of laboratory animals and the study was approved by the Norwegian Animal Health Authority. All animals had conventional microbiological status. They had free access to food (RM3 from Scanbur BK AS, Norway) and water and were kept at a 12 h light/12 h darkness cycle in rooms where the temperature was set to 23 °C and humidity to 55%–60%. All animals were acclimatized for at least 4 days before experiments. Mice, heterozygote for the aquaporin gene (AQP +/-) were bread at the animal facility, Ullevål University Hospital. The first generation offspring were genetically tested to determine the aquaporin genotype. Three groups of male mice where included in this project, AQP4 knock out mice, AQP4 -/- (first generation, n=5), AQP4 +/+ (first generation wild type, n=4) and C57BL6 strain (n=5) from B&K Universal (Sollentuna, Sweden). The animals, weighing 23–28 g, were anesthetized with pentobarbital (60 mg/kg i.p) and heparinized (500 IU i.p) before the hearts were harvested.

2.2 Fixation

Unless otherwise stated, all chemicals were purchased from Sigma—Aldrich. Hearts where excised and transferred to ice cold modified Krebs-Henseleit Buffer (KHB) consisting of (in mmol/l): NaCl 118.5; NaHCO$_3$ 25; KCl 4.7; KH$_2$PO$_4$ 1.2; MgSO$_4$/7H$_2$O 1.2; glucose/1H$_2$O 11.1; CaCl$_2$ 1.8). Redundant tissue was removed and hearts were immediately cannulated via the aorta and mounted on a Langendorff system (AD Instruments Pty Ltd, Castle Hill, NSW 2154, Australia) and retrogradely perfused with warm (37ºC), oxygenated (95% O$_2$, 5% CO$_2$) KHB at a constant pressure of 70 mmHg. The hearts were perfused for 1 minute to remove blood before they were transferred to another setup (within 10 seconds) consisting of a bottle connected to a 150 cm long tube containing 0,1 % glutaraldehyde, 4 % paraformaldehyde. The hearts were perfused with the fixatives for 20 minutes and left overnight in the same fixative. The following day, the fixative was diluted 1/10 in phosphate buffered saline (PBS) for long time storing.

2.3 Tissue preparation

The hearts was measured from apex to basis, to ensure that the same tissue sample was taken in each heart. The left ventricle was cut in 1 mm transverse sections and one slice at the same

length from apex was selected for further trimming. Figure 2a shows one transverse section and indicate the 1x1x2 mm slices prepared for electron microscopy (EM). The tissue was protected from freeze artifacts by immersion in increased concentrate of glycerol (10, 20 and 30 % in phosphate buffer). Each step lasted 30 minutes in room temperature and the tissue was left overnight in 30% glycerol.
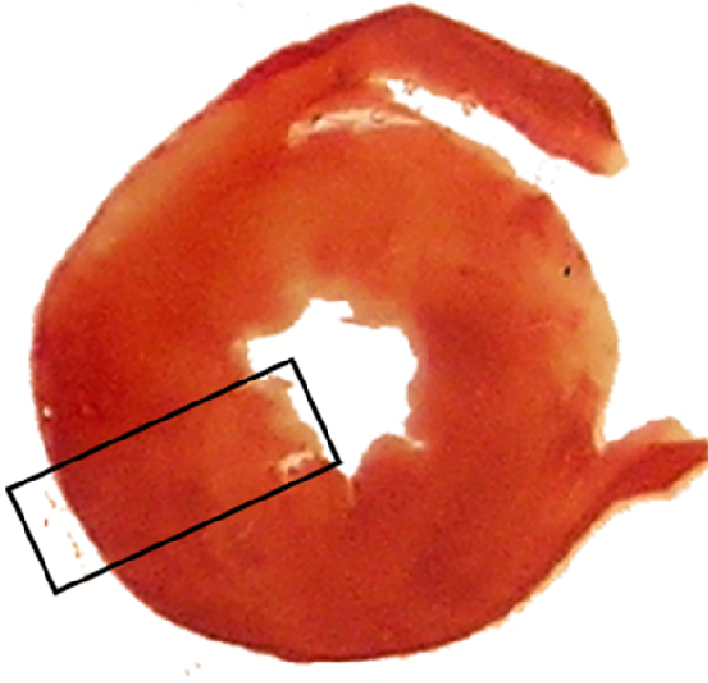

Fig. 2a. *Picture shows a transverse section of a mouse heart. The right ventricle to the right (wall ruptured) and the left ventricle to the left. The box indicates the section chosen for electron microscopy analysis.*

The following protocol is a modified procedure from Takumi et al. (1999). In short, the tissue sections where placed in a specimen pin and plunged into propane cooled to -170ºC in a cryofixation unit (Reichert KF80, Vienna, Austria) and transferred to 1,5 % uranyl acetate dissolved in anhydros methanol (-90ºC) in a computer-controlled cryosubstitution unit (Reichert). After 30 hours the temperature was raised stepwise (4 C increment per hour) from -90 to -45ºC. The sections were washed and infiltrated with lowicryl, and polymerized with UV-light for 35 hours (figure 2b).

2.4 Cutting and preparation of sections

The polymerized specimens were trimmed with a glass knife on a Leica Ultratome until we reached the tissue of interest. Some of the semithin sections (>200 nm) were stained with tryphan blue and observed in a regular light microscope to make sure that the whole tissue sample was present. When the specimen was optimal ultrathin sections (80 nm) were cut using a diamond knife. The ultrathin sections were transferred and mounted on nickel grids (figure 2b), 300 lines/inch square mesh. (Electron Microscopy Sciences, Hatfield, PA).
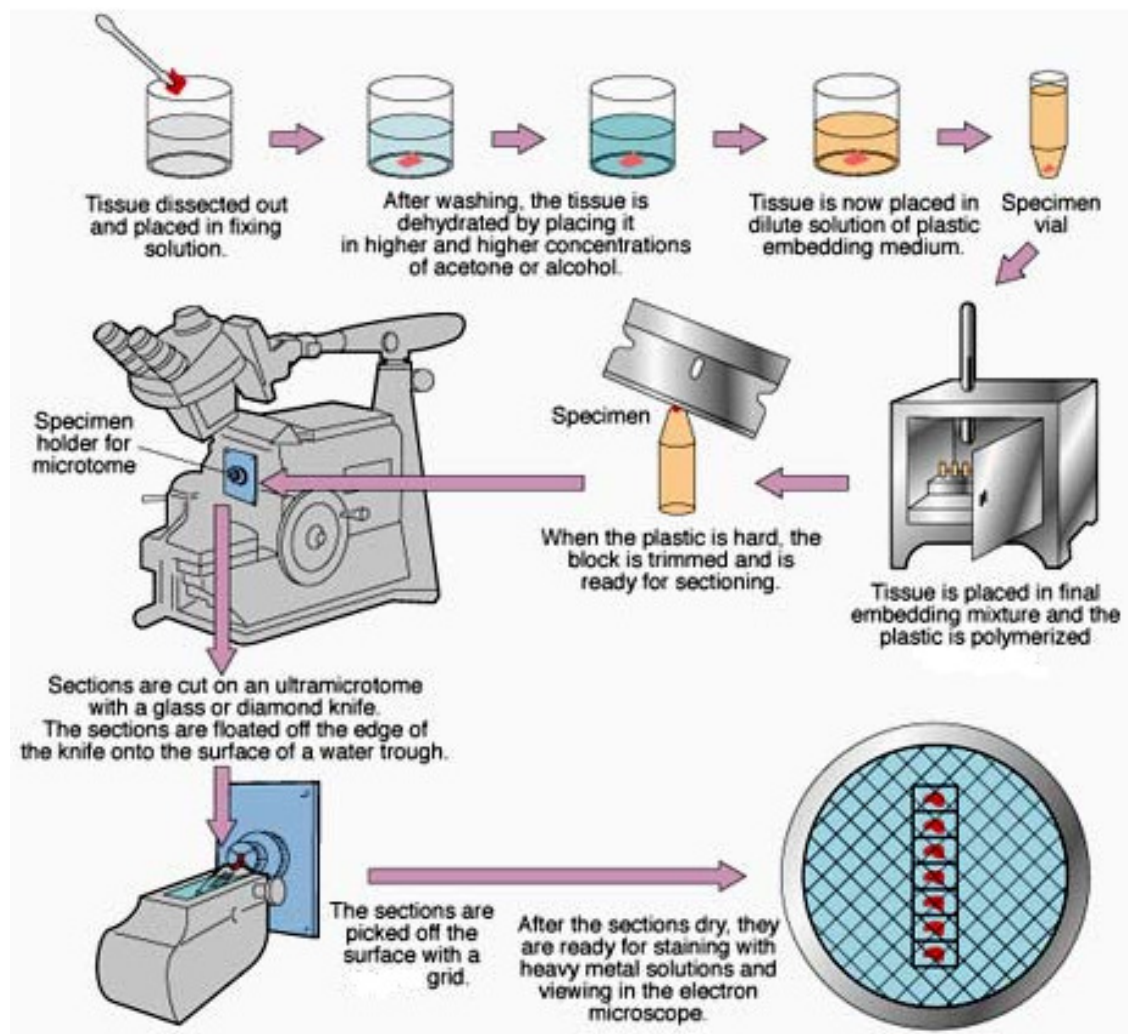

Fig. 2b. *The process of tissue preparation and cutting of sections (see text for details)*

2.5 Incubation and immunolabelling

The sections where treated according to Van Lookeren Campagne et al. (1991). In brief, grids where incubated in drops of Tris buffered saline (20 mM Trizma–base, 140 mM NaCl, 0.1%

Triton X-100 (TBST)), containing 50 mM glycine for 10 minutes, and then transferred to drops of TBST containing 2% human serum albumin for 10 minutes. Grids where then incubated for 2 hours in drops with primary antibody AQP1 (Rabbit anti-rat IgG # 1, from Alpha Diagnostic, con 1mg/ml, diluted 1:800 in TBST containing 2 % human serum albumin. Sections where washed with TBST with 2 % human serum albumin, and then incubated in drops with secondary antibody (GAR 15 nm RPN 422 anti rabbit from Amersham) conjungated to gold particles (15 nm) for 60 minutes.
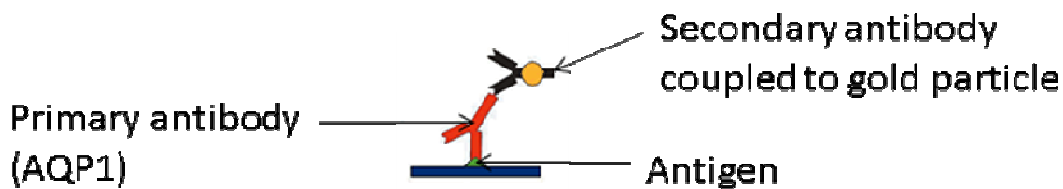

Fig. 3. *Principles of immunolabeling. The cartoon also shows the error margin of the technique. The gold particle can be observed in a radius 23.5 nm away from the protein and still be attached to the target protein.*

2.6 Staining with heavy metal solutions

The sections where incubated in 2% uranyl acetate for 1,5 minutes, washed with double destilled water and incubated in 0,3% lead citrate for 1,5 minutes, and then dried before examination in the electron microscope.

2.7 Image analysis

Pictures of the different sections where taken with a Tecnai 10 Transmission Electron Microscope (TEM) at the Anatomical Institute, Medical Faculty (UiO). Extra care was taken to obtain objectivity in the choice of pictures. Of each section (= each animal), 15 pictures of capillary. Initially, the grid was viewed at very low magnification to get an overview over the entire section. Then pictures were taken in a specific random pattern to ensure that the pictures represented the whole section. If the section covered 100 squares of the mesh in the grid, a picture was taken from every 6th mesh square (100/15). The capillary that were to be photographed were chosen at a magnification that did not reveal the gold particles. In each mesh square we chose the capillary that was closest to the left grid bar (at the same low magnification that did not reveal any gold particles. The whole process was done blinded, since the operator of the TEM did not know which tissue was present. randomly. The

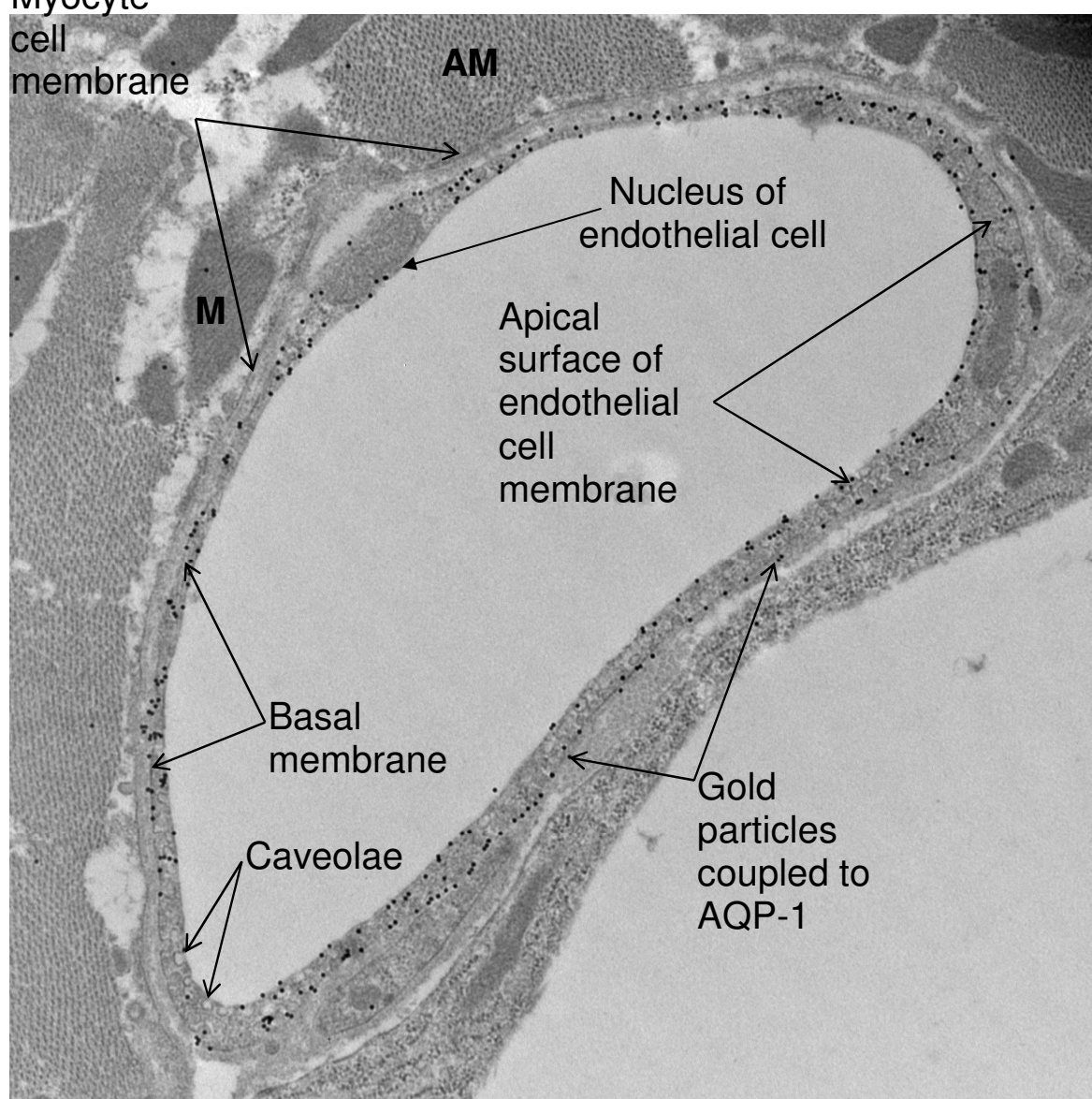protocol was repeated with myocyte membranes and t-tubuli.

Fig 4. *Cross section of a capillary with gold particles attached to aquaporin 1. The magnification is 16500 X. In the micrograph the mitochondria (M) and actin/myosin filaments (AM) are highlighted.*

Pictures were analyzed with IMGAP (ImmunoGold-Analaysis-Programme) software, created at the Centre of Molecular Biology and Neuroscience, University of Oslo, and developed in collaboration with SIS (Soft Imaging Systems Gmbh, Munster, Germany) as an extension to their product analysis. Unfortunately, the post analysis of the pictures revealed many t-tubuli

without membranes, probably due to the fixation process. These images had to be removed from the analysis and resulted in fewer included pictures of the t-tubuli.

Figures 4, 5, 6 and 7 show typical images from the analysis. The principles for detection and counting of gold particles within a user defined region of interest are highlighted below (Fig 4). In short, the region of interest was drawn on the picture in the analysis program. After the region of interest was decided, a line was drawn on the membrane of interest. The software analysis program then counted each gold particle and calculated the distance from the drawn line. The quantitative data was taken into further analysis. The histograms were made from information on all gold particles detected inside the region of interest. For detection on average AQP1 density, either a 100nm or a 23.5 nm filer was applied (with a 100 nm filter all gold particles more than 100 nm away was excluded from the analysis). Two different filters
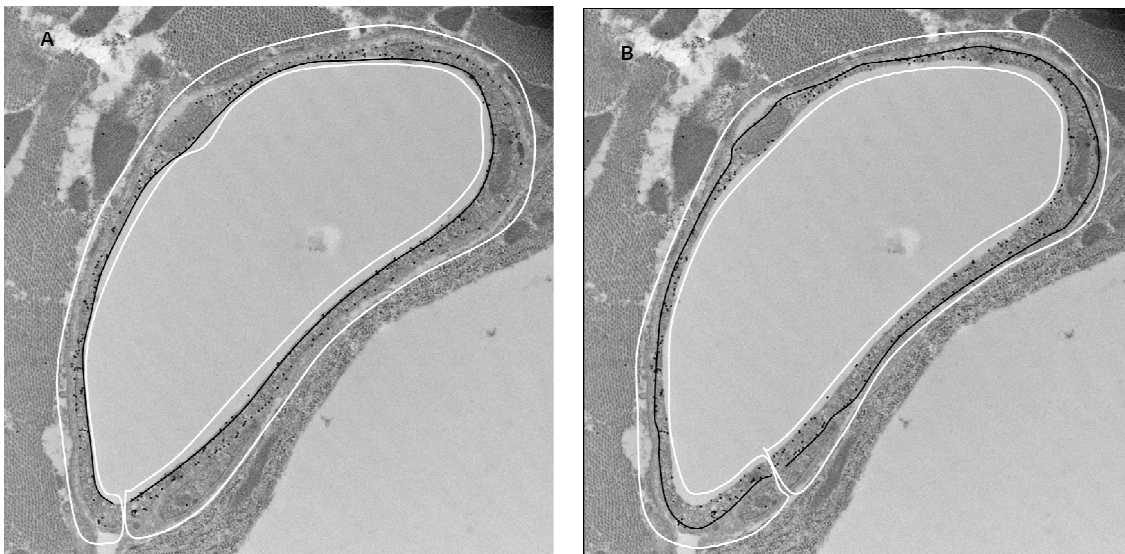

Fig 5. *The same image as in figure 4. Panel A shows the region of interest at the apical side of the endothelial cell, while panel B shows the same on the basal side. The number of included gold particles will be inside the region of interest and the distance from the black line is indicated in the histograms in the result section.*

were chosen to specify the location of AQP1 the endothelial cell. 23.5 nm represent the theoretical distance a gold particle can be from the membrane, and still be attached to a protein on the membrane.
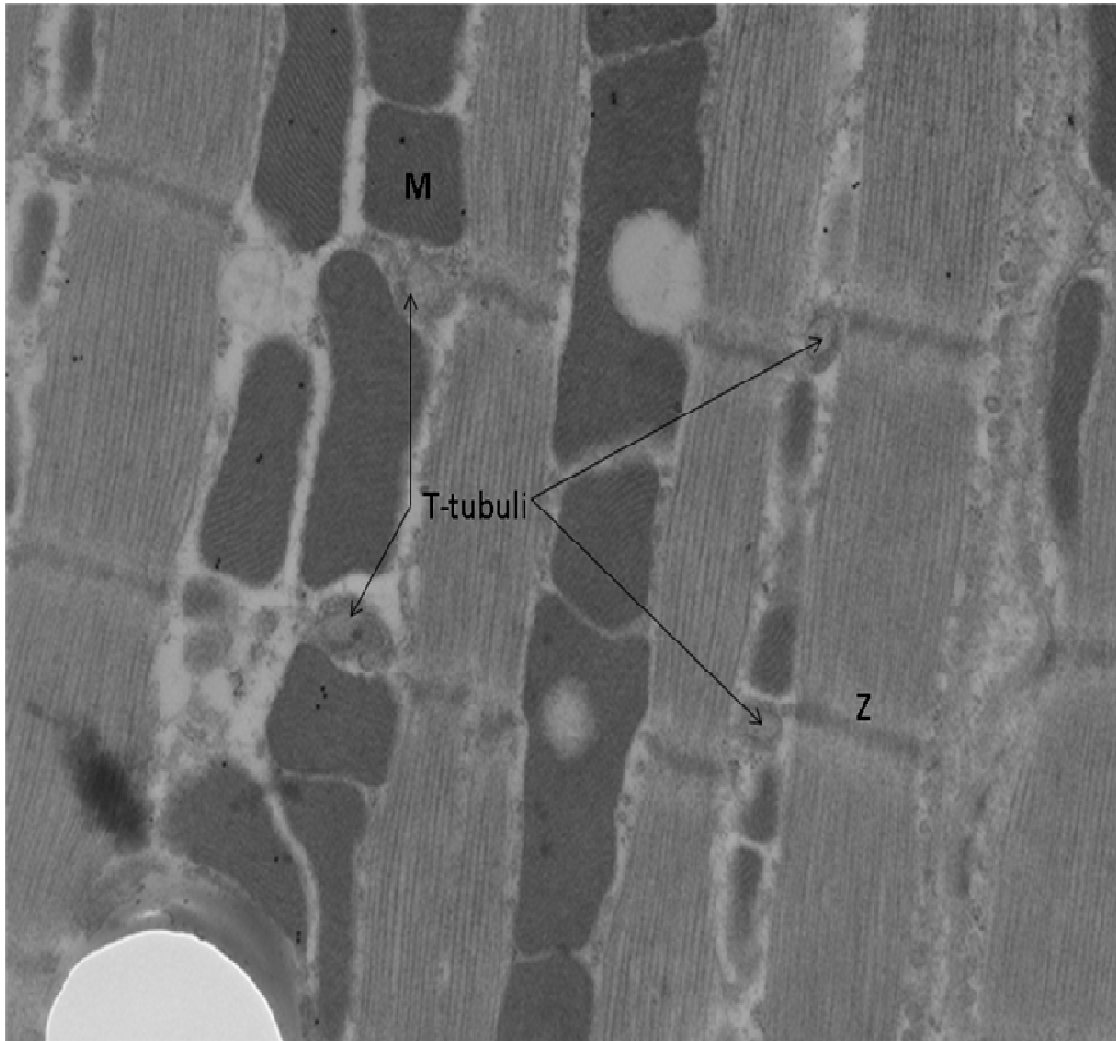
Fig. 6. *One of the pictures from the analysis showing transversely cut t-tubuli from the mouse heart. Highlighted in the image is mitochondria (M) and the z-line (Z) from the sarcomere. Magnification is 16500 x.*

2.8 Data treatment and statistics

The information from the software analysis was exported to SPSS and the further analysis was done using this or Graph Pad Prism 5.0 software. To detect significant differences, a one way ANOVA with a Bonferroni post-test was used to compare more than two groups, while a Students t-test with was used when comparing two groups.
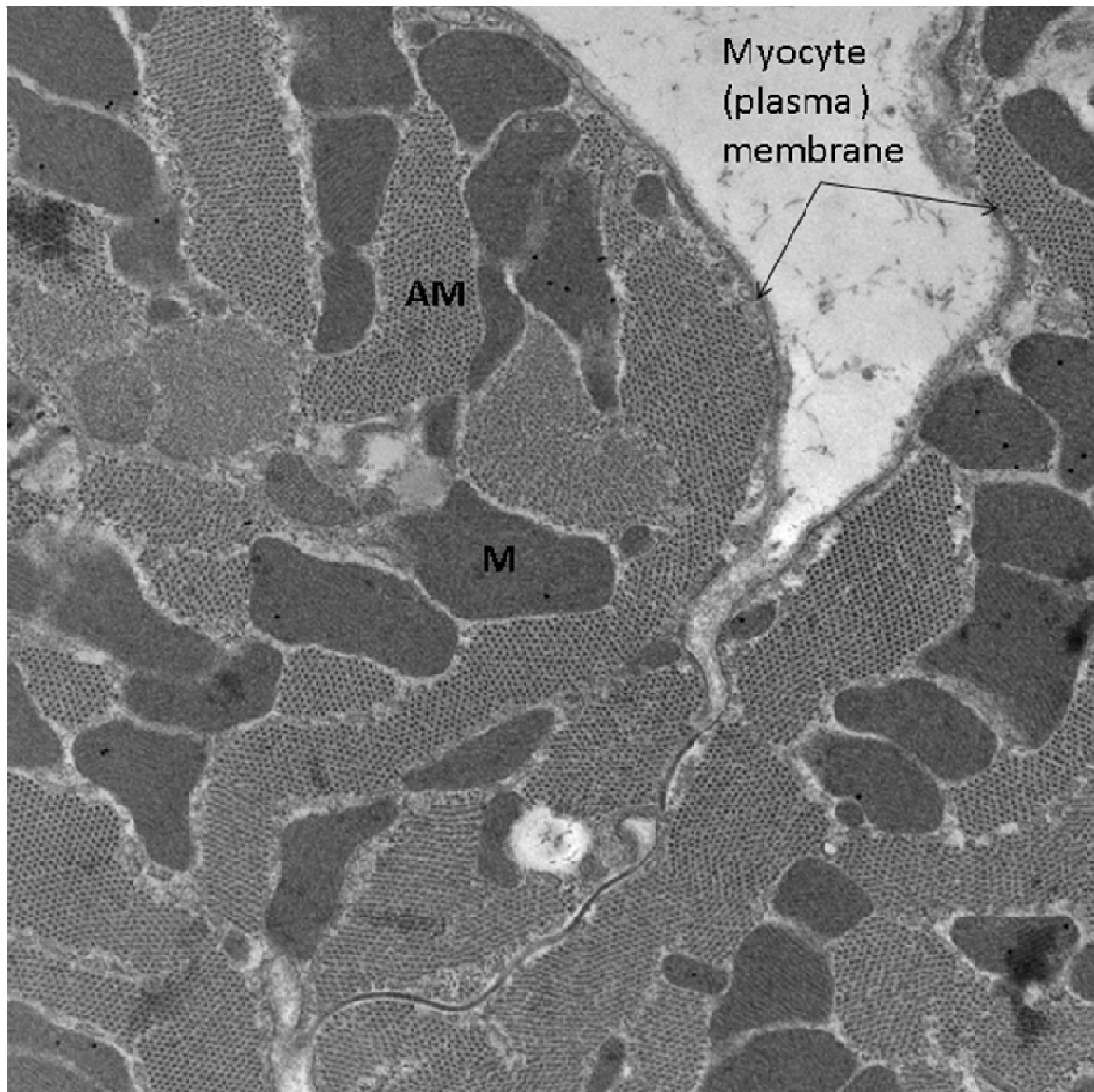
Fig. 7. *One of the pictures from the analysis showing transversely cut t-tubuli from the mouse heart. Highlighted in the image is mitochondria (M) and the z-line (Z) from the sarcomere. Magnification is 16500 x.*

3. RESULTS

3.1 Localization of AQP1

AQP1 in the heart were found mainly in the endothelial cells (Fig 4, 6 and 7). We see a small amount of background particles and very high density of gold particles on both sides of the endothelial cells of the capillaries. See also appendix I for more pictures of capillaries.

3.2 Distribution around capillaries

Figure 8A and figure 8B shows the distribution of AQP1 in the myocardial capillaries. The histograms demonstrate that AQP1 is located at or in the immediate vicinity of the endothelial cell membranes.
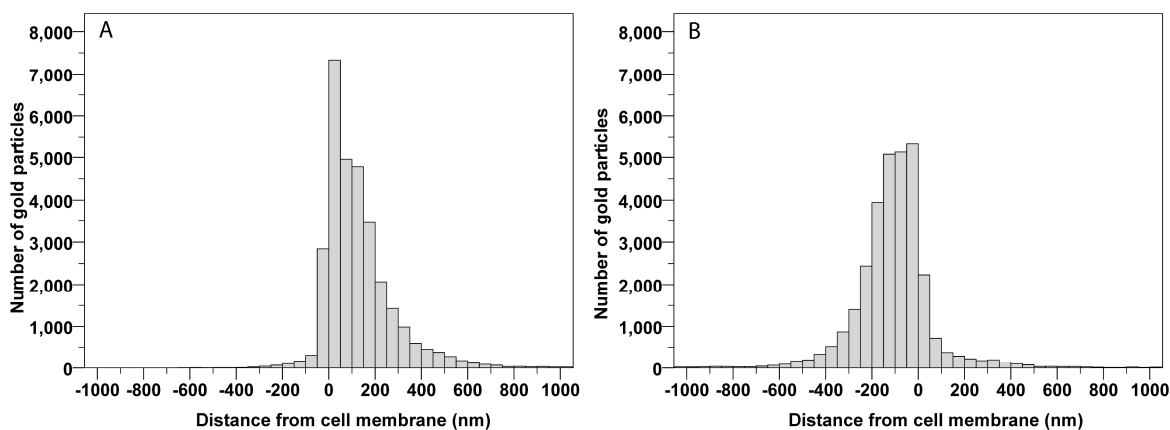

Fig. 8. *Histogram showing the frequency distribution of aquaporin-1 based on two separate electron micrograph analysis. In panel A, zero marks the luminal side of the endothelial cell membrane, and positive values indicate the number of gold particles towards the interior of the endothelial cell for all included animals (14 animals, 1 section of each animal and 15 pictures of each section). In panel B, zero marks the basal part of the endothelial cell membrane and negative values indicate number of gold particles towards the luminal endothelial cell membrane.*

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3.3 Other distribution of AQP1 in the mouse heart

We have viewed a lot of pictures stained with AQP1 specific antibodies and no other clear pattern, other than the endothelial cells in the capillaries was observed. Still we wanted to quantify the distribution on the myocyte membrane and in the t-tubuli. Figure 9 shows the linear density of AQP1 in t-tubuli and on the myocyte membrane. Although the average density was higher in the t-tubuli (mean ± SD; 0.11±0.63 particles/µm and 0.05±0.03 particles/µm t-tubuli and myocyte membrane respectively), but this may be caused by a few images with several gold particles (Fig 9). The median value indicates that there are more AQP1 on the myocyte membrane (median value 0.00 and 0.05 in t-tubuli and myocyte membrane respectively, Fig 9).
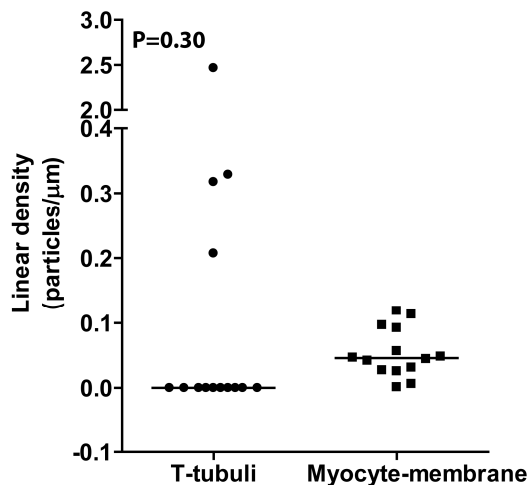
Fig. 9. *Scatter plot and median value of aquaporin-1 in t-tubule and on the myocyte membrane. Each dot represents the average from one animal.*

3.4 No difference in aquaporin-1 density between AQP4-/- and AQP4+/+

As for the quantification, there are several methods to compare the density of gold particles. One is to quantify linear density along the curves made in IMGAP, as we can see as a black line in figures 5 A, B. Another is to calculate the areal density in the region of interest. Figures 10A-D show comparison in linear density and areal density of AQP1 between the three groups (AQP4 -/-, AQP-4 +/+ and BL6) using a 100 nm filter. We did not find any difference in AQP-1 density between any of the groups.

3.5 Difference between apical and basal side

As there were no differences between groups (Fig 10) all three groups were pooled to investigate difference between the apical and the luminal side of the endothelial cell. Using a 100 nm filter we found no difference between the two sides (Fig 11A, B). However, when the same groups are evaluated with a 23.5 nm filter, there is a significant difference between the apical and the basal membrane (Fig 11C, D).
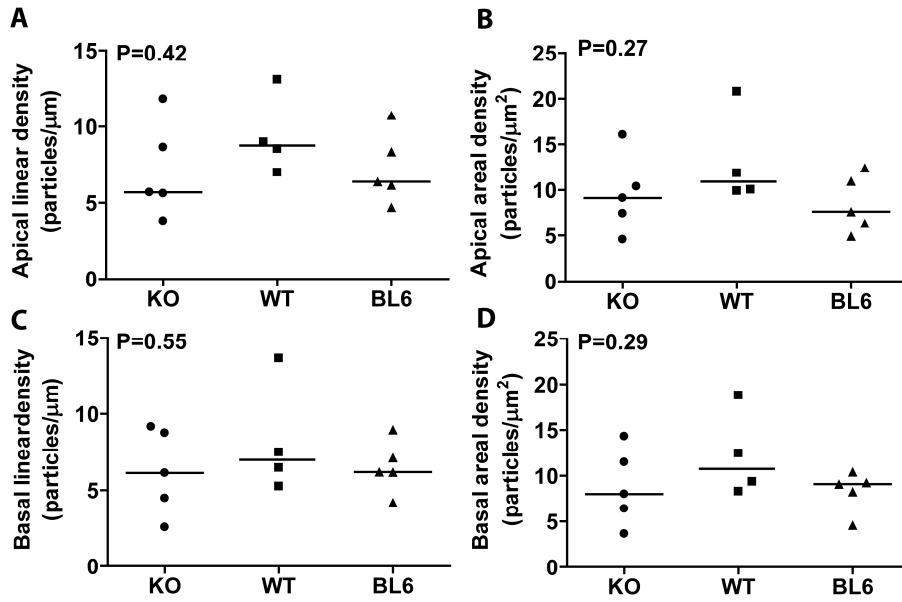
Fig. 10. *Scatter plot showing that there are no difference between AQP4 -/- (KO), AQP4+/+ (WT) and C57BL6 (BL6). Filter set at 100nm.*
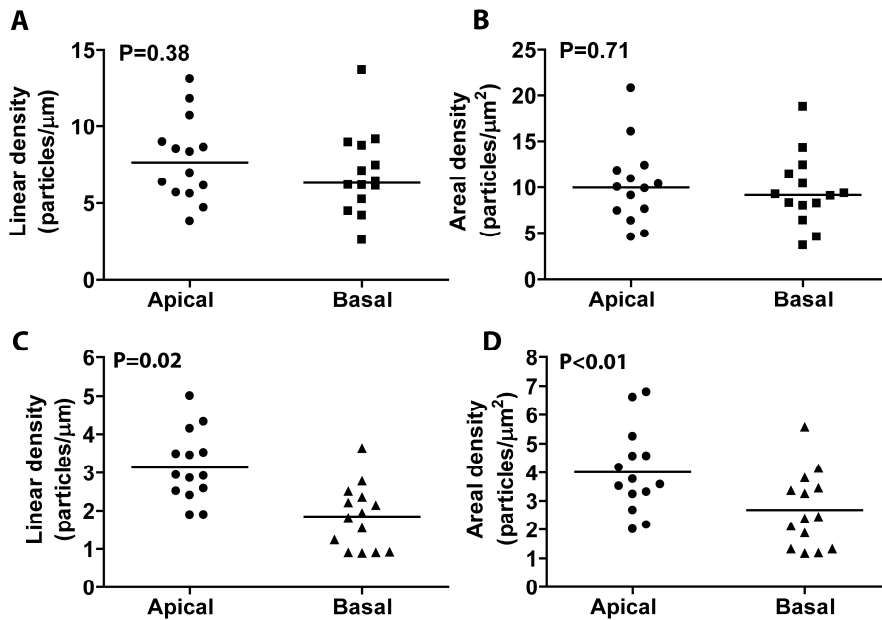

Fig. 11. *Scatter plot showing the difference in average density when all groups are pooled (AQP4 -/-, AQP4+/+ C57BL6). In panel A and B are the results with a filter set at 100nm. On panel C and D the filter is set at 23.45 nm.*

4. DISCUSSION

4.1 Summary of results:

1. AQP1 is concentrated in or close to the cell membranes along capillaries in the heart.
2. When we use a strict filter (23,5 nm) there seen to be more APQ1 in the apical surface of the endothelial cells compared to the basal membrane, but when a less strict filter is applied (100 nm) there is no difference.
3. AQP1 is not upregulated in AQP4$^{-/-}$ mice.
4. There is little or no AQP1 in cardiomyocutes and no accumulation in t-tubuli as compared to the cardiomyocyte cell membrane (plasma membrane).

4.2. Interpretation and implications of this project

Based on our findings that AQP1 is localized both at the apical and the basal membrane of endothelial cells it is tempting to suggest that AQP1 in the heart may play a major role in water transport between the intravascular and the extravascular space. This is in agreement with the general concept that AQP1 is located in the endothelium and is important for water transport, since it is highly concentrated along capillaries (Au et al 2004).

Au et al. (2004) suggest that AQP1 is co-localized with caveolin-3. Caveolins are a family of proteins that are involved in receptor-independent endocytosis (Williams and Lisanti, 2004). The caveolin gene family has three members in vertebrates: CAV1, CAV2, and CAV3, coding for the proteins caveolin-1, caveolin-2 and caveolin-3, respectively. All three members are membrane proteins with similar structure. Caveolin forms oligomers and associates with cholesterol and sphingolipids in certain areas of the cell membrane, leading to the formation of caveolae. This taken into consideration when interpreting our results, one can understand that the difference between apical cell membrane and the basal membrane ceases when analyzed with 100 nm filter, as this would include the AQP1 in caveola around both membranes, whereas when 23,5 nm filter is applied, only AQP1 placed exactly at the membranes will be included in the quantitation. This difference, i.e. the difference of apical vs basal membrane is of interest in case a translocation of AQP1 takes place in some situations. It should, however, be mentioned, that the difference at 23,5 nm probably is not of perfect accuracy, since comparison between the two kinds of membranes depends on an

extremely exact drawing along the membranes when analyzing in IMGAP. Such a degree of exactness not always is possible due to massive amounts of caveolae which renders the borders of the membranes blurred.

Au et al (2004) also suggest that AQP1 is assosiated with vinculin, which is a t-tubuli marker. They performed immunohistological staining in confocal immunofluorescence to show this. Vinculin is an actin-binding protein which is thought to contribute to the cytoskeletal scaffold that anchors the t-tubule network to the Z-line of cardiomyocytes. However, our results show no higher density of t-tubuli in the heart. It is unknown whether Au et al (2004) had positive and negative controls of their study, whereas we used the brain as a negative control, since there is no AQP1 in the brain.

AQP1 was not upregulated in AQP4$^{-/-}$mice in this study. Others have shown that AQP4 downregulation may occur in muscular diseases and that this might be associated with upregulation of AQP1 (Butler 2008). Even though there have been a lot of research in the field of AQPs, little is known of their physiological role in both normal and pathological situations.

4.3. Disadvantages and advantages of this study

Immunoelectron microscopy provides a unique possibilty to localize the distribution and subcellular localization of AQP in the heart. The technique is a gold standard for such studies.

However, there are many variables of this method which has to be optimal for all tissue samples studied; concentration of salts in buffers, concentration of antibodies, incubation time, temperature etc. There are also many potential pitfalls regarding the analysis of pictures, such as appropriate magnification, strategy for use of quantitation software etc. Furthermore it may be difficult to exact drawings of the membranes, for instance in this study to draw the endothelial cell membranes due to the presence of caveola. Another difficulty was to gather enough pictures of t-tubuli.

4.4. Conclusion

By quantitative electron microscopy it is shown that in the heart AQP1 is located in the endothelium with no or very little AQP1 in the cardiomyocytes and in the t-tubuli. In the

endothelium AQP1 is located on or very close to both the apical and the basal cell membrane. There may also be AQP1 in the caveola.

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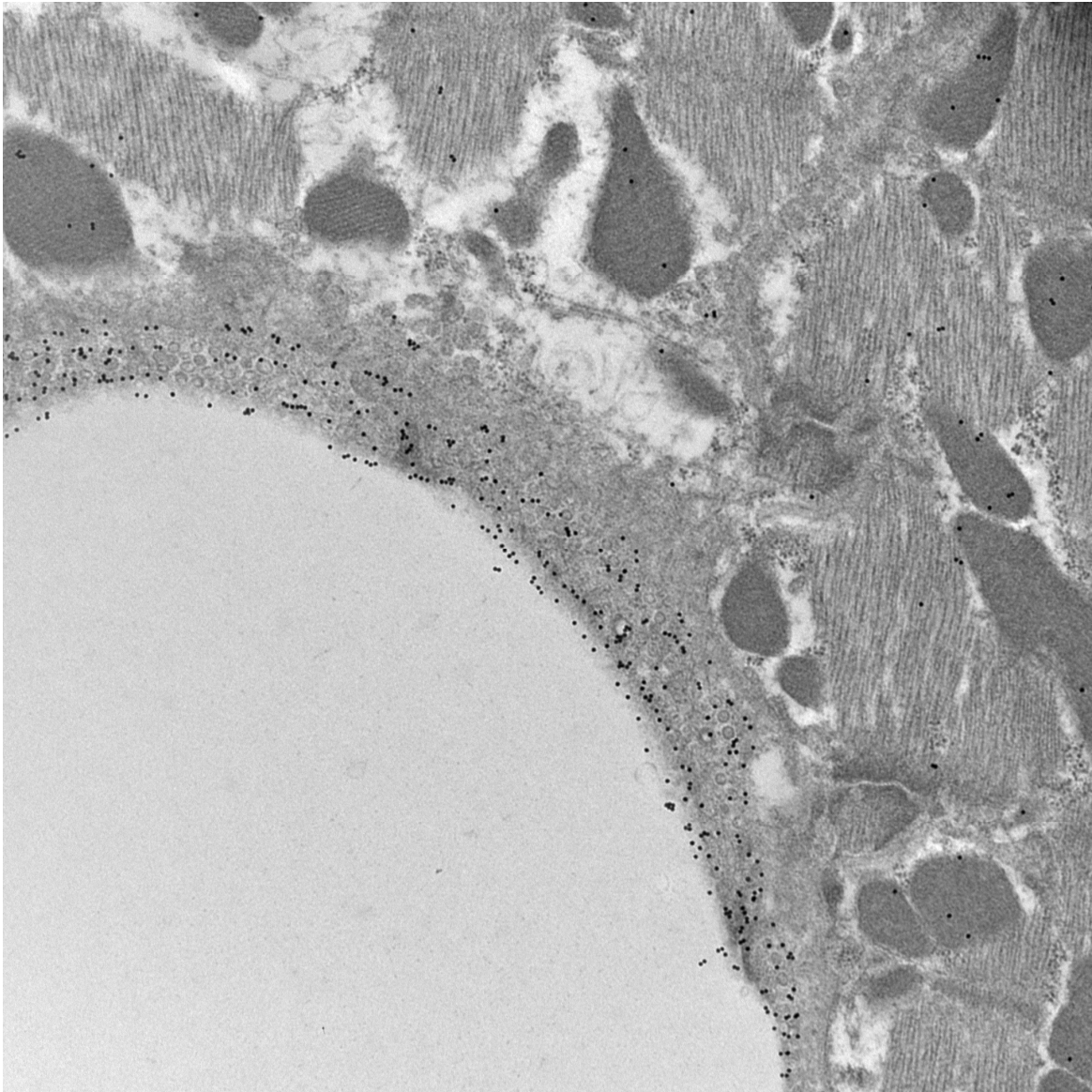
Appendix I- Pictures of different capillaries

Figure aI.1   *Massive amount of caveolae in capillary. It seems that the gold particles are*
distributed in several "layers" in the endothelial wall, i.e. the apical surface
of the cell membrane, the basal cell membrane and the basal membrane.
There is also more background here than in the previous pictures. 16500 x
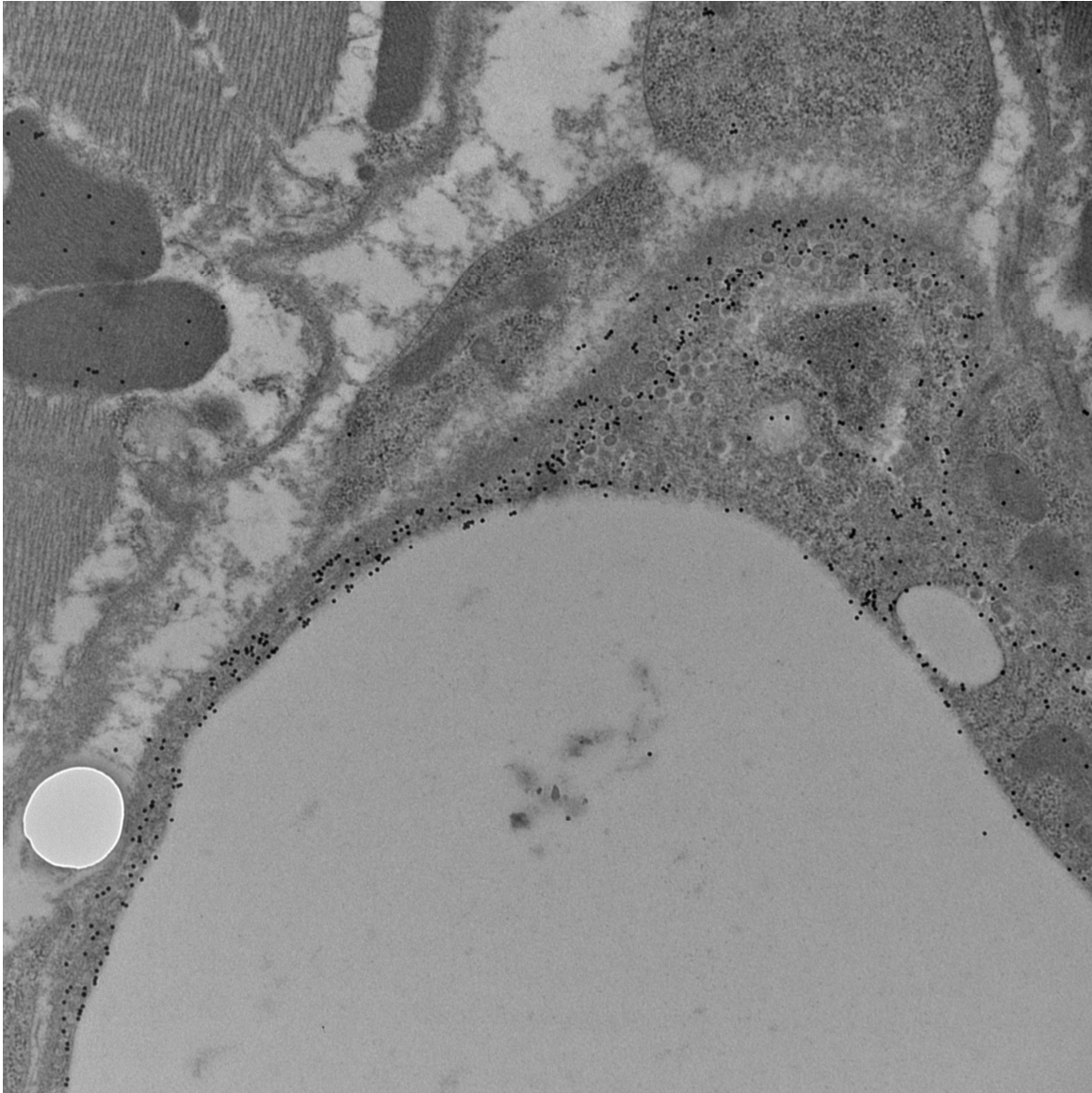*magnification.*

Figure aI.2   *Picture showing a capillary with endothelial nucleus. The white hole in the tissue at the left is a artifact which has ruptured, whereas the artifact at the right in the middle of the endothelial wall has not.16500 X magnification.*

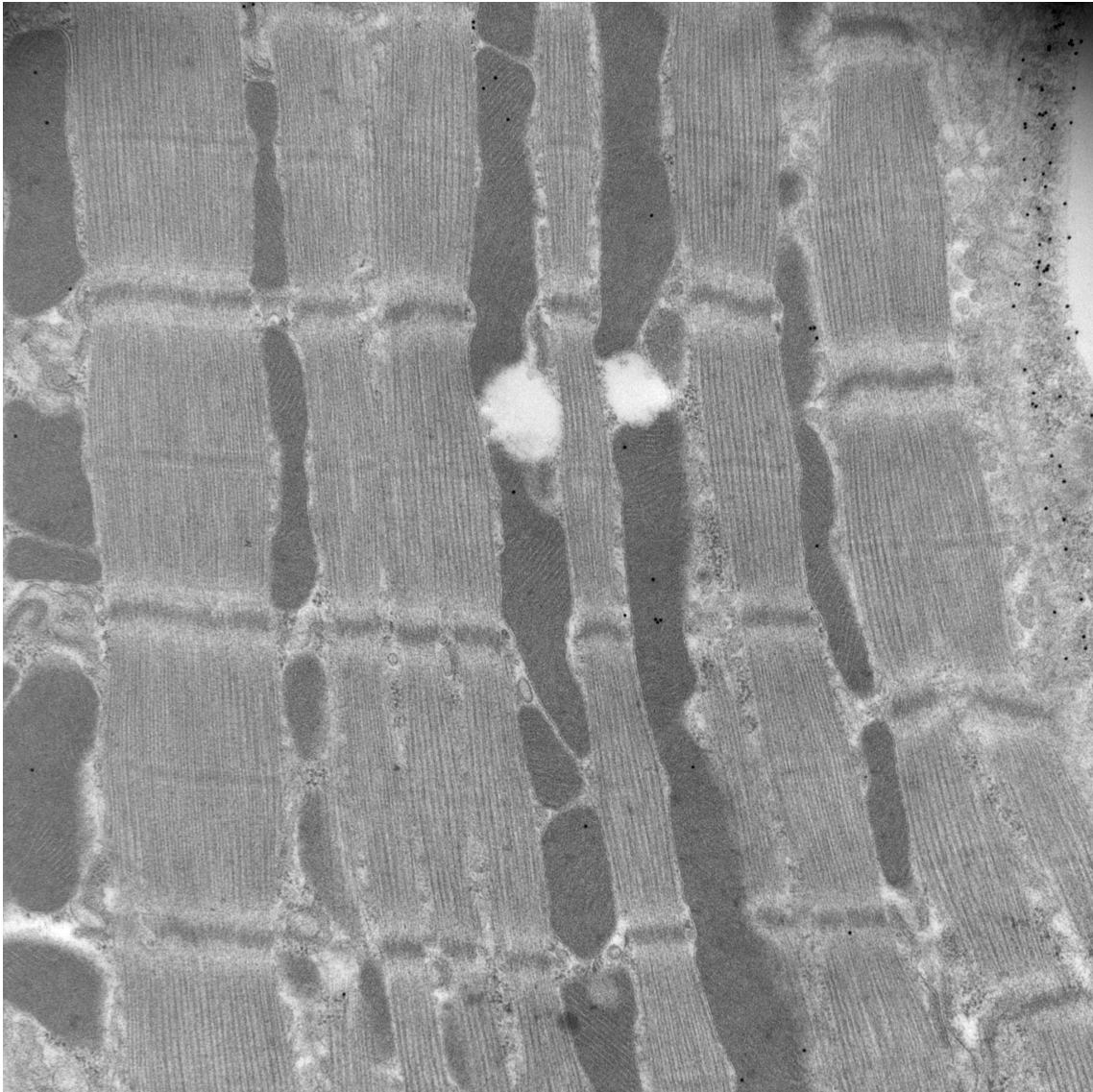**Appendix II- Pictures of different t-tubuli**

Figure aII.1 *Longitudinally cut section showing mitochondriae and a part of a t-tubuli (with a grey substance within) in the middle of the picture. There was probably t-tubuli where the two bright round structures are. 16500 x magnification.*
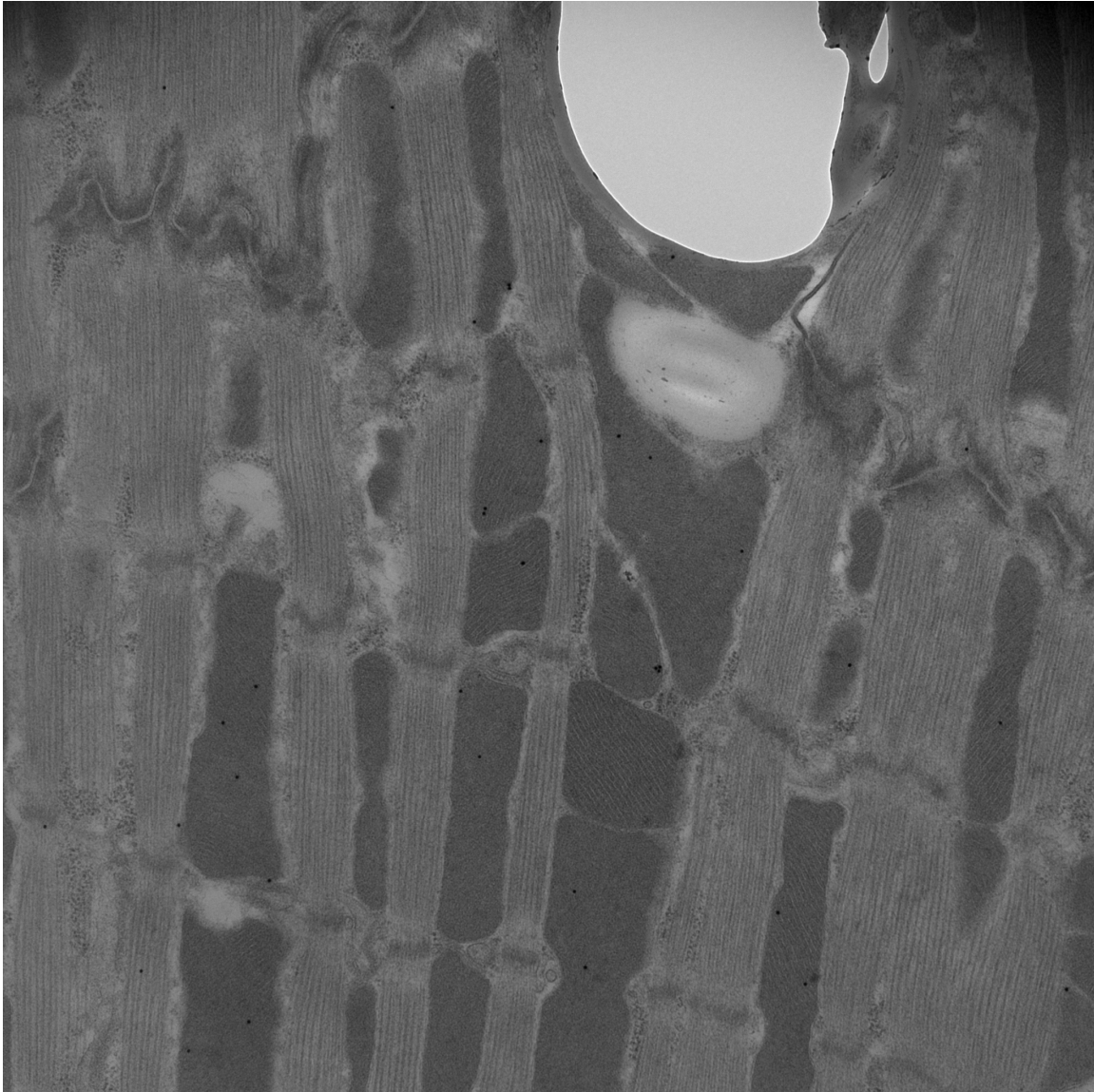
Figure aII.2 *T-tubuli in the middle of the picture, between two mitochondriae. We also see an intercalated disc, and many mitochondriae with unspesific binding of AQP1-antibody. 16500 x magnification.*