HUMAN ENDOTHELIAL CELL CONTRIBUTION TO ALZHEIMER'S DISEASE

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Dementia on maailman laajuisesti yksi merkittävimmistä syistä yksilön toimintakyvyn laskulle. Sen yleisin syy on Alzheimerin tauti. Alzheimerin tauti on etenevä muistisairaus, joka rappeuttaa aivojen kudosrakenteita ja heikentää aivojen toimintaa, johtaen lopulta vääjäämättömästi potilaan kuolemaan. Alzheimerin taudin tarkkaa aiheuttajaa ei tunneta, mutta siitä tunnetaan perinnöllinen ja ei-perinnöllinen muoto. Perinnöllisen muodon tärkeimpiin aiheuttajiin kuuluu Presenilin 1 (PSEN1) geenimutaatiot, jotka altistavat potilaan Alzheimerin taudin varhaiselle puhkeamiselle muuttamalla γ-sekretaasin toimintaa. Yksi PSEN1 geenin mutaatioista on PSEN1 Δ9, joka altistaa muiden presenilin mutaatioiden tavoin potilaan Alzheimerin taudin varhaiselle muodolle. Alzheimerin taudille altistavien geenimutaatioiden lisäksi viimeaikaisissa geenitutkimuksissa on löydetty Alzheimerin taudilta suojaava amyloidiprekursoriproteiinin (APP) mutaatio A673T, joka vaikuttaisi antavan suojaa Alzheimerin taudin puhkeamista vastaan.

Syventävissä opinnoissani tutkin näitä kahta mutaatioita ja niiden vaikutusta ihmisen endoteelisoluihin hyödyntäen ihmisen indusoituja pluripotentteja kantasoluja (hiPSC). Materiaalina tutkimuksessani käytin tutkimusryhmän aiemmin keräämiä PSEN1 Δ9 mutaatiota kantavan henkilön hiPSC soluja, saman henkilön PSEN1 Δ9 suhteen mutaatiokorjattuja hiPSC soluja, APP A673T mutaation omaavan henkilön hiPSC soluja, sekä näiden mutaatioiden suhteen terveen henkilön hiPSC soluja.

Tutkimuksen aikana nämä kantasolut sulatettiin syväjäädytyksestä, niiden määrä moninkertaistettiin kantasoluasteella, erilaistettiin verisuonen endoteelisoluiksi, värjättiin immunosytokemiallisesti varmistaen erilaistamisprosessin lopputulos, mitattiin kvantitatiisivella PCR:llä ja ELISA menetelmällä. Tulokset analysoitiin Excel tiedonkäsittelyohjelmalla ja esitetään tässä raportissa visuaalisia graafeja hyödyntäen.

Olennaisina tuloksina havaitsin APP A673T mutaatiota kantavan henkilön hiPSC soluista erilaistettujen verisuonien endoteelisolujen Amyloidi Betaa (Aß) ulospumppaavan geenin: low density lipoprotein receptor-related protein 1 (LRP1), sekä glukoosin soluunottoa toteuttavan proteiinin geenin: glucose transporter type 1 (GLUT1) geenien ekspression olevan koholla verrattuna terveen koehenkilön endoteelisoluihin, PSEN1 mutaatiota sairastan koehenkilön endoteelisoluihin, sekä PSEN1 mutaatiota sairastavan henkilön mutaatiokorjattuihin endoteelisoluihin nähden. Nämä löydökset ovat sopusoinnussa havaitun taudilta suojaavan ominaisuuden kanssa.

Sopiva jatkotutkimuksen tutkimuskysymyksiä voisi olla APP A673T mutaation omaavien henkilöiden endoteelisolujen Aß:a ulospumppaavien proteiinien toiminnan tehostumisen ja glukoosin soluun oton tehostumisen varmistaminen todellisia konsentraatioita mittaamalla, sekä geenien ekspression muutosten varmistaminen laajemmalla otannalla PCR mittauksia hyödyntäen.

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Globally, dementia is one of the leading causes of individual disability. The most common cause of it is Alzheimer's disease (AD). AD is a progressive neurodegenerative disease that degenerates patients' brain tissue, leading to a decline in brain functions and, ultimately, to inevitable death. The exact cause of AD is still unknown, but it presents itself in a sporadic and hereditary form. One of the most significant hereditary forms of early-onset AD has been linked to Presenilin 1 (PSEN1) gene mutations. These mutations in PSEN1 alter the function of γ-secrertase. One of these mutations is PSEN1 Δ9. Like the other PSEN1 mutations, this also predisposes patients to an early-onset of AD. In addition to predisposing mutations, in recent genetic studies, it has been found that the gene mutation A673T in amyloid precursor protein (APP) seems to provide a protective function against the onset of AD.

This thesis compares these two mutations and their effect on endothelial cell function. This comparison was performed by utilizing human-induced pluripotential stem cells (hiPSC). The study material included pre-collected and programmed hiPSCs from a patient carrying PSEN1 Δ9 mutation, from the same patient with a corrected PSEN1 Δ9 mutation, from a person carrying APP A673T mutation, and from a person carrying neither of these mutations.

During this thesis, these stem cells were thawed from deep-freezing, and multiplied at stem cell level. These cells were then differentiated into blood vessel endothelial cells (EC) and stained using immunocytochemistry to validate successful differentiation. The mature ECs were then analyzed using quantitative real time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). Finally, the results were analyzed using Microsoft Excel and are presented in visual graphs in this report.

The most significant findings were increased gene expression of efflux pump protein low-density lipoprotein receptor-related protein 1 (LRP1), and glucose intake protein glucose transporter type 1 (GLUT1) in the endothelial cells of the APP A673T mutation carrying person, when compared to the other previously mentioned cell lines.

Suitable follow-up studies could provide measurements to verify the improved Aß efflux pumping and glucose intake in ECs and the central nervous system (CNS) of APP A673T mutation carriers. These studies could be targeted to provide verification on the level of genetic activity and on the functional level as actual differences in concentrations in ECs and in the CNS.

ABBREVIATIONS

Aß = amyloid beta

AD = Alzheimer's disease

APP = amyloid precursor protein

BACE1 = beta-site APP cleaving enzyme 1

BBB = blood-brain barrier

CNS = central nervous system

hEC = human endothelial cell

ELISA = enzyme-linked immunosorbent assay

GLUT1 = glucose transporter type 1

hiPSCs = human induced pluripotential stem cells

LRP1 = low density lipoprotein receptor-related protein 1

mRNA = messenger ribonucleic acid

NO = nitric oxide

NOS3 = nitric oxide synthase 3

Pgp = permeability glycoprotein

PSEN1 = presenilin 1

qRT-PCR = quantitative real time polymerase chain reaction

RAGE = receptor for advanced glycation end products

ROCK = Rho kinase inhibitor

Table of contents

1 INTRODUCTION

Worldwide, dementia is one of the most prevalent causes of disability. It is a syndrome that presents significant deterioration of memory, cognition, and behavior. Dementia is a common syndrome with approximately 50 million people worldwide suffering from it. On top of this, there are approximately 10 million new cases diagnosed every year. Nonetheless, despite how common dementia is, it is still not considered as a normal part of aging but rather as a pathological phenomenon (WHO 2020, 2020). The most common cause of dementia is Alzheimer's disease (AD). It appears to be the primary cause for approximately 60-70 % of all dementia cases (Memory loss diseases. Current Care Guidelines, 2020).

Several different theories attempt to explain the development of AD (Hardy & Allsop, 1991). Two main theories explaining the AD are the amyloid theory and the neurofibrillary tangle theory. Together, these pathological mechanisms induce neuronal apoptosis and loss of synapses, especially in the brains' mesial temporal lobules. This process leads to the typical phenotype of AD (Memory loss diseases. Current Care Guidelines, 2020; Niikura et al., 2006). In the amyloid theory, the main idea is that there is evidence of amyloid beta (Aß) plague build-up in AD patients' brain parenchyma. Aß plague build-up interrupts normal cell function and contributes to neuronal cell death. In complement to the amyloid theory, it is thought that the blood-brain barrier (BBB) may play a role in Aß build-up in the brain parenchyma. Thus, leading to an onset of AD (Zenaro et al., 2016). BBB carefully regulates human brain homeostasis with highly selective semi-permeable qualities. It also protects the central nervous system (CNS) from pathogens by not allowing them to enter the it. It accomplishes this by forming a continuous barrier between the peripheral blood circulation and the CNS (Cai et al., 2018a). Among other molecules, the BBB also regulates the influx and efflux of Aß. As a conclusion, one of the potential causes of Aß build-up in the brain parenchyma of AD patients may be the BBBs reduced ability to pump Aß out of the brain parenchyma into the circulating bloodstream.

Nonetheless, from a therapeutical point of view, correcting or even overcompensating the effects of AD in BBB has not been studied thoroughly. Therefore, targeting research for therapeutic advances to the dysfunction of BBB is a potential research target in the combat against AD (Cai et al., 2018b).

To obtain the needed endothelial cells for this thesis, human induced pluripotential stem cells (hiPSCs) were selected as most suitable. They are ethically more sustainable in supply than collecting mature endothelial cells directly from the vessels of selected patients. Moreover, they are also easier to multiply.

The endothelial cells used in this study were differentiated from hiPSCs collected from a patient with presenilin 1 (PSEN1) Δ 9 mutation, its isogenic control in which the PSEN1 Δ 9 mutation was corrected, from a person with APP A673T mutation, and from a healthy donor. These cells were differentiated to human endothelial cells (hEC). Aß concentration was measured from the cells and from their growth medium. Further, the gene expression for pump proteins related to Aß and glucose metabolism were measured, as well as the gene expression for nitric oxide (NO) synthesis.

2 THEORETICAL BACKGROUND

2.1 Alzheimer's disease

2.1.1 Alzheimer's disease

AD is the most common cause of dementia. It is the primary cause of approximately 60-70 % of dementia cases (Memory loss diseases. Current Care Guidelines, 2020). It is believed to be caused by excessive extracellular Aß-plaque build-up in brain parenchyma. (Keene et al., 2018) Another pathological mechanism is hyperphosphorylated tau-proteins, which destroy microtubules inside neurons, leading to apoptosis of the affected cells (Memory loss diseases. Current Care Guidelines, 2020).

2.1.2 Onset of Alzheimer's disease

The most significant risk factor for AD is biological age. Age 65 is considered as a threshold value between an early-onset AD and a late-onset AD. Approximately 10 % of AD cases are early-onset, and 90 % late-onset cases. Among the early-onset cases, 5 % can be explained by a specific gene mutation. The most important genetic causes for early-onset AD are chromosome 21 trisomy - also known as Down Syndrome, mutations of PSEN1 and presenilin 2 genes. These affect the brain through increased Aß build-up in the brain parenchyma (Cacace et al., 2016).

2.1.3. Genetics of Alzheimer's disease

Mutations in the gene PSEN1; encoding one of the four proteins of the γ-secretase are a known risk factor for an early-onset AD (Kelleher & Shen, 2017). γ-secretase is a protein that cuts amyloid precursor protein (APP) into two soluble particles. The reason why a mutation in PSEN1 gene causes AD and cognitive decline is yet to be confirmed. Nonetheless, it is known that mutations in this gene increase the ratio of Aß42/Aß40 in the brain parenchyma extracellular space by shifting the cutting point of the APP, cut by the γ-secretase (Duff et al., 1996). As Aß42 is thought to be a significant contributor to Aß aggregation in the brain, it has been suggested that shift in this ratio speeds up the Aß plaque development and is linked to AD and cognitive decline (Kelleher & Shen, 2017).

APP gene mutation A673T protects against AD and cognitive decline (Xia et al., 2021). It has been suggested that this is due to an impaired ability of the A673T mutated APP protein to attach to a protein called beta-site APP cleaving enzyme 1 (BACE1) (Jonsson et al., 2012). BACE1 cleaves APP into Aß in the extracellular space (Sathya et al., 2012). This mutation lowers the turnover rate of BACE1, resulting in less APP forming into Aß and therefore reducing Aß aggregation (Jonsson et al., 2012).

2.2 Blood-brain barrier in Alzheimer's disease

Human brain blood supply is based on arterial supply and venous outflow. Capillaries connect these two. In the human brain, these capillaries are not fenestrated like in the rest of the body. Instead, they form a continuous barrier between the blood supply and brain parenchyma. Capillaries form from blood vessel endothelial cells, and in the brain, they are also surrounded by CNS glial cells called astrocytes. These astrocytes form arms that enclose the capillaries inside them. This structure is called the BBB. It is semi-permeable filter, and it effectively regulates molecular transportation across it, carefully maintaining the homeostasis in the brain parenchyma (Soinila et al., 2016).

In an AD patient's brain, the BBB has been recorded to have reduced ability to pump Aß out from the brain tissue. This reduced ability increases the concentration of Aß in the patient's brain tissue, causing an acceleration of Aß plaque build-up in the extracellular matrix. Further, in AD patients, an increase of oxidative stress in the BBB has been recorded. This oxidative stress may partly explain why BBB dysfunction appears in AD patients (Cai et al., 2018).

2.3 Related proteins

2.3.1 LRP1

LRP1 is an efflux pump protein found in the human BBB. It transfers Aß from brain tissue into endothelial cells of the BBB. It is located mainly on the abluminal side of the BBB. The expression of LRP1 reduces significantly in aging people and AD patients. This leads to an increase of Aß in the brain parenchyma, possibly resulting in the onset of AD (Ramanathan et al., 2015). The effects of APP A673T mutation on the LRP1 gene expression have not been studied in BBB endothelial cells.

2.3.2 RAGE

RAGE is a significant influx protein pump for Aß in the BBB. It transports Aß back to the brain parenchyma. In AD patients, expression of RAGE has been recorded as elevated. It seems to provide the BBB a capability to reabsorb the Aß that has been transferred into the bloodstream, increasing the concentration of the Aß in the brain (Ramanathan et al., 2015).

2.3.3 GLUT1

Glucose is the main energy source for human neurons. Human brain of an adult consumes averagely 20% of all the energy consumed in a human body per day (Mergenthaler et al., 2013). The human brain is highly dependent on GLUT1 in terms of receiving glucose. The expression of this gene has been decreased in AD patients' brain microvasculature (Ramanathan et al., 2015). This process leads to glucose deficiency in the brain parenchyma (Winkler et al., 2015). The decrease of glucose further causes the brain tissue to go into a hypometabolic state, affecting LRP1 gene expression in the BBB by decreasing it. It occurs as a compensatory mechanism to preserve glucose for more critical functions of the brain parenchyma cells (Ramanathan, et al. 2015). In individuals with familial AD, it was observed that they have a reduced glucose intake to the hippocampus, parietotemporal cortex, and posterior cingulate cortex (Winkler et al., 2015).

2.3.4 Pgp

Permeability glycoprotein (Pgp) is a pump protein found on the luminal side of the BBB endothelial cells. It transfers Aß42 & Aß40 out from the endothelial cells into the blood circulation. Thus, co-operating with LRP1 to pump Aß out of brain tissue. Loss of Pgp gene expression has been linked to an accumulation of Aß in the brain parenchyma. Pgp gene expression in AD patients has been shown to vary between the different locations of the brain microvasculature (Jeynes & Provias, 2013).

2.3.5 NOS3

Nitric oxide synthase 3 (NOS3) is a protein producing NO in endothelial cells. In AD, NO is believed to cause oxidative stress in several brain areas (Serretti et al., 2007). NO is produced by neurons, especially in pathological stages like AD. Produced in adequate amounts it acts as a vasodilator. However, when NO concentrations increase, it may cause apoptosis and cytotoxic cascades in neurons and the BBB endothelial cells. Soluble guanylate cyclase, a receptor of NO, has a crucial role in synaptic transmission and plasticity of the cerebral cortex and hippocampus areas. These areas are both involved in memory functions of the brain (Malinski, 2007).

3 HYPOTHESES

The mutation in the PSEN1 gene predisposes the carriers to early onset AD and cognitive decline (Steiner et al., 1999). For comparison, this study used hiPSC lines obtained from a patient carrying PSEN1 Δ 9 mutation and its isogenic line with the PSEN1 Δ 9 mutation corrected, hiPSC obtained from person carrying an APP A673T mutation that is considered protective, and a line from a healthy donor. The results are expected to show changes in the endothelial profile depending on the genetic background.

Aims of this thesis are:

- Differentiate existing hiPSCs into hECs.
- To validate successful differentiation by methods of immunocytochemistry.
- To analyse gene expression of LRP1, Pgp, RAGE, GLUT1, and NOS3 using quantitative real time polymerase chain reaction (qRT-PCR).
- To compare the gene expression between AD, AD iso, APP A673T, and control cell line.
- To attempt to define if these pump protein gene activities could explain PSEN1 mutation predisposition for AD and APP A673T mutation protective qualities against AD.
- To measure Aß concentrations in the cells and in their growth medium using enzyme-linked immunosorbent assay (ELISA).
- To compare Aß concentrations between AD, AD iso, APP A673T, and control cell line.

4 MATERIALS & METHODS

4.1 Cell lines

Obtaining hECs from a patient with a PSEN1 Δ 9 mutation, a person carrying APP A673T mutation, and a person without either of these mutations was crucial in order to perform this study. This goal was achieved by using hiPSCs. HiPSCs can be cultured and differentiated into the target cell type using a preferred method in laboratory conditions (Karagiannis et al., 2019). The hiPSCs used in this study had been previously collected from skin biopsy fibroblasts and reprogrammed into hiPSCs. These cells were collected from patients with PSEN1 Δ 9 mutation, a person with APP A673T mutation, and a person without either of these mutations. Additionally, a genetically corrected PSEN1 Δ 9 cell line was used from the same donor as PSEN1 cell line. See *table 1* (Lehtonen et al., 2018; Oksanen et al., 2017). In this thesis, they were matured into hECs prior to analyzing them.

TABLE 1: Summary of human induced Pluripotent Stem Cell lines used in this thesis.

Patient	Cell line name	Age	Sex	Mutation	Purpose	References
1	AD	47	F	PSEN1 Δ 9	Mutation predisposing to AD	(Oksanen et al., 2017)
1	AD iso (isogenic)	47	F	corrected PSEN1 Δ 9	Isogenic Control	(Oksanen et al., 2017)
2	control	64	M	-	-	(Oksanen et al., 2017)
3	protective	65	M	APP A673T	A mutation reducing the risk for AD	(Lehtonen et al., 2018)

4.2 Differentiation

All hiPSC:s were first placed in small clusters in E8 media with Rho kinase inhibitor (ROCK) 1:2000. After 24 hours of incubation the growth medium was replaced with StemDiff APEL with 6µM CHIR [CHIR-99021 Cayman (3 mM): 6µM (dilution: 1:500, 2 µl/ml)] + Penicillin / streptomycin (dilution:

1:200)). After 24 hours of incubation, the medium was replaced with StemDiff APEL medium supplemented with 25ng/ml BMP4, 10ng/ml FGF and 50ng/ml VEGF. After 48 hours of incubation, the cells were lifted with tryple and seeded on matrigel coated plates in EC growth medium MV2 with an additional 50 ng / ml VEGF. Finally, the MV2 medium was changed every 2 days for 4-6 days until only hECs grew on the matrigel-coated plates. A visual representation of this differentiation protocol can be seen in *figure 1.*

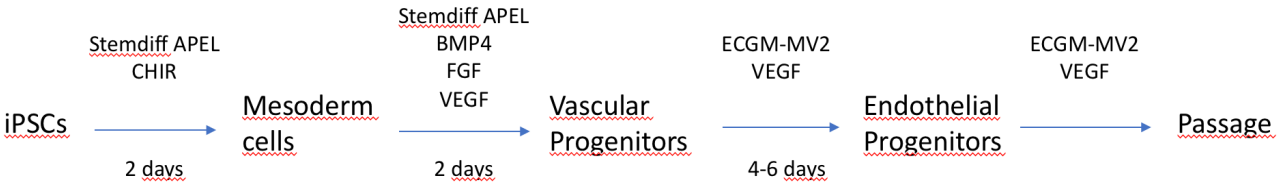

FIGURE 1: Timeline of the differentiation protocol used to differentiate hECs from hiPSCs.

4.3 Immunocytochemistry

Immunocytochemistry was used to characterize the differentiated hECs. This is done because, after differentiation, there are usually multiple different mature and progenitor cell types present. The last step of the differentiation protocol is purification. Immunocytochemistry is used to validate the outcome of this purification. The goal is to produce a homogenous population of hECs.

Before cells can be imaged, they are fixed onto a plate. The fixing protocol is presented underneath. All used antibodies are listed in *table 2*.

4.3.1 Protocol used for Immunocytochemistry fixation:

24 well plate was used. 80 000 cells were plated to each well, 6 wells for each cell line. They were washed once with PBS. Then 4 % Formaldehyde was placed into each well and incubated at room temperature for 20 minutes. The cells were washed with PBS 2-3 times. They were then covered with 750 ml PBS + Tween20 + NaN3 until staining. The cells were stored at + 5°C on the plate, sealed with parafilm.

TABLE 2: Table of primary and secondary antibodies used for immunocytochemistry.

Target protein	Primary antibody	Secondary antibody	Characterizing function
Von Willebrand factor	rabbit anti-Von Willebrand factor (1:400), ab6994 (abcam)	goat anti-rabbit 488 (1:400), Molecular Probes A11011	Von Willebrand factor is only found in Megacaryocytes and endothelial cells. It functions as an attachment site for thrombocytes during a rupture of the endothelial layer of all blood vessels (Bryckaert et al., 2015).
Claudin-5	Mouse-Claudin 5 Monoclonal Antibody (4C3C2) (1:150), catalog#: 35-2500 (Invitrogen)	Alexa Fluor 568 goat anti-mouse IgG (H+L) (1:300), Molecular Propes, cat. A11004	Claudin-5 is a tight junction protein found on the surface of endothelial cells to bind them together and prevent molecular traffic between the endothelial cells (Morita et al., 1999).
VE-cadherin	rabbit anti-VE-cadherin FITC (1:100), ab 33321 (abcam)	-	VE-cadherin is exclusively expressed in endothelial cells (Corada et al., 2001). It is a binding protein between endothelial cells to form a united endothelium layer (Abu Taha & Schnittler, 2014).

4.3.2 Protocol for VE-cadherin staining

ECs were grown on fibronectin-coated glasses in 24-well plates. Fixed cells were washed 5 times with PBS, 500 µl each time. Primary antibody diluted to 1:150 was added. Cells were incubated at

4°C overnight and were wrapped in a dark sealing. After incubation, cells were washed 5 times with PBS, 500 µl each time. After washing, cell nuclei were stained with DAPI, diluted 1:2000 in water, for 5 minutes. Then, cells were washed with PBS 5 times, 500 µl each time. Cells were left in 500 µl of water. Coverslips were mounted on glass with a drop of fluoromount-G. Slides were allowed to dry at 20°C. Slides were then stored in a darkened container at 4°C. Images were taken within 2 months after staining.

4.3.3 Protocol for Claudin-5 and von Willebrand factor stainings

ECs were grown on fibronectin-coated glasses in 24-well plates. They were stored in 5 % normal goat serum in PBS. Cells were washed 5 times with PBS, 500 µl each time. ECs were permeabilized with 0.1% Triton X-100 in PBS at 20°C for 30 minutes. Then, unspecific binding sites were blocked by incubating the ECs in 5 % normal goat serum in PBS for 1 hour. Then, the primary antibody was added each to their own sets. For Claudin-5 staining, Mouse-Claudin 5 Monoclonal Antibody diluted to 1:100. For von Willebrand factor, rabbit anti-Von Willebrand factor diluted to 1:400. Primary antibody was incubated at 4°C overnight. After incubation, ECs were washed 5 times with PBS, 500 µl each time. Then, the secondary antibody was added. For Claudin-5 staining, Alexa Fluor 568 goat anti-mouse IgG (H+L) was diluted to 1:300 in 300 µl of PBS. For von Willebrand factor, goat anti-rabbit 488 was diluted to 1:300 in 300 µl PBS. Secondary antibody was incubated at 20°C for 1 hour in a dark container. After incubation, ECs were washed 5 times with PBS, 500 µl each time. After washing the nuclei were stained with DAPI diluted 1:2000 in water for 5 minutes. ECs were washed 5 times in PBS and the last set of PBS was left in the wells. Coverslips were mounted on glass slides with a drop of fluoromount-G. Glass slides were left to dry in 20°C and stored in a dark container at 4°C. Images were taken within 2 months after staining.

4.4 qRT-PCR

QRT-PCR was used to evaluate the differences in gene expression of LRP1, RAGE, GLUT1, Pgp, and NOS3 between all studied cell lines. QRT-PCR provides a good sensitivity and high level of scaling of the produced messenger ribonucleic acid (mRNA) and, therefore, of the cell gene expression

(Jozefczuk & Adjaye, 2011). It makes it possible to measure accurately singular gene expressions and was, therefore, chosen to be one of the methods of this thesis.

The needed cells for the qRT-PCR measurement were cultured, collected, and stored in various sets and each sample pellet was frozen to -70°C, then thawed and used to extract RNA by *RNeasy MiniKit* (Qiagen). The passaging number varied between freezing sets. List of passaging number for each set can be found in *table 3*.

TABLE 3: Passaging numbers of each set at the time of freezing.

Cell line	**freezing set 1**	**Freezing set 2**	**Freezing set 3**
AD	2 x p.1	1 x p.3	2 x p.4
AD iso	2 x p.1	1 x p.4	2 x p.5
control	2 x p.2	1 x p.3	2 x p.4
APP A673T - protective	2 x p.4	2 x p.5	1 x p.6

Protocol for cDNA synthesis from RNA:

1. mix RNA sample (500 ng) and nuclease-free water.
    - full volume 27.5 µl
    - use 0,5 ml nuclease-free tubes on ice

2. Add hexamer primer to samples.

    Sample 27,5 µl
    Random hexamer primer (Fermentas #S0142) 1µl

    In total: 28,5 µl
    - Vortex and spin

3. Incubate samples for 5 min at 65°C in qRT-PCR machine.

4. Prepare synthesis mixture

Synthesis mixture (per sample)	µl
10 mM dNTP	2
5x reaction buffer for Maxima RT	8
Ribonuclease inhibitor (40 U/ul Fermentas E00381)	0.5
Maxima reverse transcriptase (Fermentas EPO742)	1
	In total: 11.5

5. Add 11.5 µl synthesis mixture to samples.
    - Vortex and spin

6. PCR run

    25 °C 10 min
    50 °C 30 min
    85 °C 5 min

RT-qPCR protocol:

Relative quantification of the genes of interest was done in SYBR-green assays. Results are relative to healthy control sample and normalized against ß-actine gene expression. All reactions were done in triplicates.

PCR mix per reaction

10 ul Maxima SYBR green master mix (Thermo scientific)
1 ul 10 uM primer F

1 ul 10 uM primer R

3 ul H2O

5 ul cDNA synthesis (diluted 1:5)

PCRs were run in Step One Plus- machine (Applied Biosystems) in a 2-step reaction with melting curve

95C 7'

95C 15''

60C 30''

go to step 2 39x

65C 5'

65C > 95C, 0,5C incr.

After the run:

Result data was analyzed by utilizing a delta delta Ct analysis. From each 4 cell lines; 5 samples were analyzed. Out of those samples, major outliners were excluded from all the cell lines, leaving 4 samples for each cell line to be analyzed. For each cell line, a mean Ct value was determined, then a standard deviation of each sample was compared to that mean value. These Ct values were processed as follows:

dCt = Ct (gene of interest) – Ct (control gene)

ddCt = dCt (sample) – dCt (control sample)

Power = 2^{-ddCt}

A mean fold change value was determined for each sample, and a standard deviation was determined within the cell line samples. Kruskal-Wallis test was used to calculate the statistical significance of the results. Kruskal-Wallis test was chosen because the number of samples was under 30, and therefore the results may not obey the normal distribution.

4.5 ELISA

ELISA was the chosen method for measuring Aß 40 and Aß 42 peptide concentrations in the mature hECs and their growth medium. ELISA has excellent accuracy in low concentration measurements, which it achieves by utilizing antigen-antibody binds (Aydin, 2015). As Aß levels in hECs and their growth medium were expected to be low to non-existent, the ELISA method was a good choice.

In ELISA testing, 12 well plates and 2 wells for each cell line were used for PSEN1, AD iso, and control lines. At this point, the protective cell line was not ready to be used. Thus, it was not included in the test. 200 000 cells from the desired cell line were placed in each well. In addition, 1.0 ml of growth medium was placed in each well.

Invitrogen kits used in the test:

Amyloid beta 40 Human ELISA Kit
Catalog # KHB3482

Amyloid beta 42 Human ELISA Kit
Catalog # KHB3442

The plate was incubated in the refrigerator in +5 °C overnight instead of shaking it as directed in the kit protocols.

Results were analyzed by one-way ANOVA to test for differences between tested groups.

5 RESULTS

5.1 Differentiation

Every cell line used in this study differentiated successfully into hECs. However, the protective cell line had a small remnant of impurities post-differentiation. Images of the differentiation progress can be found in *figure 2*.

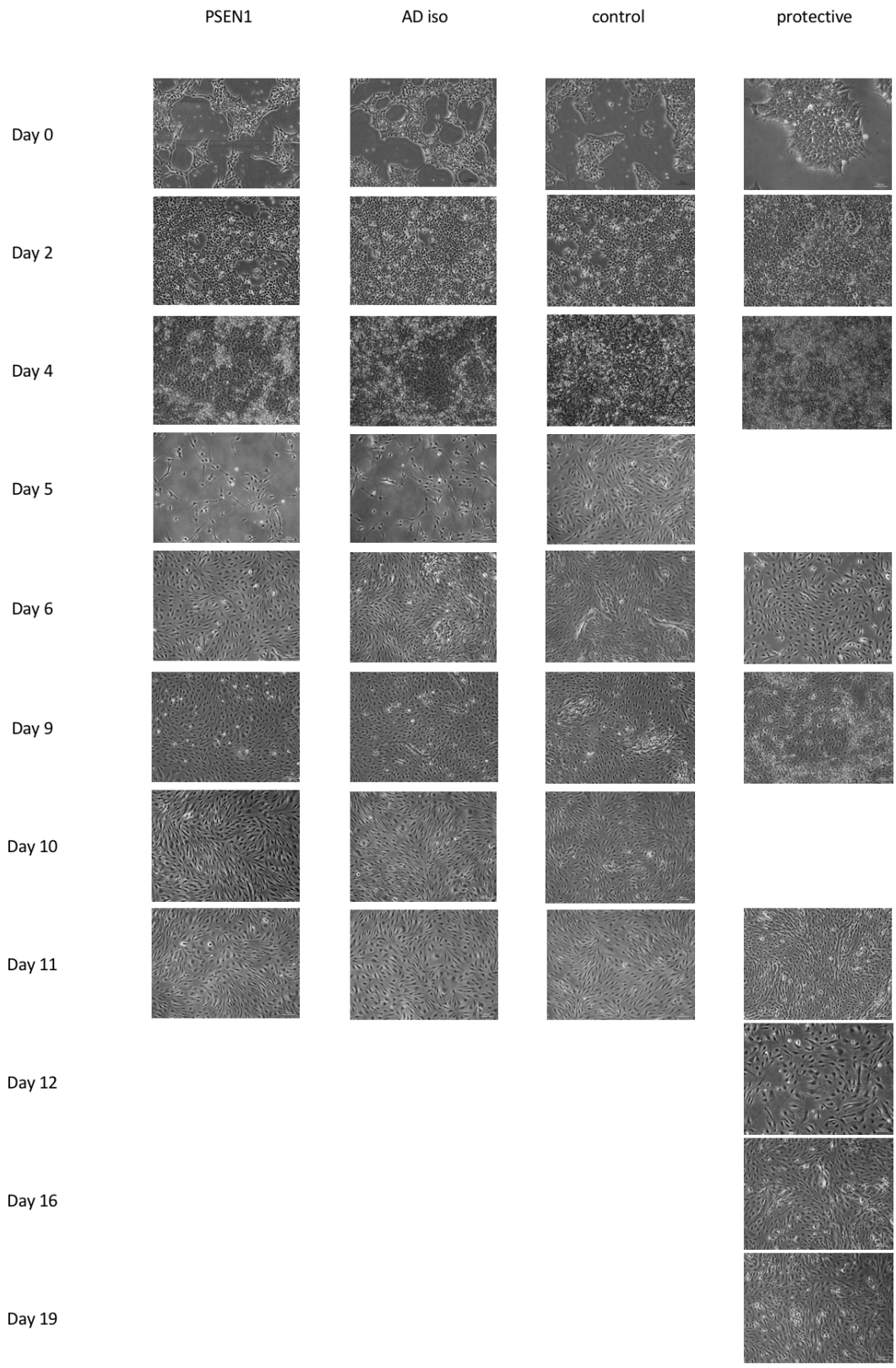

FIGURE 2: Differentiation. On day 0, 10x magnification was used. On days 2-19, 5x magnification. The change is due to the realization that 5x magnification shows the differentiation progress better than 10x, hence the magnification change on day 2.

5.2 Immunocytochemistry

Fluorescence images are presented in *figure 3*. All cell lines expressed Claudin-5, VE-cadherin, and von Willebrand factor. Thus, it can be concluded that the tested cells from each cell line were hECs. All images were taken from the most representative area of each cell line.

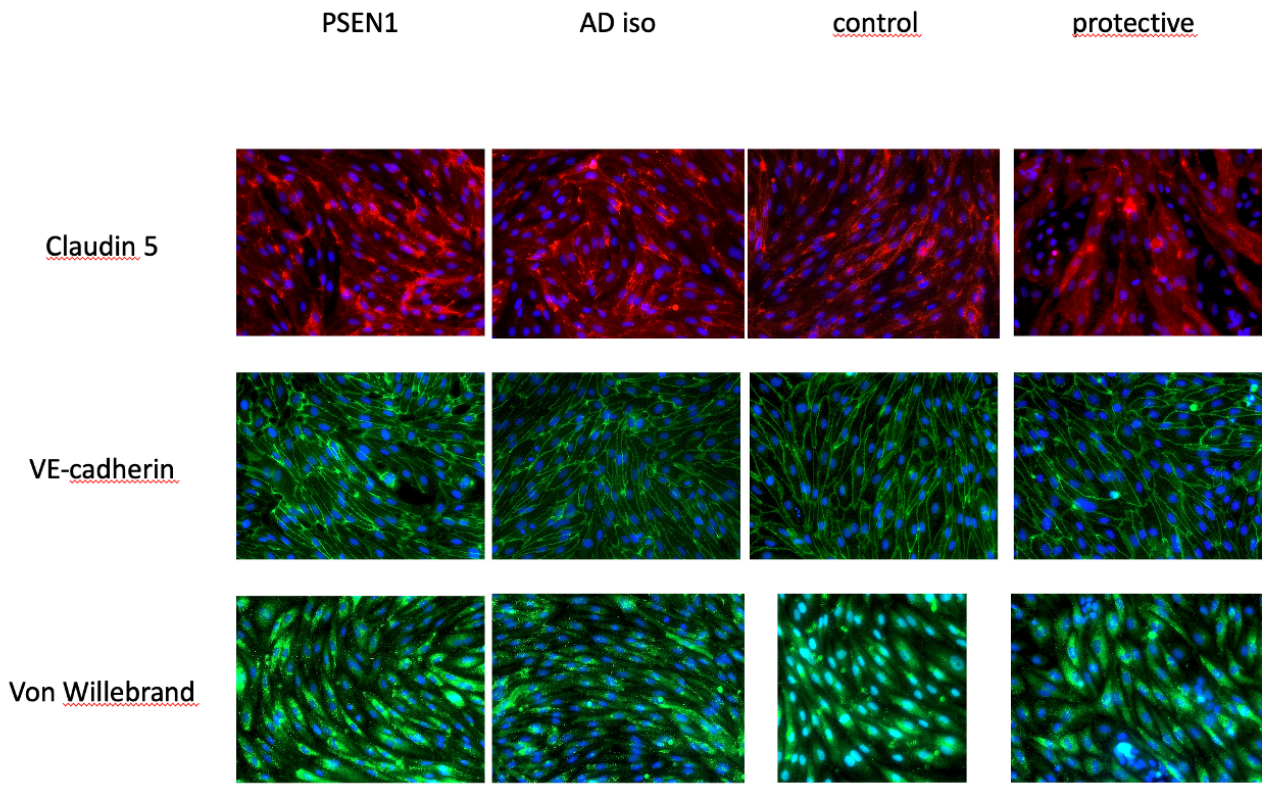

FIGURE 3: Representative fluorescence images. It can be seen that every cell line differentiated into endothelial cells expressing all 3 proteins tested by antibodies listed in table 2.

5.3 qRT-PCR

LRP1 gene expression is approximately 4-fold higher in the hECs derived from the protective line when compared with AD iso line, $p < 0.05$. The same can be visually seen between the protective line compared to AD line and control line hECs but this difference is not statistically significant (Fig.4).

No statistical difference was found among AD, AD iso, and control hECs.

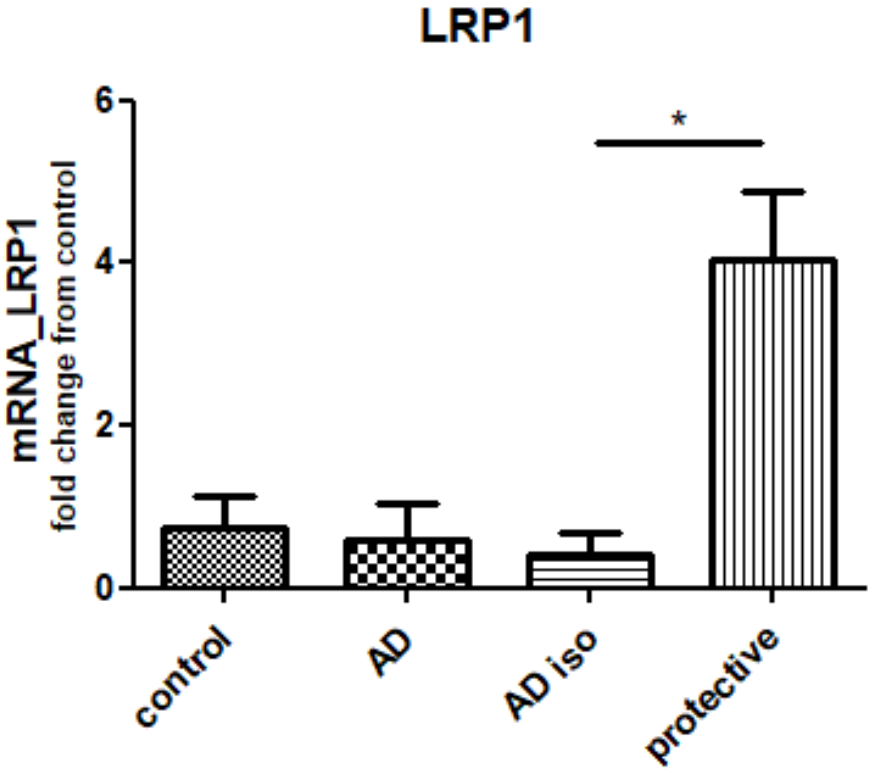

FIGURE 4: Gene expression levels of mRNA LRP1 measured from hECs derived from hiPSCs. The data is presented as mean +/- SD (n=4). *p <0.05. The statistical method used for comparing each cell line was one-way-anowa. *AD* represents PSEN1Δ 9; *AD iso* represents PSEN1 Δ 9 with corrected mutation; *protective* represents APP A673T mutation, which is considered protective.

GLUT1 gene expression is 8-fold higher in the hECs derived from the protective line than from the AD line, $p < 0.01$ (Fig.5). Also, the same difference is visible between the protective line and AD iso line, $p < 0.05$ (Fig.5). In addition, GLUT1 expression is 4-fold higher in hECs from the protective line compared to the control line. However, this difference does not have a statistical significance.

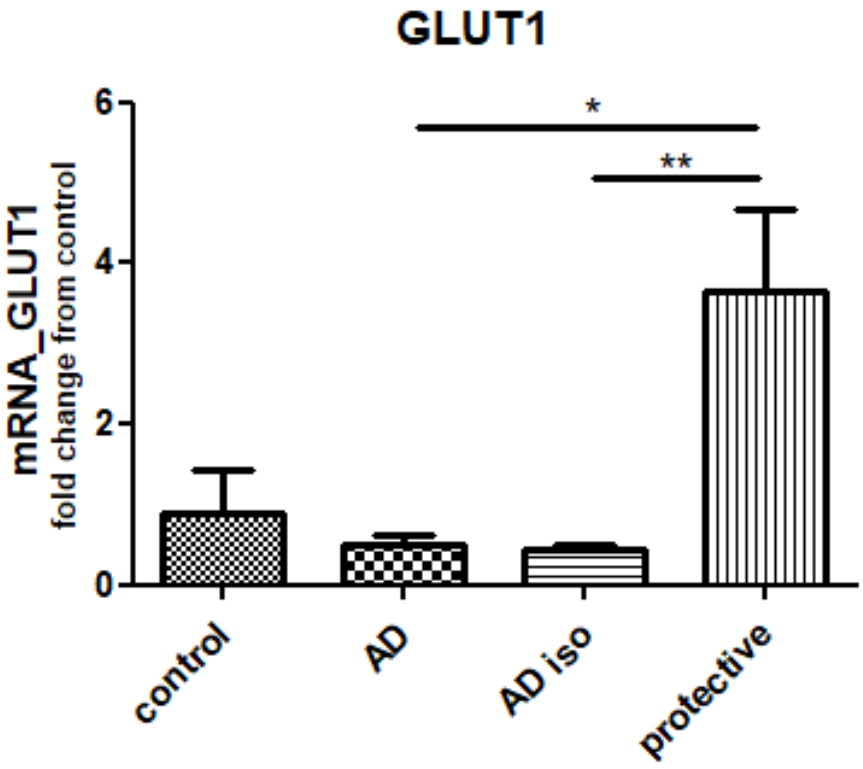

FIGURE 5: Gene expression levels of mRNA GLUT1 measured from hECs derived from hiPSCs. The data are presented as mean +/- SD (n=4). **p <0.01, *p <0.05. The statistical method used for comparing each cell line was one-way-anowa. *AD* represents PSEN1Δ 9; *AD iso* represents PSEN1 Δ 9 with corrected mutation; *protective* represents APP A673T mutation, which is considered protective.

In gene expression of RAGE, there were no differences found between hECs cell lines (Fig.6).

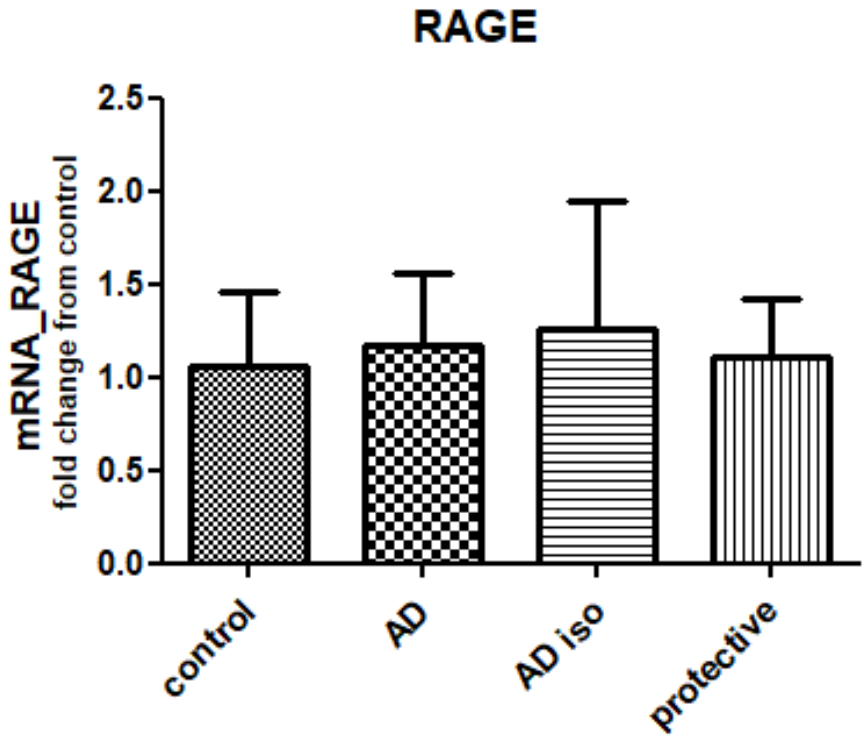

FIGURE 6: Gene expression levels of mRNA RAGE measured from hECs derived from hiPSCs. The data is presented as mean +/- SD (n=4). The statistical method used for comparing each cell line was one-way-anowa. *AD* represents PSEN1Δ 9; *AD iso* represents PSEN1 Δ 9 with corrected mutation; *protective* represents APP A673T mutation, which is considered protective.

In gene expression of Pgp, there were no statistically significant differences between hECs cell lines (Fig.7).

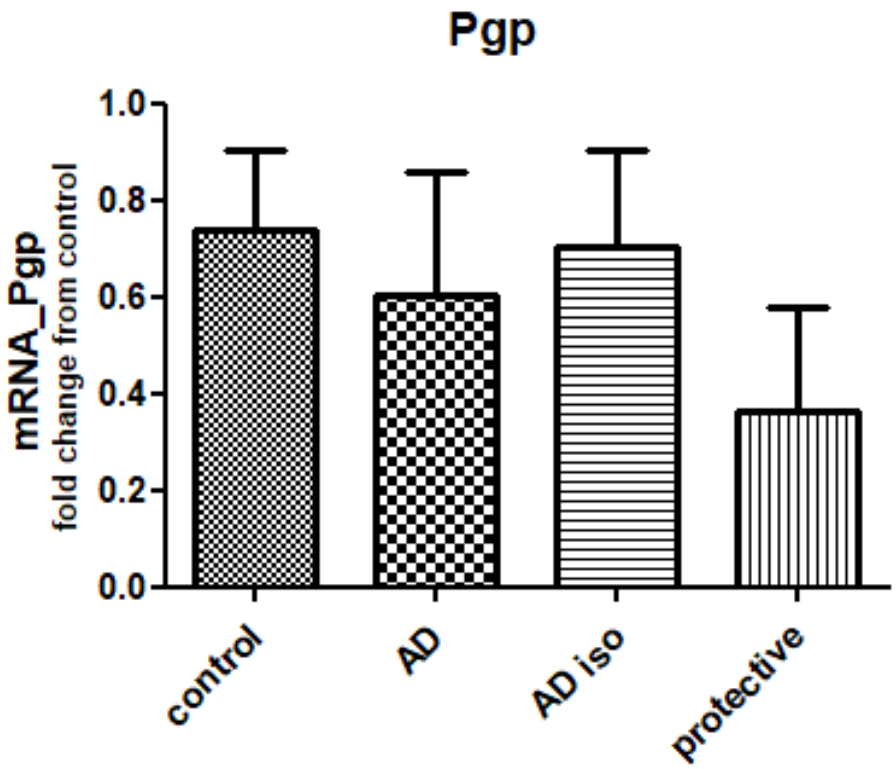

FIGURE 7: Gene expression levels of mRNA Pgp measured from hECs derived from hiPSCs. The data is presented as mean +/- SD (n=4). The statistical method used for comparing each cell line was one-way-anowa. *AD* represents PSEN1Δ 9; *AD iso* represents PSEN1 Δ 9 with corrected mutation; *protective* represents APP A673T mutation, which is considered protective.

NOS3 has a statistically significant 5-fold increase in hECs from the protective line compared with control hECs, $p < 0.05$ (Fig.8). Between control and AD / AD iso hECs, there is approximately a 2-fold increase, although this is not a statistically significant change. Also, between AD line / AD iso line and protective cell line, there were no statistically significant changes. However, approximately 2-fold higher expression can be seen in a visual examination.

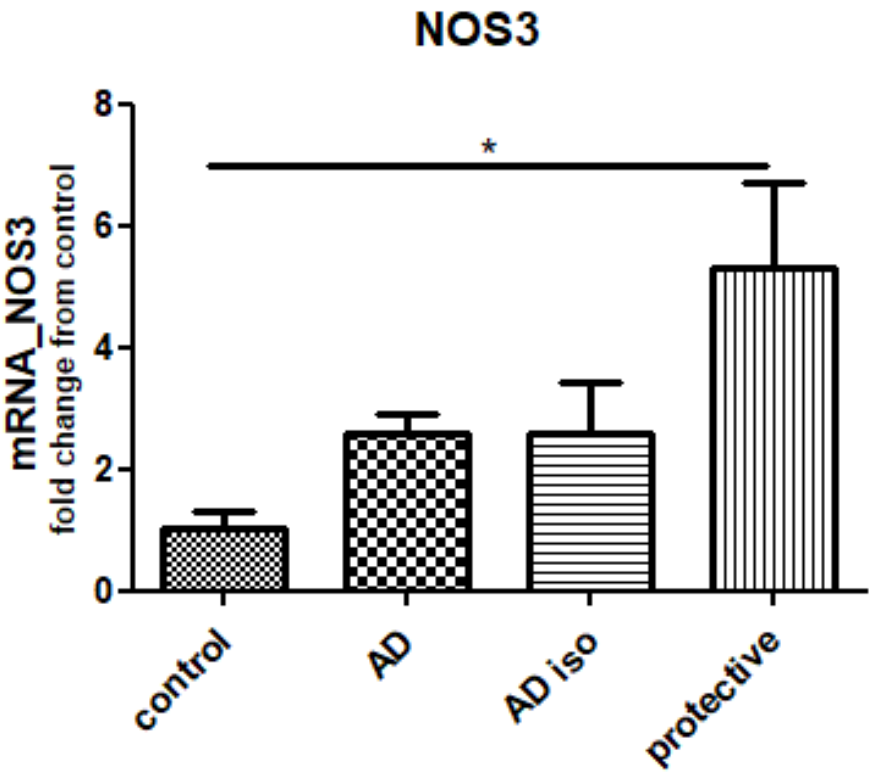

FIGURE 8: Gene expression levels of mRNA NOS3 measured from hECs derived from hiPSCs. The data is presented as mean +/- SD (n=4). *$p < 0.05$. The statistical method used for comparing each cell line was one-way-anowa. *AD* represents PSEN1Δ 9; *AD iso* represents PSEN1 Δ 9 with corrected mutation; *protective* represents APP A673T mutation, which is considered protective.

5.3 ELISA

Concentration measures of Aß 40 and Aß 42 were taken from the cell hyaloplasm and growth medium using ELISA method. There were no detectable differences between hECs derived from control, AD, and AD iso cell lines.

6 DISCUSSION

Worldwide dementia is a significant health problem. AD is the leading cause of it. The most important risk factor for it is aging. To a patient, AD is eventually fatal when it has progressed far enough. Globally, the number of patients suffering from AD is increasing due to the constantly increasing life expectancy. In addition, life expectancy seems to continue to increase when more people have access to decent health care. Furthermore, the constant development of medical knowledge and treatments also advances the life expectancy of people that already have access to adequate healthcare. Therefore, further understanding of AD is needed to combat this continuously growing problem.

Hyperphosphorylated tau-protein and Aß in brain parenchyma have been under great interest for developing treatments against AD. However, a curative treatment is yet to be found. Therefore, it is imperative to widen our research to understand the pathological importance of BBB dysfunction in AD and treatments targeting it. This study was a pilot study to examine potential BBB hEC contribution to the onset of AD and protective functions against it.

Immunocytochemistry showed that nearly all cells tested were endothelial cells. In the AD iso cell line, some impurities were present among the hECs. As a result, all the cell lines consistently expressed Claudin-5, VE-cadherin, and Von-Willebrand factor, of which VE-cadherin is specific for hECs (Corada et al., 2001). The weakness of immunocytochemistry is the enormous number of cells to be inspected, compared to the area that can be viewed reliably under a microscope. Also, what can be observed through a microscope only gives a rough estimate of the ratio between hECs and other cells.

QRT-PCR was performed successfully, and the main results of this thesis were concluded from the difference in gene expression between cell lines. It is to be noted that, within the qRT-PCR method, there were differences between passage number of each set. This may produce minor variations within the results of each cell line. Since qRT-PCR requires precise pipetting technique; it can be assumed that some statistical error in the results is due to human inaccuracy in pipetting. This phenomenon is further emphasized due to the small number of samples used in this thesis.

As concluded from the qRT-PCR results, the statistically significant findings are as follows:

- LRP1 gene expression in the hECs derived from the protective line is increased 4-fold compared to other cell lines.
- GLUT1 gene expression in the hECs derived from the protective line is increased approximately 8-fold compared with AD and AD iso lines.
- NOS3 gene expression in the hECs derived from the protective cell line is increased 5-fold compared to the control line.

In qRT-PCR, none of the gene expressions show a significant difference between hECs derived from control, AD, and AD iso lines. On the other hand, the hECs derived from the protective line demonstrated significant differences to the previously mentioned cell lines in expression levels of LRP1, GLUT1, and NOS3. Thus, it can be concluded that the APP A673T gene mutation likely alters the gene expression of the hECs. These findings are concurrent with current knowledge about the overall impact of APP A673T reducing the likelihood of developing AD. Possible mechanisms to explain the protective qualities could be the improved LRP1 efflux pump efficiency of Aß from the hECs into the bloodstream. Furthermore, a genetically globally increased GLUT1 expression prevents the brain parenchyma from ending into a hypometabolic state towards the senile end of life. These findings are concurrent both with the hypometabolic state theory and the amyloid theory. On the contrary, NOS3 gene expression increase in the protective cell line opposes protective function regarding AD. The current understanding is that NO increases vascular inflammation, predisposing the patient to AD rather than protecting the patient from it. A possible explanation for this is that the LRP1 expression increase and GLUT1 expression increase overcome the NO expression increase, leading as a whole to a clinically protective function regarding the APP A673T mutation. Another possible explanation is that the current understanding of high NO concentration increasing the risk for AD is incorrect and needs to be re-evaluated.

The increased GLUT1 and LRP1 gene expression in APP A673T mutated endothelial cells suggests that this gene mutation alters the amount of Aß and glucose in ECs. Nonetheless, this study does not prove ECs of people with APP A673T mutation to have a lower concentration of Aß in their ECs,

nor in the CNS. The same applies to the glucose concentrations in the ECs or the CNS of the APP A673T mutation carriers.

Results of ELISA show no differences in Aß 40 / 42 concentrations between any of the hECs. This was partly expected as the hECs were grown without astrocytes and pericytes. Nonetheless, understanding Aß movement to and from hECs could be restudied when other cell-type components of BBB are present.

This study provides limited proof of the results and can be mainly used to target more extensive studies on the same topic. Further studies could improve the current study's reliability and statistical significance in several ways. Increasing the number of hiPSC donors and the hECs cell samples will improve the accuracy of the results. Furthermore, robotic pipetting systems would offer the highest quality available to these measurements.

New study questions could examine other protective mutations against AD and be used to further examine the significance of APP A673T in efflux Aß pumping, glucose intake and NO synthesis. These studies may be conducted on the level of genetical activity, as well as on functional levels, measuring the actual concentrations of Aß and glucose in the ECs and CNS of APP A673T mutation carriers.

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