Bachelor's project

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biomedical Laboratory Science

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Does exercise training or exercised blood affect gene expression in the brain of a rat model of Alzheimer's disease?

Bachelor's project in Biomedical Laboratory Science
Supervisor: Nathan Scrimgeour, Atefe R. Tari and Wenche Slettahjell Prestvik

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CERG
Cardiac Exercise Research Group

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Preface

This paper is a final project for our bachelor's degree in medical laboratory technology at the Norwegian University of Science and Technology (NTNU). The bachelor thesis has been prepared in collaboration with the Cardiac Exercise Research Group (CERG).

The project has given us an insight of the process and execution of medical research and new knowledge of the global health issue that Alzheimer's disease is. We have developed a deeper understanding of the beneficial effects that exercise has on lifestyle related diseases. Writing this paper has been challenging and interesting, and our learning curve has been steep. We have enjoyed the opportunity to try our hand as researcher.

We want to take this opportunity to thank our amazing bachelor supervisors; Nathan Scrimgeour, Atefe R. Tari and Wenche Slettahjell Prestvik, for helpful guidance and constructive feedback. A special thanks to Nathan Scrimgeour for your patience, expertise, and follow-up throughout the process.

Trondheim, 19.mai 2020

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Abstract

Alzheimer's disease is the most common form of dementia worldwide, and the prevalence is only expected to increase in the future, as a result of an aging global population. Alzheimer's disease is a chronic neurodegenerative disease which leads to memory loss, cognitive impairment and loss of bodily functions. As the disease progresses, Alzheimer's patients become more and more dependent on care, elevating the psychosocial impact on the patient and their family. The cost of diagnostics, treatment and care is also a large economic burden for both patients, families, and society. Over time the neuron damage of the disease may become so severe that it can be fatal.

Cardiac Exercise Research Group (CERG) aims to study the effect of exercise on lifestyle related diseases in order to promote good health throughout life. This paper is written as part of their research project on the effects of exercise on Alzheimer's disease. We have based our work on the following hypothesis:

"Does exercise training or exercised blood affect gene expression in the brain of a rat model of Alzheimer's disease?"

The experiment was originally planned to be executed in CERG's laboratory using an Alzheimer's disease rat model and quantitative PCR to look for alterations in gene expression as result of exercise. Due to the COVID-19 pandemic, and national restrictions, we were not allowed access to the laboratory. As a result of this, our paper takes a more theoretical approach and is written as a research plan.

The paper presents five genes that we have found to be associated with Alzheimer's disease, and we have designed PCR primers for each of these that may be used when executing the experiment at a later date. Calculations and results presented in the paper are based on fictive values that show possible outcomes of the experiment and troubleshooting and mitigation for the different scenarios are discussed. Finally, we have made some recommendations for further research.

Sammendrag

Alzheimers sykdom er den mest utbredte formen for demens i verden. Prevalensen er forventet å øke i fremtiden som et resultat av en aldrende verdensbefolkning. Alzheimers sykdom er en kronisk neurodegenerativ sykdom som forårsaker hukommelsessvikt, kognitiv svikt og tap av kroppsfunksjoner. Alzheimers pasienter blir gradvis mer og mer avhengig av pleie etter hvert som sykdommen utvikler seg. Dette medfører psykososiale belastninger både for pasienten og pårørende. Diagnostikk, pleie og behandling av Alzheimers pasienter medfører store økonomiske kostnader for pasienter, pårørende og samfunnet. Over tid kan Alzheimers sykdom medføre død som følge av den omfanget av den neurodegenerative skaden.

Cardiac Exercise Research Group (CERG) forsker på effekten trening har på livsstilssykdommer for å fremme god helse gjennom hele livet. Denne oppgaven er skrevet som en del av deres forskningsprosjekt om effektene trening har på Alzheimers sykdom. Vi har tatt utgangspunkt i følgende problemstilling:

"Har trening eller trent blod effekt på genuttrykket i hjernen hos rotter med Alzheimers sykdom?"

Eksperimentet var opprinnelig planlagt utført ved CERG sine laboratorier. Utgangspunktet for forsøket var å bruke en rotte modell med Alzheimers sykdom, og ved hjelp av kvantitativ PCR se etter endring i genuttrykk som resultat av trening. På grunn av COVID-19 pandemien og påfølgende nasjonale restriksjoner var det ikke mulig å utføre forsøket ved laboratoriet. Som følge av dette har denne oppgaven fått en teoretisk vinkling og blir lagt frem som en mulig gjennomføringsplan for forsøket.

Oppgaven presenterer fem gener som er assosiert med Alzheimers sykdom. Vi har designet PCR primere for hvert gen som kan benyttes under utførelsen av eksperimentet. Beregninger og resultater presentert i oppgaven er basert på fiktive verdier som viser mulige utfall av eksperimentet. Mulige feilkilder og forbedringstiltak som kan gjennomføres blir diskutert. Til slutt har vi kommet med anbefalinger til videre forskning på området.

Contents

1.0 Introduction

1.1 Alzheimer's Disease

Dementia is a collective term for neurodegenerative brain diseases that cause memory impairment and reduce cognitive function. The most common neurodegenerative disease is Alzheimer's Disease (AD), making up 50-70% of dementia cases (1). AD is characterized by gradual and progressive impairment of memory and cognitive function and is most common in elderly patients, although early-onset AD may occur. Early onset AD accounts for 5% of AD cases and affects people under the age of 65 (1). Typical AD-symptoms include episodic memory loss, behavioral change, attention impairment and language deficits (2). Over time, AD can damage the brain so severely that essential bodily functions such as mobility, swallowing and even breathing may be compromised, leading to fatal outcomes (3, 4). AD may also be the underlying cause of death (3, 4), often with aspiration pneumonia as the primary cause of death (3).

AD pathophysiology is characterized by amyloid plaques and neurofibrillary tangles. Amyloid plaques are diffuse or compact accumulations of insoluble amyloid-β in the brain, which together with neurofibrillary tangles are associated with neurodegenerative processes (2). Neurofibrillary tangles consist of neurons filled with hyperphosphorylated tau proteins that give the neuron its tangled shape (2). Deposits of amyloid-β may also cause reduced blood flow and this amyloid angiopathy may lead to complications such as vascular dementia or stroke (4). Ultimately, AD is caused by extensive damage and loss of neurons in the brain (3). In order to definitively diagnose AD, the presence of amyloid plaque deposits and neurofibrillary tangles in the brain is required. This diagnosis can only be definitively made following post-mortem analysis (2, 3). Other observations, analyses of biomarkers in spinal fluid, and cognitive tests are used to diagnose patients with probable AD on a clinical basis.

Age is commonly accepted as the main risk factor for AD (5). Due to the aging population worldwide, the prevalence of AD is increasing. In 2015 the number of AD patients was estimated to be around 47 million people, and it is estimated that this number will reach 75 million by 2030 and 131 million by 2050 (5). Low-income and middle-income countries are expected to have the largest increase in AD incidence (1). In Norway, 80-100 thousand people

are diagnosed with dementia diseases, and 60% of these are estimated to have AD (6). The costs of diagnostics, treatment and care, loss of income and psychosocial factors are all burdens that affect society, patients, and their families. Developing new treatment to reverse or prevent AD is important to meet these increasing challenges.

The hippocampus has a significant role related to learning and memory functions and shows some of the earliest damages in the development of AD (7). The hippocampus is situated in the medial temporal lobe of the brain and consists of structures such as dentate gyrus (DG) and Cornu Ammonis regions (CA1, CA2 and CA3) (8, 9). The DG is known as one of the major areas for neurogenesis in the adult brain (10). In AD patients, the neuronal density in CA-regions, especially the CA1-region, is significantly decreased (7).

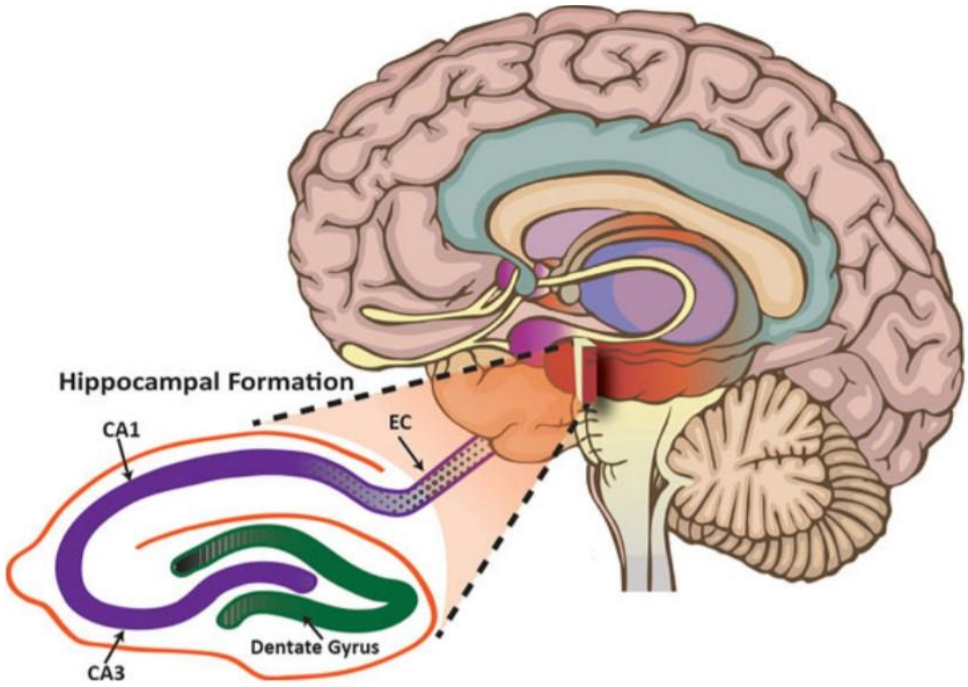

Figure 1: Schematic overview of hippocampal structure in the human brain. (Source: http://www.creative-diagnostics.com/blog/wp-content/uploads/2019/02/The-structure-of-hppocampus.jpg, April 28 2020)

Neurogenesis, angiogenesis, and synaptic plasticity are associated with good brain health. Neurogenesis is the process where new neurons are formed in the brain. Angiogenesis is the process of forming new blood vessels. The process of generating new neurons in the brain is dependent on a sufficient supply of oxygen and other bloodborne factors, thus angiogenesis is

closely linked to neurogenesis (11). A nerve signal is communicated between neurons when an electric impulse leads to release of neurotransmitters, which pass over the synapse and is converted back to an electric impulse in the adjacent neuron (12). Plasticity refers to the brain's ability to adapt to new information. Synaptic plasticity is the efficiency of the communication between neurons and is important for memory storage. An AD brain is characterized by neuron degeneration which reduces brain density and decreases connection between neurons, reducing synaptic plasticity (11, 13). In addition, inflammation caused by neurodegeneration can induce stress-activated pathways and exacerbate AD pathophysiology (14-16). Over time, atrophy widely damages parts of the brain to an extent where the total brain volume is reduced (17) leading to loss of bodily functions beyond memory and cognition.

1.2 Physical activity and AD risk

There are several risk factors associated with development in AD, and these can be sorted into modifiable, semi-modifiable and non-modifiable risks. Modifiable risks for AD are common to other lifestyle-related diseases and include physical inactivity, obesity, hypertension, diabetes mellitus type 2, smoking and high consumption of alcohol (5). These factors are also known as risk factors for cardiovascular diseases (CVD) and affect the brain's vascularity and neurons negatively as a result of reduced blood circulation (18). Factors such as low educational attainment, low social exposure and lack of cognitive stimulus are seen as semi-modifiable risks (5). Case studies show that modifiable and semi-modifiable risk factors account for about 30-50% of AD cases, making it possible that some cases may be preventable (19, 20). Non-modifiable risks include genetic disposition and age, which as mentioned is the main risk factor for AD (5). Genetic disposition is also a non-modifiable risk factor, but it only accounts a very small percentage of AD cases.

There is a strong connection between cardiovascular health and brain health. Many of the risk factors related to AD are also risk factors for CVD (19), and most of these can be modified with exercise. Exercise training that promotes cardiovascular health, increases brain blood flow, leading to better circulation of oxygen and nutrients which are essential for the brain function (21). In addition, exercise training can lead to decrease in concentration of systemic blood-borne inflammatory cytokines, that can pass through the blood-brain barrier and contribute to AD pathophysiology (16). Studies have successfully managed to identify certain blood-borne

factors, such as brain-derived neurotrophic factor (*BDNF*) and vascular endothelial growth factor (*VEGF*) (22-24), that are released from skeletal muscles and other tissues as a result of exercise and can cross the blood-brain barrier and promote brain health through angiogenesis, neurogenesis and increased synaptic plasticity (25-28). Exercise seems to have beneficial impact on the occurrence of these factors and may therefore have rejuvenating or prophylactic effect on dementia (22).

Change in physical activity (PA) habits may affect an individual's level of fitness (29). PA can be measured using a health maker called cardiorespiratory fitness (CRF). CRF defines the circulatory, respiratory and muscular systems' ability to supply oxygen during PA (29, 30), quantified by VO_2max. PA, or more specifically aerobic exercise, is proven to have beneficial effect on the brain health in older adults (31) and may therefore potentially prevent or partially reverse dementia and AD symptoms (32, 33).

1.3 Systemic mediation of brain health

In a study by Villeda et al., where old mice were exposed to blood from young mice through parabiosis or systemic administration, young blood had a rejuvenating effect on age-related degeneration of the brain (13). Young blood improved synaptic plasticity in the hippocampus and increased spine density of functionally and morphologically mature neurons in older rodents. Old mice treated with blood from young mice showed enhanced cognitive function compared to untreated old mice or those who were treated with blood from other old mice, when submitted to fear conditioning testing and to a spatial learning water maze test. The findings indicate that blood-borne molecules found in a young, healthy systemic environment can pass through the blood-brain barrier and induce positive changes in the old brain (23).

1.4 Genes associated with brain health

BDNF is a nerve growth factor which stimulates synaptic plasticity and neurogenesis in the adult brain (22, 34). It increases resistance to brain insult and is involved in processes related to learning and mental performance (22). Evidence indicates that reduced *BDNF* is linked to AD (35). High levels of *BDNF* injected into animals with AD have shown to enhance memory and learning due to reversal of synapse loss and improving cell signaling (36). A study using

rats showed that the level of *BDNF* mRNA in the hippocampus was increased after several days of voluntary wheel-running (22).

VEGF is a pleiotropic factor that works on the brain in many ways (24). It serves an important role in promotion and coordination of angiogenesis in mammals of all ages. This factor also works to regulate hemostasis in the brain, by regulating vasculature to comply with the demand for oxygen and metabolites. *VEGF* may also have an important function in neurogenesis and this process is dependent on sufficient blood flow. Results from an animal study on reversibly transgenic mice, expressing either a loss of cerebral *VEGF*-function or gain of cerebral *VEGF*-function, indicate that *VEGF* also has an important regulating function of plasticity in mature neurons and, as an effect of this, on contextual and spatial memory (24).

Early growth response 1 (*EGR1*) is a transcription factor that works as a regulator of synaptic plasticity and neuronal activity (37), and is associated with neurogenesis. Tumor necrosis factor (*TNF*) is a marker for inflammation (38). *PLAU* is the gene encoding for urokinase plasminogen activator (UPA1) which is involved in angiogenesis (39, 40). UPA1 may also have degenerative effect on Aβ-plaques (41, 42)

1.5 Aim of the study

Inspired by the study done by Villeda et al., the Cardiac Exercise Research Group (CERG) at the Norwegian University of Science and Technology (NTNU) has a broad hypothesis that exercise causes changes in blood that may improve brain health and neurogenesis. In order to examine alterations in gene expression related to brain health, this research plan aims to look at five genes that are biomarkers for angiogenesis, neurogenesis, inflammation and synaptic plasticity in an AD rat model. The targeted genes are *BDNF*, *VEGF*, *EGR1*, *TNF* and *PLAU*.

2.0 Materials and methods

2.1 Animals

In order to test the hypothesis that blood from trained rats may reduce AD symptoms or have rejuvenative effects on the brain, this experiment used an animal model with Wistar rats as plasma donors for McGill-R-Thyl-APP transgenic (Tg) rats. Wistar rats are one of the most commonly used rats in medical research and were chosen as donors because of their shared genetic background with the Tg rats. The Tg rat line has been genetically developed from Wistar rats to express AD-like amyloid pathology by inserting a gene for a modified human amyloid-β precursor protein (AβPP) into the rat's DNA (43). These rats are provided by Menno Witter's lab at the Kavli Institute for Systems Neuroscience / Centre for Neuronal Computation. The homozygous Tg rats develop accumulation of human amyloid-β (Aβ) proteins intraneuronally as early as one week postnatal. As the rats age, the Aβ proteins accumulate progressively, resulting in full AD-like amyloid pathology over time. At three months of age, the Tg rats show significantly reduced cognitive function as a result of Aβ-accumulation.

2.2 Study design

Wistar donor rats, aged two months, were randomized and divided into two groups; sedentary and exercised donors. Over a span of six weeks prior to plasma donation, the sedentary rats were kept without physical training while the exercised group followed a training program. The program consisted of aerobic intervals on a treadmill, where the rats were trained for 60 minutes per day, 5 times per week, in intervals alternating between 4 minutes at 85-90% of VO_2max and 2 minutes at 50% VO_2max (44). The rats' VO_2max was measured before starting the program, and every two weeks in order to adjust training intensity correctly. After six weeks, plasma was obtained from blood that was collected by cardiac puncture while the donor rats were under isoflurane anesthesia prior to euthanasia.

Plasma from the donor groups was injected into the tail vain of McGill-R-Thyl-APP rats (AD-rats), beginning at age two months, for a total of ten times over four weeks. In addition to the two groups of AD-rats receiving either plasma from sedentary or exercised donor rats, a third group was injected with saline as a control. During week five and six after the first injection, several cognitive and physical tests were run on the AD-rats and three more injections were administered. The control group was injected with saline the same amount of times as the test

groups. A schematic overview of the study design is presented in figure 2. In addition, a fourth group consisting of AD-rats followed the same exercise program as the Wistar rats over six weeks without receiving injections.

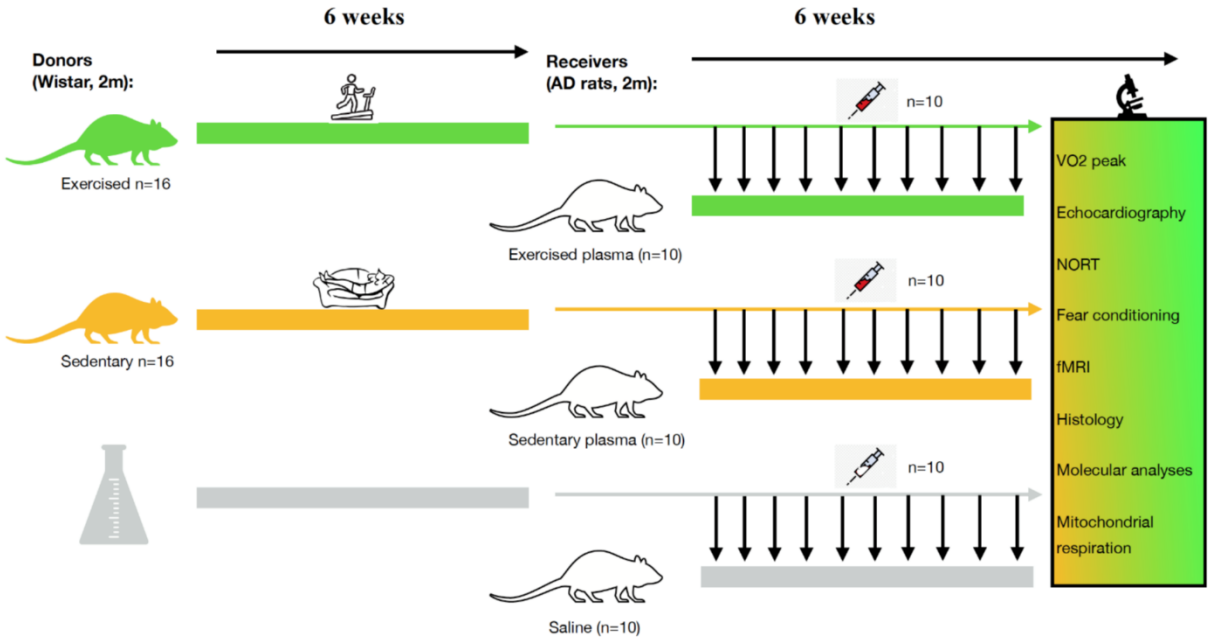

Figure 2: Schematic overview of study design (Source: CERG)

2.3 RNA extraction and reverse transcription

Brain tissue extraction was done immediately after euthanizing the four groups of AD-rats. The hippocampi, or more specifically CA region and DG, were dissected from the brains, snap frozen, and stored at -80°C.

In this study the frozen tissue will be homogenized using the Precellys 24 Tissue Homogenizer (Bertin Technologies). RNA will be extracted from the frozen tissue using RNeasy Mini Kit (Qiagen), following manufacturer's protocol unless otherwise specified. The tissue samples used are smaller than the maximum 30mg limit of the kit and will therefore be used whole. After RNA has been extracted from the brain tissue, the RNA- yield will be measured using a NanoDrop 2000/2000c Spectrophotometer (ThermoFisher Scientific). The value for pure nucleic acid is expected to lie in the rage of 2.0-2.2 measured at 260/230nm. Mini Kit (Qiagen), following manufacturer's protocol unless otherwise specified. The frozen tissue samples used are smaller than the maximum 30mg limit of the kit and will therefore be used whole. After

RNA has been extracted from the brain tissue, the RNA- yield will be measured using a NanoDrop 2000/2000c Spectrophotometers (ThermoFisher Scientific). The value for pure nucleic acid is expected to lie in the rage of 2.0-2.2 measured at 260/230nm.

Following the isolation of RNA, the next step will be reverse transcription (figure 3). This process uses the enzyme reverse transcriptase to make a complementary DNA (cDNA) from the single-stranded RNA template molecule (45). The cDNA reverse transcription process will be conducted using Quantitect Reverse Transcription kit (Qiagen) according to manufacturer´s protocol. In order to preserve the unstable structures of cDNA and remaining RNA, samples are kept at -20ºC.

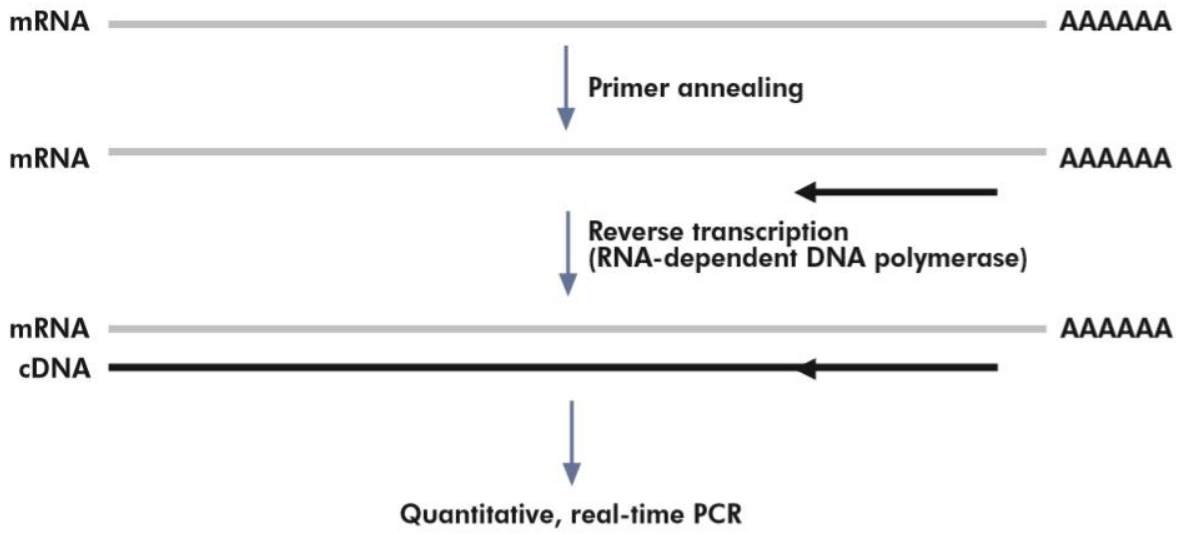

Figure 3: cDNA synthesis. Quantiscript Reverse Transcpriptase in first-strand cDNA synthesis. (Modified from: QuantiTect Reverse Transcription Handbook, March 2009, Qiagen)

2.4 Quantitative PCR

The cDNA samples will be analyzed using quantitative PCR (qPCR). PCR is a technique for amplifying targeted DNA sequences by multiple cycles of DNA synthesis (45). Each cycle consists of three steps; denaturation, annealing and extension. In the denaturation step the cDNA sample is heated briefly at 94°C to separate it into two single strands. Next the temperature will be lowered down to 50-60°C so specific primers can anneal to the template on both sides of the DNA sequence to be amplified. The annealing temperature can be adjusted to

fit the melting temperature (Tm) of the primers, but temperatures around 60°C result in more specific bindings and is therefore preferred annealing temperature. The temperature will then be raised to 72°C in the extension-step where DNA polymerase will bind nucleotides from the master mix to complementary bases on template DNA, to make double stranded DNA.

Preparations of the samples will be done using Quantitect SYBR Green PCR Kit (Qiagen) following manufacturer's protocol. The kit's master mix contains magnesium ions (Mg^{2+}). Correct concentration of Mg^{2+} is critical because it binds to DNA, primers and nucleotides in the amplification reaction and will affect the enzyme activity (46). Mg-concentration can be adjusted to increase the melting temperature of dsDNA, but this will lead to less specific bindings and is therefore not recommended. The master mix also contains SYBR Green I, which is a fluorescent dye that binds nonspecifically to double-stranded DNA products (47). In real-time PCR, data is collected during the amplification at the end of each cycle by recording the amount of fluorescence, rather than at the end. The amount of fluorescence is proportional to the amount of PCR-product. To quantify the amplification, a threshold line is set in the linear region of the amplification plot over background fluorescence. The number of cycles necessary for the fluorescent signal of the sample to cross this threshold is called cycle threshold (Ct). The Ct value is inversely proportional to the amount of amplification (46). This implies that lower Ct-value means higher sample concentration. The Ct value should not exceed 35 (48).

Preparations will be amplified, and fluorescence measured using Bio-Rad C1000 thermal cyclers with CFX96 real time modules. Each target will be tested in triplicate in each sample and a control sample with RNA-free water.

The specificity of the target can be verified by making a melt curve of the amplification (figure 4). This is achieved by slowly increasing the temperature above the Tm of the primer and measuring the fluorescence, which is then plotted as a function of the temperature. If primers are specific, this results in a single melting peak that is characteristic for the amplicon and serves to distinguish it from amplification artefacts with different Tm. If the melting curve shows more than one peak, this indicates that primers are non-specific. In this case fluorescence comes from

amplification of several sequences, and one cannot know for sure which one comes from the target, or if the target has been amplified.

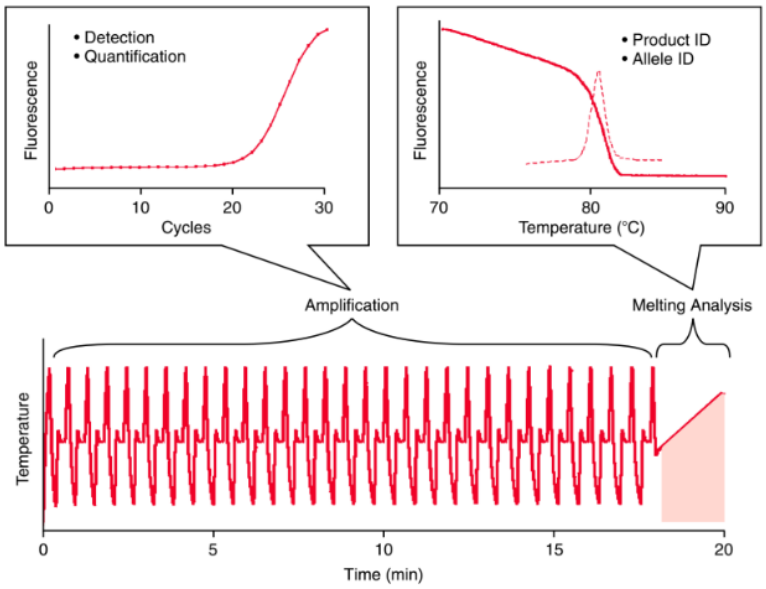

Figure 4: Illustration of amplification plot and melting analysis by real-time PCR. The bottom panel shows the PCR temperature profile, followed by a temperature ramp for melting analysis (shaded area). Fluorescence is measured each cycle and the information is shown in an amplification curve and allows quantification of target. Fluorescence monitored through the melting phase can be used to verify target identification. (Source: (49), Page 938)

2.5 Primer design and validation

Primers for each of the targeted genes and housekeeping gene were designed using Primer-BLAST (50). A housekeeping gene is a gene that is involved in the maintenance of basal cellular functions and is expected to be found in the organism's cells under normal conditions (42). Under intervention conditions a housekeeping gene is not expected to change and is used as an internal control. In this experiment hypoxanthine guanine phosphoribosyltransferase (*HPRT*) will be used as a housekeeping gene (41).

Search parameters such as the PCR product length, exon junction span and splice variant handling were altered to give primers with higher probability of success. The remaining parameters were left as default settings. According to the protocol used for the RT-qPCR process, the optimal product length is between 100 and 150 bp and it should not exceed 500bp (51). Setting restrictions for the PCR product length limits the amount of primers suggested by

Primer-BLAST, so in order to find good primers the primer length parameter for the search was set to 70-250 bp. The exon junction span was set to find primers spanning an exon-exon junction, in order to avoid unwanted amplification and contamination of genomic DNA (gDNA) (50, 52). This setting ensures that both forward and reverse primer hybridizes to a splice between two exons in cDNA, making the primer bind to both the 3'end of one exon and the 5'end of the adjacent exon, while not binding to gDNA where there are introns between the exons (52). The box for splice variant handling can be checked off to allow primer to amplify mRNA splice variants. This generates primers that may not be specific to a particular splice variant mRNA if exons in the gene are too similar to distinguish one from the rest (50). The primer may then bind to different splice variants in the relevant gene, making the primer gene-specific rather than transcript-specific. This will not affect the results in the experiment, as we are interested in the gene rather than a specific transcript. This parameter was checked off for all primer designs for this experiment, except for *VEGFa*.

For each target, three possible primer pairs are proposed (table 1) in order to have a backup if a primer fails to go through validation. The proposed primers were chosen by comparing the primers from Primer-BLAST to a set of criteria associated with a good primer. It is critical that the primer has a 100% nucleotide sequence match to the specific gene. The Tm of the primers should be at 60ºC±3, and the difference between the pair should not exceed 3ºC (52). This temperature only allows more specific bindings, and a low difference between the pair ensures that both primers anneal at the same temperature. Another requirement is that the GC content of the primer should be between 40-60% and the 3'end of the primer should preferably end with a C or G. The reason for this is that the G and C nucleotides form three hydrogen bonds and therefore binds more specifically and gives more stability to the final product than the double bonds between T and A. In order to avoid possible primer-dimer formation it is important to avoid primers with high self-complementarity (SC) (52). High SC in the 3'end of the primer may lead to undesirable hairpin-structures and should therefore be avoided. PCR primer design is an inexact science. Even primers that on paper fit all the criteria may not work when put to the test, so primer validation is required for each target.

To validate the primers cDNA will be extracted from a random brain section that is not intended for the actual experiment, so as to not waste the limited material. The cDNA sample will be

diluted exponentially with RNA-free water to a 10-fold dilution. With this series as a template, each primer pair will then be analyzed individually using qPCR, and Ct from each sample will be plotted against the $\log_{10}$ concentration of PCR product. Efficiency of the amplifications is calculated with formula 1. The slope is found by linear regression of the tested samples.

$$Primer\ efficiency = -1 + 10^{\frac{-1}{slope}} \qquad (1)$$

Primer efficiency of 100% means that there is an exact doubling of the targeted sequence every cycle (53). Efficiency between 90-110% are considered acceptable for qPCR reactions. Efficiency exceeding 110% implies impurities in the test sample that inhibit the PCR process. Such impurities can occur if there is carry-over from the sample or contaminants from RNA-isolation. The RNA-yield measured after extraction is meant to check if the sample is pure, so that we may avoid efficiency over 110%. Efficiency under 90% can imply that reaction concentration or conditions are not optimal, or that there is something wrong with the primer design, resulting in targeted sequence not doubling every cycle.

2.6 Relative quantification of amplification and statistics

The delta-delta Ct method ($2^{-\Delta\Delta Ct}$ method) is used to calculate the relative changes in gene expression in qPCR (54) and is done in five steps. Firstly, the average Ct for each technical replicate of each sample is calculated. Then ΔCt will be calculated with formula 2 and shows the difference in Ct-values between the housekeeping gene for a given sample, and the gene of interest. A control ΔCt average is calculated by averaging the biological replicates of the control group (saline). This control average is then used to calculate ΔΔCt for each sample, with formula 3.

$$\Delta Ct = Ct\ (gene\ of\ interest) - Ct\ (housekeeping\ gene) \qquad (2)$$

$$\Delta\Delta Ct = \Delta Ct\ (sample) - \Delta Ct\ (control\ average) \qquad (3)$$

Lastly, the method presents the fold change for an amplification (formula 4), which shows the relative amplification of the target gene in the sample compared to the control. In order for the

$2^{-\Delta\Delta Ct}$ calculation to be valid, the efficiency of the target and the control need to be approximately the same (54). Therefore, it is important to calculate the efficiency of each primer.

$$Fold\ change = 2^{-\Delta\Delta Ct} \quad (4)$$

The geometric mean of the individual fold changes is calculated to give the average fold change of a group. The geometric mean can be used to present the fold change for each group in a bar graph.

Standard deviation (SD) is a measure for variability in a population from which a sample is drawn. In a population with normal distribution, 95% of the individuals will have values within limits of ±2 SD, while 5% are spread equally above and below these limits (55). SD is also a valid measure for variability when the 5% outside the 2 SD fall on the same end of the limit. SD describes how widely scattered the measurements are. When presenting data in a bar graph SD can be used as error bars.

Because we were not able to execute the experiment there were no actual results for these calculations, but a spreadsheet (excel) for the $2^{-\Delta\Delta Ct}$ method has been made using fictive values to show how the results would look (appendix 1). The spreadsheet can be used when the experiment will be executed, simply by changing the Ct results for each gene.

2.7 One-way ANOVA with Bonferroni post-hoc correction

ANOVA is an analytical method used to compare more than two groups in an experiment, to see if one group varies from the rest. In this test the null hypothesis (H_0) is that all the groups are the same. Rejecting this hypothesis means that one of the groups differs from the others.

In cases where H_0 is rejected because one group differs, a multiple comparisons test, also called a post-hoc test, can be used to figure out which of the groups deviate from the others. In a statistical hypothesis test, there is always a slight probability that an extreme result will be

observed if H_0 is true. If H_0 is statistically rejected even though it was actually true, this error is signified by a probability value called type I error (α). If there is a 5% α error when comparing two groups with a t-test, the real probability of trueness of H_0 is 95%. When comparing many pairs, the real probability increases exponentially with each pair, which again inflates the α (formula 5). The α increases with increase in the number of comparisons.

$$Inflated\ \alpha = 1 - real\ probability\ of\ trueness = 1 - (1 - \alpha)^n \qquad (5)$$

The increase in α will increase the number of false negative cases, where cases are detected as not being statistically significant, but they really are different. To remedy this error rate, one can use a Boneferroni adjusted post-hoc test. This is often used when it is important not to make any type I errors at all. By using this adjustment, a threshold value can be set for α. Most commonly the threshold value for α is set to less than 0,05. This value of 0,05 is chosen because this is the p-value we are looking for when comparing two groups with a t-test. If the H_0 hypothesis is true for all tests the probability that the group of tests includes one or more false positives is 0,05. With an increase in number of tested pairs, the significance for the error is spread over a number of hypothesis tests, as an adjusted α (formula 6).

$$Adjusted\ \alpha = \frac{\alpha}{k\ (number\ of\ hypothesis\ tested)} \qquad (6)$$

The one-way ANOVA with Bonferroni post-hoc correction gives the advantage of an intuitive calculation. The weakness of the test is that as the increased number of compared hypotheses the significance of the error becomes very small and may result in a high false negative rate. This type of ANOVA is recommended for experiments where one is looking for one or two important comparisons amongst multiple comparisons, where one false positive may be an issue.

Seeing as there were no real results for the experiment, the ANOVA test was not performed, but this would need to be done in the actual experiment in order to discuss the results.

3.0 Results

3.1 Primer design

The following primers were designed using Primer-BLAST and were compared to the chosen set of criteria (table 1). The primers are proposed in order so the one considered to be best is listed first of the three. Values marked in green are very close to what is considered to be optimal for the criteria. Red is used to mark values that deviate more from desired value.

Table 1: Primer design (Primer-BLAST)

Gene	NM-code	Primer pair	Sequence (5' --> 3')	Product length	Tm	Self-complementarity (SC)	GC%
HPRT	012583.2	2	F: TCCCAGCGTCGTGATTAGTG	166	59,83	3,00	55,00
			R: ATGGCCTCCCATCTCCTTCA		60,33	4,00	55,00
		10	F: CAGTCCCAGCGTCGTGATTAG	229	60,80	3,00	57,14
			R: ATCCAGCAGGTCAGCAAAGA		59,31	4,00	50,00
		8	F: TGGATACAGGCCAGACTTTGT	154	59,01	4,00	47,62
			R: TGCCGCTGTCTTTTAGGCTT		60,25	3,00	50,00
BDNF	001270630.1	1	F: GTCGCACGGTCCCCATTG	246	61,12	3,00	66,67
			R: ACCTGGTGGAACTCAGGGT		60,07	5,00	57,89
		7	F: TGGCTGTCGCACGGTC	250	59,63	3,00	68,75
			R: CCTGGTGGAACTCAGGGT		58,09	5,00	61,11
		9	F: GCTGTCGCACGGTCCC	249	60,49	3,00	75,00
			R: ACCTGGTGGAACTCAGGG		58,09	5,00	61,11
VEGFa	001110333.2	2	F: CAGAAAGCCCATGAAGTGGTG	169	59,46	4,00	52,38
			R: CTTCATCATTGCAGCAGCCC		59,90	5,00	55,00
		1	F: AGAAAGCCCATGAAGTGGTGA	243	59,57	4,00	47,62
			R: GCTGGCTTTGGTGAGGTTTG		59,97	2,00	55,00
		10	F: CAGAAAGCCCATGAAGTGGTGA	85	60,82	4,00	50,00
			R: GGAAGATGTCCACCAGGGTC		59,75	4,00	60,00
EGR1	012551.2	2	F: TTCAATCCTCAAGGGGAGCC	70	59,37	4,00	55,00
			R: CGATGTCAGAAAAGGACTCTGTG		59,32	4,00	47,83
		3	F: TTTCAATCCTCAAGGGGAGCC	72	59,37	4,00	52,38
			R: GCGATGTCAGAAAAGGACTCTGT		59,32	4,00	47,83
		5	F: GGACAACTACCCCAAACTGG	235	58,09	3,00	55,00
			R: GTCAGAAAAGGACTCTGTGGTC		58,61	4,00	50,00
TNF	012675.3	9	F: TTCTCATTCCTGCTCGTGGC	199	60,39	2,00	55,00
			R: CCGCTTGGTGGTTTGCTAC		59,42	2,00	57,89
		2	F: GATCGGTCCCAACAAGGAGG	137	60,11	4,00	60,00
			R: CTTGGTGGTTTGCTACGACG		59,49	3,00	55,00
		7	F: TGGGCTCCCTCTCATCAGTT	106	60,25	4,00	55,00
			R: CGCTTGGTGGTTTGCTACG		59,79	2,00	57,89
PLAU	013085.3	1	F: TGGTGATGCTCCGTTTGGTT	223	60,18	2,00	50,00
			R: TCCTCCTGAATCTCCCGAGC		60,76	4,00	60,00
		2	F: CAGGACTGCTCTCTCAGCAAA	214	60,00	4,00	52,38
			R: CCCAGCAAGGACTGATGAGG		60,11	4,00	60,00
		9	F: CCAGCGTCAGTACCATGAGA	84	59,18	4,00	55,00
			R: CAAGTTCACTGCCACCTTCAG		59,39	4,00	52,38

3.2 Primer validation

Because of the COVID-19 pandemic of 2020, the primer validation was not possible to execute. The numbers in the following data analysis are fictive, given to us by our bachelor supervisor Nathan Scrimgeour, and made to exemplify possible results and outcomes for the experiment.

Table 2 explains six example scenarios for validation of a primer pair, with a ten-fold dilution of the sample. These values were used to make standard curves with linear regression, and a melt curve is provided for examples 5 and 6. This is shown in figure 5.

Table 2: Fictive Ct results of ten-fold dilution for validation of a primer pair.

DNA concentration	**$\log_{10}$**	**Example 1**	**Example 2**	**Example 3**	**Example 4**	**Example 5**	**Example 6**
10^{0}	0	-	36,35	28,53	24,77	24,77	24,77
10^{-1}	-1	-	39,95	32,41	29,79	28,09	28,09
10^{-2}	-2	-	-	36,25	32,81	31,4	31,4
10^{-3}	-3	-	-	39,98	35,23	34,71	34,71
10^{-4}	-4	-	-	-	39,95	37,87	37,87

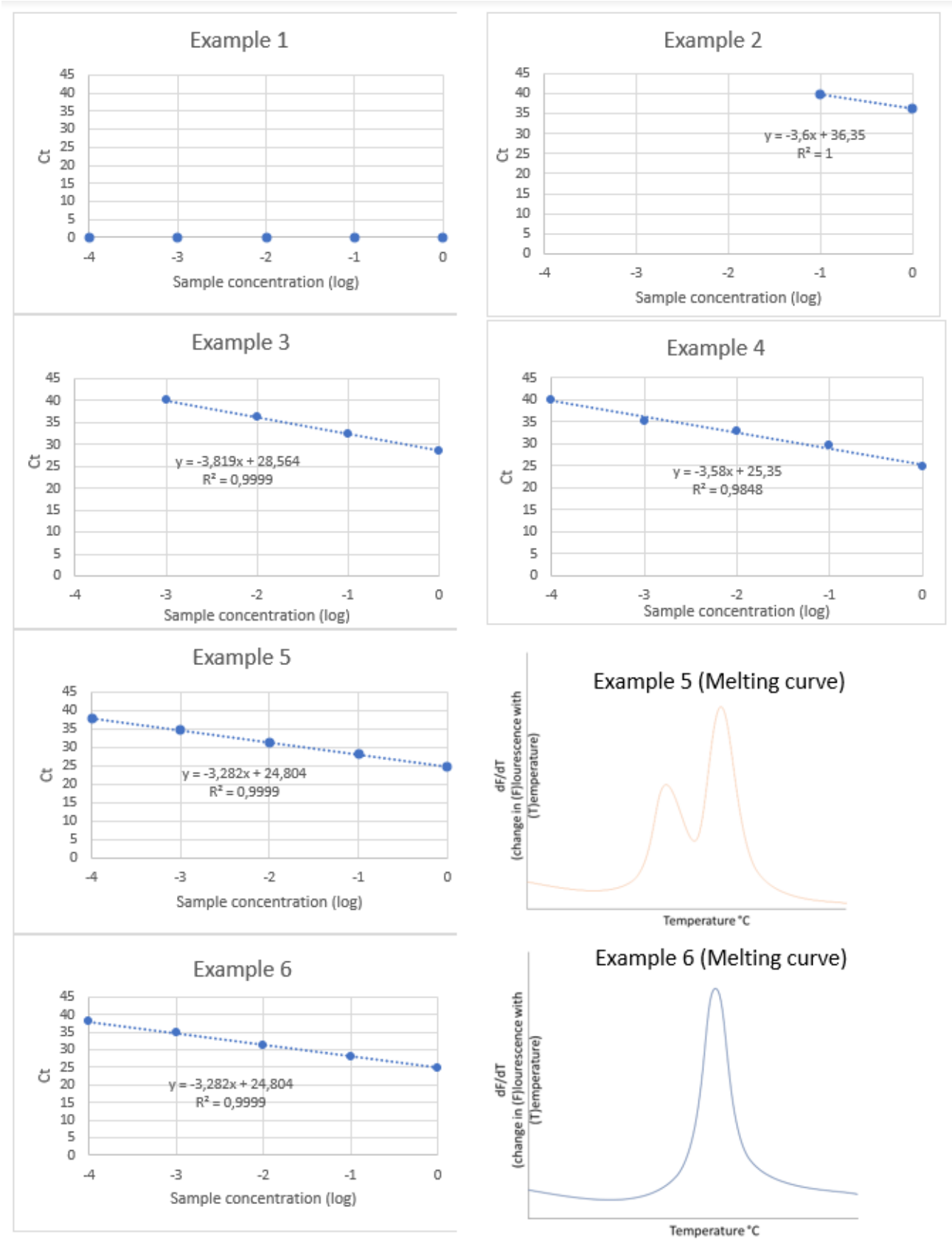

Figure 5: Examples of standard curves and melt curves for possible scenarios for primer pair validation. (Excel)

Table 3 summarizes key calculations that are relevant to the validation of the primer pair in the examples. Values for R^2 and primer efficiency are commented in separate columns.

Table 3: Schematic overview of slope average, R-squared and primer efficiency calculation.

	Slope	R^2	Comment	Primer efficiency (%)	Comment
Example 1	-	-	No amplification	-	No amplification
Example 2	-3,600	1,0000	-	89,57	Low
Example 3	-3,819	0,9999	OK	82,75	Low
Example 4	-3,580	0,9848	Low	90,25	OK
Example 5	-3,282	0,9999	OK	101,69	OK
Example 6	-3,282	0,9999	OK	101,69	OK

3.3 Delta-delta Ct

The bar graph in figure 6 is based on the fictive Ct-values for *BDNF* and *HPRT* given by our bachelor supervisor (calculations in appendix 1). It shows the geomean of the fold change for each group, which is the relative amplification of the targeted gene in the test group compared to the control group. The graph also includes SD error bars, showing the variability within each group.

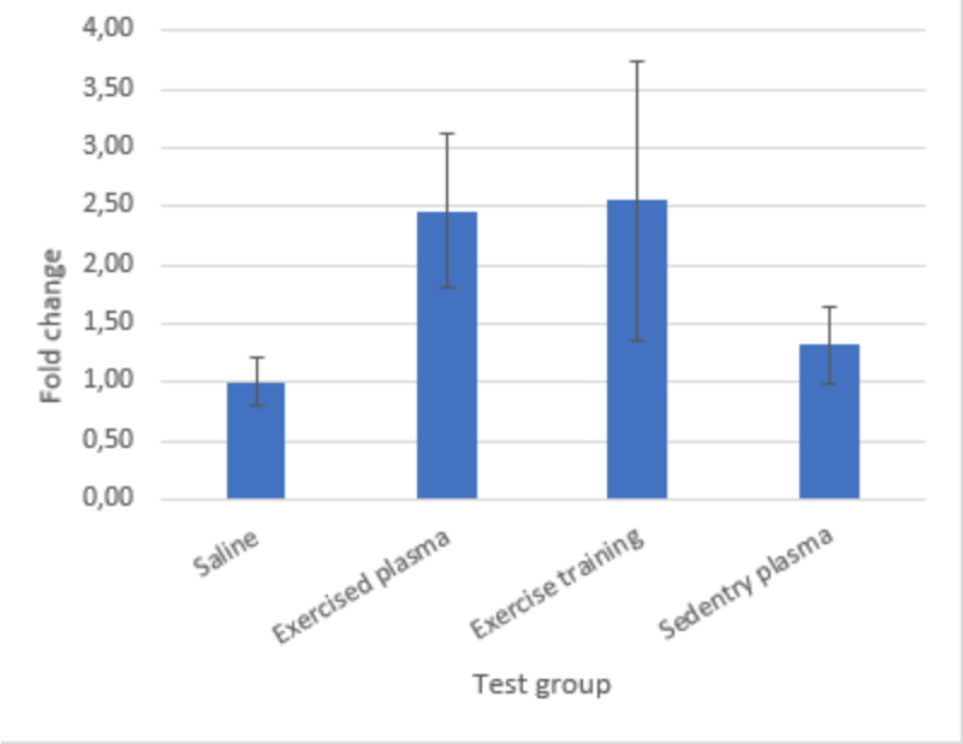

Figure 6: Expression of BDNF in rats after exercised training and in rats given exercised or sedentary plasma measured by qPCR. Fold change was calculated using ΔΔCt-method. The control rat was given saline and the housekeeping gene was HPRT (the calculations is enclosed in appendix 1). Data is presented as mean ± SD.

4.0 Discussion

4.1 Validation of primer designs

Example 1 shows a scenario where there has been no amplification during the qPCR process. This can happen if there is a problem with either the sample, reaction mix or primers. In order to find the source of the problem and rectify it, one should start by verifying that the experiment has been done correctly in terms of volume and concentration of reagents and sample etc. If there is still no amplification after rerunning the test, there may be a problem with the sample from the RNA extraction or reverse transcription. New cDNA should be procured. If there is still no amplification, this can indicate that the primer(s) do not bind to DNA template. A new primer should then be tested.

In example 2, only two Ct-values have been detected for the highest concentrations of the sample. These values are a bit high (>35), indicating that the sample has low abundance of the targeted gene. To remedy the problem, it may be possible to increase sample concentration by putting more RNA into the reverse transcription step. Another possible fix could be to not dilute the sample as much, for example a two- or five-time serial dilution, instead of ten. If so, logscale must be adjusted accordingly. Also, it is possible that the transcript can be very lowly transcribed in the tissue samples, so the target may not be a valid in this tissue.

Example 3 shows four Ct-values, but there has been no detected amplification for the most diluted sample. Most likely this is because the sample concentration is too low in this last dilution. One can still calculate the R^2 as long as there are three or more Ct-values. Example 3 shows Ct-values for three dilutions in the series. R^2 is acceptable, but the efficiency is too low. This means that the target gene has not been doubled each cycle, making it hard to quantify the amplification. Low efficiency is often associated with non-optimal PCR conditions or primers that are not annealing properly. The problem may be fixed by adjusting conditions or changing primers.

The Ct values in example 4 have a low R^2-value, indicating that the dilution of the samples have not been accurate. Relatively low R^2 means that the linear regression does not fit well to the data and can indicate problems with sample preparations. This situation may occur, but as

long as at least the first three dilutions are accurate, one can proceed with these and exclude the inaccurate dilutions. In this example, the inaccuracy appears already in the first dilution (10^{-1}). This will affect the regression line too much, so the results cannot be accepted. The dilution process and qPCR should be redone.

In example 5 the standard curve, R^2 and primer efficiency are acceptable. The melting curve shows that there are two melting points. This indicates that the primer pair may be nonspecific to the targeted sequence, leading to fluorescence from two different sequences. It is not possible to know which amplification belongs to the target. To remedy this scenario, one should change the primer pair.

Example 6 shows optimal results for both the standard curve, R^2, primer efficiency and melt curve.

4.2 Delta-delta Ct method and statistics

The bar graph in figure 6 shows the average expression of *BDNF* in the test groups compared to control (saline) and emerges from the fictive $2^{-\Delta\Delta Ct}$ calculation which is enclosed in appendix 1. The graph presents the scenario this experiment hopes to attain, where the test groups receiving exercised blood or exercise show increased *BDNF* expression. As *BDNF* is positively associated with neurogenesis and synaptic plasticity, these results would point to a link between exercise and good brain health. The SD error bars in the figure show the variability internally in each test group and point to high variability within the exercised plasma- and exercised group compared to the saline control and sedentary group.

One-way ANOVA with Bonferroni post-hoc correction should be used in the actual experiment to compare the test groups and identify if a group differs from the others, and if so which one.

4.3 Strengths and limitations

One of the strengths of the study is the use of a rat model. This gives more control over the different aspects of the experiment. One can control the amount and intensity of training of both

the donor rats and the exercised AD-rats, as well as the lack of training in the sedentary rats. In a human study this is harder to control, as the participants may change their own PA habits as a result of being in the study, regardless of which test group they have been sorted into. Animal studies also opens for more tests to be run during the study and a far greater scope of post mortem analysis.

There are some technical challenges and limitations of the experiment that must be evaluated. Many of these apply to human error and mistakes made in sample preparations that for example may lead to contamination of the sample. There is also a limited amount of tissue sample available for this experiment. This means that there is a limited chance to repeat the experiment if mistakes are made. It also limits the number of targets that can be analyzed, as each target is run separately and in triplicates of each sample. Given the limited amount of sample tissue available, one should still proceed with samples that have been measured to have low RNA-yield, but the PCR efficiency must be monitored as this may affect the results. The limited amount of tissue will also affect potential follow up experiments as there may not be any more tissue left after this experiment. CERG has some other sample material (blood and paraformaldehyde fixed brains) collected from the rats used in this experiment, which can be used in a follow up study to look for blood-borne molecules or proteins in brain tissue etc. Alternatively, one can extend the study by including more rats in order to get more brain material.

Each test group in the study consists of 3-4 samples per group. This number is considered to be a bit low and may be underpowered for detecting statistically significant changes. In a worst-case scenario the real effect of an experiment can be missed as a result of this.

When evaluating the results from this experiment in relation to human pathology, it is important to consider that there are certain translation factors between a rat model of AD and AD in humans. There are interspecies-related differences that may affect the translation. Also, the rat model used only shows Aβ-pathology, and does not account for effects of tau proteins in AD-pathology. The model therefor does not look at the full AD-pathology and gives a non-holistic presentation of the disease.

4.4 Future directions

If the experiment indicates alteration in gene expression as a result of treatment, new experiments should be conducted in order to confirm the results and also check if exercise has an effect on protein- or functional level. This can for example be done using Western blot to look for proteins. Another possible method for detecting proteins in tissue is immunohistochemical staining. A follow up experiment to confirm results can also be done by including more rats in the study. This gives greater statistical power to detect smaller changes when looking for reproducibility. One could also set up an experiment to see what effect training has on blood borne molecules that can be associated with good brain health. It may be possible to identify which molecules have been changed and see if any of them have positive effect in isolation. It is hoped that positive findings in this rat model of AD may eventually lead to a clinical study, to see if the effects of training or trained blood also can be found in humans at risk of developing AD.

If the study shows no change in gene expression for the chosen genes this may mean that the null hypothesis is true. Alternatively, this result may be attributed to experimental errors or that the chosen genes were not valid targets. One should evaluate the process of the experiment and look for errors and consider other genes to target that are related to neurogenesis, angiogenesis, synaptic plasticity and inflammation.

5.0 Conclusion

This paper has been written in association with CERG and their project hypothesis that exercise causes changes in blood that may improve brain health and neurogenesis. Because of the COVID-19 pandemic the experiment could not be executed in the lab, and this is therefore a paper describing the research plan and possible outcomes.

To look for alterations in gene expression related to AD we chose to target five genes associated with neurogenesis, angiogenesis, synaptic plasticity or inflammation. Primer pairs were designed for the targeted genes; *BDNF*, *VEGF*, *EGR1*, *PLAU* and *TNF*. A primer pair was also designed for the housekeeping gene; *HPRT*. These primers can be used when proceeding with the experiment, but they need to be validated first. The examples and calculations described in the results of this paper are based on fictive Ct-values and exemplify possible outcomes that may occur. The spreadsheet in appendix it 1 can be used when executing the experiment by simply changing the fictive Ct-values with actual Ct-values.

When proceeding with the experiment, the actual results will hopefully be similar to those in example 6 and the $2^{-\Delta\Delta Ct}$ calculation for the genes that are positively linked to brain health. $2^{-\Delta\Delta Ct}$ calculation related to *TNF* should be inverse to the calculations for the other genes, as *TNF* is associated with inflammation and will negatively affect brain health. Such findings would give good indication that gene expression related to neurogenesis, angiogenesis, synaptic plasticity and inflammation can be altered at transcript level with exercise to improve brain health.

Ultimately, as part of CERGs larger project, this experiment may serve as a step towards the goal of preventing or treating AD by identifying mechanisms to stimulate neurogenesis, angiogenesis, synaptic plasticity and reduce inflammation.

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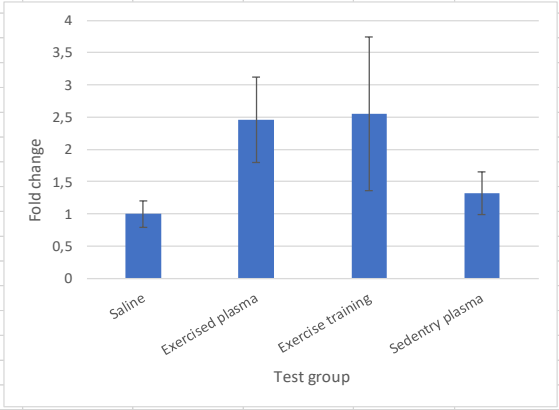
Appendix

Appendix 1: $2^{-\Delta\Delta Ct}$ calculation

	HPRT (housekeeping gene)					BDNF (gene of interest)								Fold change	Average fold		Error
	rep1	rep2	rep3	Average Ct	SD	rep1	rep2	rep3	Average Ct	SD	**ΔCt**	Control ΔCt	**ΔΔCt**	2^(-ΔΔCt)	change	Geomean	SD
saline 1	25,26	25,62	25,52	25,47	0,2	26,03	25,79	25,77	25,86	0,1	0,40		-0,22	1,17			
saline 2	27,59	26,99	26,86	26,93	0,1	27,83	28,00	27,88	27,90	0,1	0,98		0,36	0,78			
saline 3	24,90	25,02	25,02	24,98	0,1	25,66	25,37	25,36	25,46	0,2	0,48	0,62	-0,14	1,10	1,02	1,00	0,21
ex plas 1	26,89	26,80	26,95	26,88	0,1	26,27	26,19	26,25	26,24	0,0	-0,64		-1,26	2,40			
ex plas 2	25,49	25,42	25,54	25,48	0,1	25,09	25,20	25,19	25,16	0,1	-0,32		-0,94	1,92			
ex plas 3	29,39	29,25	29,57	29,40	0,2	28,54	28,15	28,30	28,33	0,2	-1,07		-1,69	3,23	2,52	2,46	0,66
ex train 1	26,01	26,07	25,63	25,90	0,2	24,49	24,57	24,43	24,50	0,1	-1,41		-2,03	4,07			
ex train 2	25,45	25,88	25,46	25,60	0,2	25,10	25,19	25,17	25,15	0,0	-0,44		-1,06	2,09			
ex train 3	25,86	25,78	25,80	25,81	0,0	25,35	25,59	25,47	25,47	0,1	-0,34		-0,96	1,95	2,70	2,55	1,19
sed plas 1	25,17	25,17	24,79	25,04	0,2	25,17	25,50	25,23	25,30	0,2	0,26		-0,36	1,29			
sed plas 2	25,64	26,14	25,46	25,55	0,1	26,08	26,03	26,21	26,11	0,1	0,56		-0,06	1,04			
sed plas 3	26,39	25,82	26,26	26,33	0,1	26,01	26,38	26,14	26,18	0,2	-0,15		-0,77	1,70	1,34	1,32	0,33

SD cutoff is 0,2. Ct values marked in red are outliers giving higher SD than acceptable for the sample and are removed from all calculations. Formulas for average Ct and SD have been editied to exclude these values.



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