Alterations in growth factor levels in plasma after stroke or exercise

Elise Fritsch

Master Thesis for the title of Master in Pharmacy

Department of Pharmaceutical Biosciences

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45 credits

The Faculty of Mathematics and Natural Sciences

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In a time ruled by home office and strict social restrictions due to COVID-19, I have been truly grateful for being able to go to the laboratory, having a relatively normal everyday study life. Still, I must admit that the current situation also has had its downsides, making this year a bit lonelier and more independent than I had imagined my master year to be.

First of all, I would like to thank my supervisor associate professor Cecilie Morland, for giving me the opportunity and for your guidance through my thesis. Your optimism and positive attitude throughout have been highly appreciated. I would also like to thank Linda Thøring Øverberg for letting me be a part of your PhD project and for good (and some frustrating) times at the laboratory.

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Oslo, May 2021
Elise Fritsch

Abstract

Worldwide there are 80 million people who live with the impacts of stroke. Post-stroke impairments such as physical disability, cognitive decline, and depression are major burdens not only for the affected individual, but also for their relatives and for the society. Patients with the same type of brain injury show great differences in their cognitive and motor function recovery, and to this day the cause of this is not known. Neither is it known how to predict recovery after stroke. In this present study, we investigated how different growth factors (GFs) and irisin change after stroke or exercise, with the goal of providing clinically relevant knowledge of factors secreted to the blood which may affect the brain during the rehabilitation phase.

We compared plasma levels of the above-mentioned factors in plasma from stroke patients (n=48) taken the day after admission, and in plasma from age-/gender matched controls (n=48). The control group was exposed to high intensity interval exercise (4x4 minutes running/walking), and blood samples were taken at baseline (before), immediately after, and 30 minutes after the exercise. To detect the circulating concentrations of the different factors, sandwich enzyme-linked immunosorbent assays (ELISAs) were performed. Through our research we found significantly lower levels of brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) in the stroke patients compared to the baseline levels in the control group. When measuring basic fibroblast growth factor (bFGF) and irisin, we did not detect significant differences in the levels found in the stroke patients, when compared to the baseline levels in the control group. In response to exercise, the levels of BDNF and EGF significantly increased. This tendency was observed in both genders but was more prominent in men than in women, and for BDNF it was only statistically significant for the males. For Irisin and bFGF, on the other hand, no significant changes in the levels was discovered in response to exercise.

The results from this study lays the foundation for further studies on alterations in GFs and other biomarkers secreted in the blood, as a response to stroke and exercise. This may prove to represent critical information when aiming for more individually tailored treatment services after stroke, contributing to lessen the major disabilities seen after stroke, hence also the major burden on the effected individuals and the societal costs.

Abbreviations

Ahus	Akershus University hospital
ADL	Activities of daily living
Abs	Antibodies
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMI	Body mass index
CA	Capture antibody
CBF	Cerebral blood flow
CDT	Clock drawing test
CNS	Central nervous system
CV	Coefficient of variation
DA	Detection antibody
DAG	Diacylglycerol
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
FGF	Fibroblast growth factor
FNDC5	Fibronectin type III domain containing protein 5
GF	Growth factor
HADS	Hospital anxiety and depression scale
HCAR1	Hydroxycarboxylic acid receptor 1
HIF	Hypoxia inducible factor
HIIT	High intensity interval training
HR	Heart rate
HSPG	Heparan sulfate proteoglycans
Ig	Immunoglobulin
IP_3	Inositol-1,4,5-triphosphate
K2EDTA	K2 ethylendiaminetetraacetic
MAP	Mitogen-activated protein kinase
MMPs	Matrix metalloproteases
MMSE	Mini-mental state examination
MMSE-NR3	Norwegian Revised mini-mental state examination
mAbs	Monoclonal antibodies
mRS	Modified rankin scale
NSCs	Neural stem cells
NHR	Norwegian stroke registry
NIPH	Norwegian institute of public health
NP	Neuropilin
NVU	Neurovascular unit
pAbs	Polyclonal antibodies
PA	Physical activity
PBS	Phosphate buffered saline

PI3K	Phosphatidylinositol 3-kinase
PLC-γ	Phospholipase C-γ
PlGF	Placental growth factor
$p75^{NTR}$	p75 neurotrophin receptors
RD	Reagent diluent
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
TIA	Transient ischemic attack
TMT	Trail making test
TMT-NR3	Norwegian Revised TMT
Trk	Tropomyosin receptor kinase
TrkB	Tropomyosin receptor kinase B
VEGF	Vascular endothelial growth factor
WSO	World stroke organization
SGZ	Subgranular zone
STAT	Signal transducers and activators of transcription
Streptavidin-HRP	Streptavidin conjugated to horseradish-peroxidase
SVZ	Subventricular zone
VO_2max	Maximal oxygen uptake

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1. Introduction

Stroke is the second leading cause of death and a major cause of disability worldwide. According to World Stroke Organization (WSO), there are 13.7 million new stroke incidents each year, and their annual report estimated 80 million people to live with the impact of stroke in 2019 [1]. Incidence and mortality of stroke differ between countries, geographical regions, and ethnic groups. While the numbers are declining in high-income countries, stroke incidence in low- and middle-income countries has more than doubled over the last four decades [2].

In Norway, stroke is the leading cause of disability and institutional long-term care, and the third leading cause of death [3]. In 2019, the Norwegian Stroke Registry (NHR) reported 9,022 acute stroke cases. Of these patients, 55 % were men and 45 % were women, with a median age of 73 and 79, respectively [4]. Today the stroke mortality rate has decreased to less than 10 % due to effective treatment, but more than half of stroke survivors will experience greater or lesser degree of permanent disability [4, 5]. A common after-effect of stroke is various degrees of weakness or paralysis, ranging from a droopy eye to the entire side of the body being affected [6]. Physical disability can cause problems with even the simplest of daily activities. Stroke is also responsible for a significant cognitive decline, and represents an important contributing factor to about half of all dementia cases [3]. In addition, one third of the stroke survivors experience post-stroke depression [7]. Post-stroke cognitive impairment can affect the patient's ability to live independently even after recovery from physical disability and leads to an enhanced risk of recurrent strokes. Both cognitive impairment and depression are associated with further cognitive deficiency, greater functional disability, higher mortality and, finally, with worse rehabilitation outcome [8, 9].

Many post-stroke patients will have a need for long-term rehabilitation and care. Stroke is therefore a major health-related and economic burden, for both the affected individual and the society [3, 5]. There are few studies related to the costs of post-stroke care in Norway, but a study performed in 2018 estimated a monthly cost per patient to $2,147 [10]. In another study from 2007, the total societal costs were estimated at 7 - 8 billion Norwegian kroner per year, not included informal care and nursing from relatives [5]. The stroke incidence will probably increase with 50 % over the next 20-30 years as the population ages. As a consequence, societal costs of stroke are expected to increase further in the years to come [3].

1.1 Aetiology of stroke

The term ”stroke” is a broad concept, which simplified can be defined as lack of oxygen caused by impaired blood flow leading to cell death in the brain [2, 11]. The two major causes of stroke are an occlusion of a precerebral or cerebral artery, also known as ischemic stroke, or a hemorrhage. Both cause ischemia, which rapidly leads to necrosis in the stroke core. Ischemic stroke is responsible for 80-90 % of all strokes [2, 4, 12]. Transient ischemic attack (TIA) is a type of stroke caused by a temporary blockage of- or decrease in blood flow to the brain, which does not cause acute infarction [11]. In this thesis, the term “stroke” will be used to refer to classic ischemic stroke. When referring to hemorrhage strokes or TIA, this will be specified.

Ischemia is defined as a condition in which the blood flow, and thus the availability of oxygen and nutrients, is restricted or reduced in a part of the body [13, 14]. An ischemic stroke occurs when a blood vessel in the neck (precerebral artery) or brain (cerebral artery) is blocked, resulting in an insufficient cerebral blood flow (CBF) [15]. A blood cloth usually causes the blocking and can be due to thrombosis; the formation of a cloth within a blood vessel of the brain or neck, embolism; the formation of a clot in the periphery that travels with the blood stream to the brain, or a severe narrowing of an artery in or leading to the brain, called stenosis. The most common cause of stenosis is atherosclerosis, a condition with narrow arteries due to deposits of plaque in or on the artery walls [6].

If the lack of blood flow is limited to a specific region of the brain, the condition is called a focal stroke. If the hypoxic injury affects the entire brain, it is referred to as global stroke; global strokes are often more severe than focal strokes. Because different arteries supply different areas of the brain, the localization of the occlusion and the size of brain area supplied by the occluded artery will determine which functions are affected. The degree of damage also depends on whether the blocking is partial or total, and whether reperfusion occurs or not [6]. General health level and habits regarding physical activity before the stroke will also influence the outcome [16].

1.2 The neurovascular unit

The brain amounts for only 2 % of the human body mass but receive 15 % of the cardiac output and 20 % of the oxygen and glucose supply [17, 18]. Both oxygen and glucose are needed to generate adenosine triphosphate (ATP), which is the currency for cellular energy. It has been

estimated that one single cortical neuron utilizes approximately 4.7 billion ATPs per second in the resting human brain [19]. Despite the high energy demand, the brain has very few energy reserves of its own. A small storage of glycogen synthesized from glucose is found in astrocytes. Astrocytic glycogen can be broken down to lactate during hypoglycemia and lactate can be transferred to adjacent neurons or axons where it is used as metabolic fuel [20, 21]. In addition, newer studies have observed lipid droplets in the brain, but it is uncertain whether these are used as energy reserves [22]. The brain is therefore depended on a continuous blood flow to receive glucose and oxygen to maintain its high energy demand.

The CBF is regulated by a complex mechanism of communication between cellular and extracellular components that together form the neurovascular unit (NVU) [23]. In addition to control the CBF, a specialized part of the NVU is also responsible for the maintenance of the blood-brain barrier (BBB) [24], a highly selective semipermeable barrier made up by a continuous endothelial monolayer connected by tight and adherens junctions [25, 26]. This barrier is a protective mechanism, helping the central nervous system (CNS) maintain a stable environment protected from the fluctuating conditions of the blood [27].

The NVU consist of neurons and their axons, glial cells: astrocytes, oligodendrocytes, microglia and microvessels: endothelial cells, myocytes and pericytes (Figure 1) [23]. Neurons are able to detect very small variations in their supply of nutrients and oxygen, and transform this information into electrical and chemical messages that they send to adjacent interneurons or astrocytes [23]. Oligodendrocytes that are localized in close proximity to neuronal axons support axonal function both by supplying glucose [28] and by producing myelin sheaths that facilitates fast axonal transport of action potentials [29]. Astrocytes function as a connection between the neurons and the blood vessels. Astrocytic end feet are in contact with pericytes and the outer surface of the endothelial cells, connected by the basal lamina [26]. In response to changes in neuronal activity, astrocytes can signal to the endothelium and/or pericytes in order to regulate the CBF. Astrocytes also contribute to a variety of other functions in the brain, including synapse formation and plasticity [30]. The pericytes which are smooth muscle-like cells also act as macrophages [26]. Since they extend their processes around the basolateral surface of the endothelial cells, they are also able to influence on the vascular diameter, and therefore local CBF by contraction [30, 31]. In lager vessels, myocytes are also present and recognized as the main effectors of contractility [23].

The last cell type found in the NVU is microglia, known as the main immune cells of the CNS [32]. Microglia constantly screen the microenvironment within the NVU and can detect signals associated with an altered brain homeostasis. In addition, they communicate with neurons and play an important role in supporting neuronal functions [33]. There is also growing evidence for interactions between microglia and astrocytes and pericytes, as microglia are reported to promote astrocyte differentiation [34], and to regulate the detachment of pericytes via production of reactive oxygen species (ROS) [35]. As a consequence of the intricate cross-talk between the different cell types of the NVU, damage to one of the components may affect the function of the entire NVU.

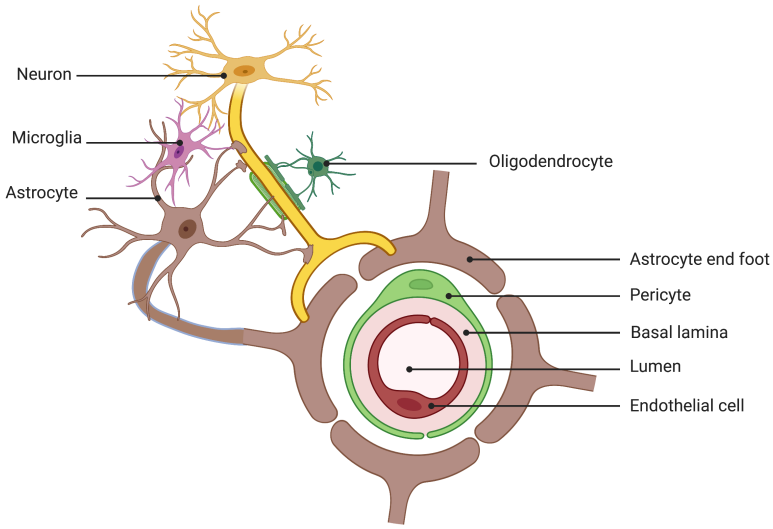

Figure 1 Components of the neurovascular unit (NVU). *The NVU consist of neurons, astrocytes, oligodendrocytes, microglia, endothelial cells and pericytes. (Figure created with BioRender).*

1.3 Pathophysiology of stroke

The large need for oxygen and glucose makes the brain exquisitely sensitive to ischemia. Small infarctions, well below the size that causes clinical signs in other organ systems, can cause severe symptoms from the brain [36].

A stroke results in a depletion of cellular ATP at a rate higher than it can be synthesized [37]. The resulting loss of energy stores results in ionic imbalance, neurotransmitter release, and inhibition of reuptake of excitatory neurotransmitters [36]. Especially accumulation of extracellular glutamate is important in the pathophysiology of stroke, causing excitotoxicity that result in activation of proteases and lipases, which in turn degrade membranes and proteins essential for cellular integrity [37, 38]. The mitochondrial production of ROS increases as a

consequence of limited oxygen supply and the glutamate induced excitotoxicity, and contributes to further cellular damage and apoptosis. As the neurons have relatively low levels of endogenous antioxidants, the brain is especially vulnerable to ROS when compared to most other organs [38, 39].

The injury after stroke can be divided into the irreversibly damaged infarct core and the ischemic penumbra surrounding it. Neurons in the core rapidly undergo necrosis, while the penumbra still receives some oxygen from the surrounding collateral arteries and may be rescued if adequate perfusion is restored in time [40, 41]. The degree of neural death depends on how long brain cells are deprived of blood and is proportional to the degree of loss in perfusion. If perfusion does not occur, the core size usually increases, while the salvageable penumbra decreases with time. Early reperfusion is essential to prevent extensive neural damage [36, 41].

Although rapid reperfusion is essential to prevent permanent damage to the brain, the reperfusion phase itself also may induce detrimental effects on the brain: immediately after the CBF is restored to the affected area, and glucose and oxygen flows freely, mechanisms from the ischemic phase are still ongoing. Increased access of oxygen can lead to a further surge in production of ROS, activating matrix metalloproteases (MMPs). MMPs degrade collagen and laminins in the basal lamina which disrupts the integrity of the vascular wall and increases BBB permeability [39]. Increased permeability can cause neuroinflammation as it allows the entry of molecules and immune cells that are normally prohibited by the BBB.

The brain responds to ischemic injury with both acute and prolonged inflammatory processes. Various cells that generate inflammatory mediators are recruited into the ischemic brain [36]. Microglia are the first line of cells reacting to the damage, and the primary source of proinflammatory cytokines and chemokines. Release of these cytokines and chemokines causes local activation of microglia and mobilization of leukocytes [42]. In addition, oxidative and nitrative stress triggers recruitment and migration of neutrophils and other leukocytes to the cerebral vasculature, and these cells release enzymes that further increase basal lamina degradation and vascular permeability. These events can lead to parenchymal hemorrhage, vasogenic brain edema and further neutrophil infiltration into the brain [39].

The inflammatory response can be both detrimental and beneficial, depending on the balance between different components of the immune response, the degree of the response and when it occurs. Different pro- and anti-inflammatory mediators as well as other factors involved in tissue damage and/or repair may affect stroke outcome differently depending on whether they increase in the acute phase of a stroke or if they increase hours or days after the stroke.

1.4 Neural plasticity after stroke

As a response to experience, the nervous system has an ability to modify itself, functionally and structurally. This capacity underlies the brain's ability to learn and adapt to changes in the environment, and is referred to as "neural plasticity" [43]. Animal studies have revealed that neural plasticity can also be triggered by brain damage, and it is therefore considered as a mechanism for recovery after stroke [44].

Compared to other organs such as the liver and skin, the brain has a limited capacity for tissue regeneration after damage [45]. However, tissue repair does occur in the early stages of the recovery phase (3–30 days) [46]. Much of the recovery experienced at later time points, for instance during rehabilitation, is actually due to the brain rewiring itself. This implies that the brain finds new pathways so that other brain regions take over the function of brain regions that are lost to the stroke [47]. The recovery phase after stroke begins shortly after the acute injury has stimulated restorative processes and evolves over several weeks [48]. The most rapid recovery usually occurs during the first three to four months following the injury [49].

Neuronal plasticity includes processes such as angiogenesis and neurogenesis [50], tightly coordinated by communication within the NVU [51]. The two processes are largely stimulated by growth factors (GFs) and influence each other [52]. Both angiogenesis and neurogenesis have proven to be important contributing factors for functional recovery after stroke [53].

1.4.1 Neurogenesis

Neurogenesis is the process where new functional neurons are produced from neural stem/progenitor cells (NSCs). NSCs are multipotent cells which generate neurons and glial cells. Neurogenesis includes proliferation of endogenous NSCs, migration, and differentiation into mature functional neurons [53]. In the adult brain, neurogenesis occurs mainly at two

distinct regions: in the subventricular zone (SVZ) of the lateral ventricles and subgranular zone (SGZ) in the dentate gyrus of the hippocampus [54].

Ischemic stroke causes low levels of oxygen and varying degrees of neuronal death, which are strong stimulants for increased proliferation of progenitors/NSCs [55-57]. Brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) are two GFs reported to be upregulated in the brain following stroke [58, 59], and known to induce the proliferation and differentiation of NSCs [60, 61]. While basic fibroblast growth factor (bFGF), another GF that is upregulated in the brain following stroke [62], is involved in the protection and enhancement of NSCs [63]. As a result, stroke-generated neuroblasts are produced and migrate towards the ischemic boarder guided by chemokines and cytokines secreted by resident activated microglia and astrocytes [64]. Once the neuroblasts reach the site of injury, they differentiate into new mature neurons, replacing lost or damaged ones [64].

Stroke is associated with increased neurogenesis [56], but the extent and relevance of injury-induced neurogenesis is still not well understood. According to a review paper on neurogenesis and behavioral recovery, there is an impression that stroke-induced neurogenesis contributes to better recovery from stroke [65]. However, the majority of these studies are done on young rodents, while the typical stroke patient is elderly with several co-morbidities [66]. Although stroke-induced neurogenesis has been observed in elderly mice [67], neurogenesis may not be able to produce a full recovery of the damaged brain after stroke [68] as the number and capacity of NSCs decrease with age [69].

Furthermore, the majority of the stroke-generated neurons have been reported to undergo programmed cell death during the first two weeks after their formation [70] due to non-optimal conditions in the stroke core and boarder. Nevertheless, neurogenesis is still thought to be a key process in post-stroke recovery and repair of the damaged brain region [64]. This depend on survival of at least some of the newborn neurons, which to a large degree depend on GFs. One important source of GF, nutrients and oxygen to supply the survival of new neurons is new vasculature. The microvascular endothelial cells secrete GFs and chemokines, which may support the survival of the newly formed neurons [50]. Thus angiogenesis is directly linked to neurogenesis.

Newly formed blood vessels as a result of angiogenesis, are thought to enhance neurogenesis by enhancing proliferation of NSCs by expression of several extracellular signals, then supporting the migration of the cells toward the border of the ischemic core. The latter is done by supplying oxygen, nutrients, and soluble factors as well as serving as a scaffold for migration [71]. Induced oxygenation in the ischemic core will also facilitate the differentiation of the migrated NSCs into mature neurons [72].

1.4.2 Angiogenesis

Angiogenesis is defined as the formation of new microvessels by branching off from pre-existing vessels [53]. The process begins with local degradation of the basement membrane surrounding the capillaries, allowing invasion of underlying endothelial cells. The endothelial cells proliferate and form a three-dimensional structure that join with other similar structures to build a network of new blood vessels [73, 74].

Under hypoxic conditions macrophages and other immune cells are recruited to the hypoxic region where they synthesize and secrete pro-angiogenic factors, such as VEGF and bFGF [75] [76]. After brain ischemia, endothelial cells start to proliferate at the border of the ischemic core, and after 4-7 days active angiogenesis takes place [77, 78]. Post-ischemic angiogenesis can be beneficial by contributing to neurogenesis as previously described, and by leading to enhanced oxygen and nutrient supply to the affected tissue [72, 79].

Angiogenesis can be measured by counting the number of blood vessels or measuring the percentage of tissue volume occupied by blood vessels. Both of these measures are often referred to as the microvessel density [80]. Several studies show that increased microvessel density in the penumbra of patients surviving acute ischemic stroke correlates with longer patient survival [78, 81, 82], suggesting that active angiogenesis is beneficial for the ischemic brain. Even so, it is also important to remember that the endothelial barrier function is severely compromised during the angiogenetic process. Factors such as VEGF and MMPs contribute to loosen the other-ways tight connection between the endothelial cells to allow endothelial sprouting. This leads to an increased vascular permeability which disrupts BBB function and in turn can lead to edema increased infarction size. Increased vascular permeability also increases the risk of hemorrhagic formation, further worsening stroke outcome. Finally, increased vessel density in the early phase after stroke may also contribute to enhanced

inflammatory reaction, further increasing neuronal damage [83]. The timing of angiogenesis after stroke therefore appears to be crucial. This has not been investigated experimentally, but one may speculate whether angiogenesis will be harmful if it occurs during the acute phase, while beneficial if occurring in the chronic phase.

1.5 Growth factors in the regulation of plasticity

GFs are a group of proteins that stimulate cellular processes, including cell survival, growth, proliferation and differentiation by inducing replicative DNA synthesis and cell division [84, 85]. Together, the combined action of GFs contribute to the regulation of angiogenesis and neurogenesis by binding to their respective receptors, mainly receptor tyrosine kinases (RTKs) [86]. Following ligand binding, RTKs usually form homodimers and phosphorylate each other, leading to the activation of several downstream signaling cascades, and subsequently initiating gene expression and protein synthesis [84]. The three major pathways activated are the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and the phospholipase C-γ (PLC-γ) pathway [87]. The pathways regulate partly overlapping functions.

GFs are normally expressed in adult brain, and many of them are upregulated in response to brain ischemia as important regulators of protection and recovery [88]. The components of the NVU and immune cells, release various GFs to build a functional capillary system that supplies oxygen and other nutrients to the tissue, and to promote the viability of neurons, providing a sustained protection under conditions of oxygen deprivation [89, 90]

1.5.1 Vascular endothelial growth factor

The VEGF family comprises five members that mainly binds the tyrosine kinase receptors: VEGFR1-3, in addition to non-tyrosine kinase receptors: neuropilin-1 (NP-1) and -2 (NP-2). The NPs lack intrinsic enzymatic activity and are therefore termed co-receptors for the VEGFs [91]. VEGF-A, the most potent angiogenic factor within the VEGF family, primarily binds to VEGFR1, VEGFR2, NRP-1 and NRP-2 [92]. VEGF-A is in the following referred to as VEGF.

In the brain, several cell types express VEGF, such as endothelial cells, neurons, astrocytes, and microglia [93]. Secreted VEGF regulates angiogenesis in the brain by the combined action of VEGFR1 and VEGFR2, where activation of the latter increases angiogenesis, and activation

of the former decreases it. The main effect of VEGFR1 is to prevent the binding of VEGF to VEGFR2 [91].

Hypoxia is an important stimulus of VEGF expression. A hypoxic or oxygen-deprived cell produces hypoxia inducible factor-1α (HIF-1α), which under non-hypoxic conditions is rapidly degraded [94]. HIF-1α upregulate VEGF transcription, but also induces erythropoietin (EPO) [95], which in turn increases the secretion of VEGF. Inflammatory cytokines may also contribute to the regulation [41]. Both hypoxia and inflammation are present during an ischemic stroke, and both VEGF and its receptors VEGFR-1 and VEGFR-2 are upregulated [96]. As early as 1 hour after stroke, a significant upregulation of VEGF is seen in the ischemic core and in the penumbra, peaking at 24-48 hours after stroke onset [97, 98]. VEGFR2 and VEGFR1 are upregulated around the infarct border on endothelial cells, astrocytes, and neurons by 48 hours after the ischemic stroke [91].

Activation of VEGFR2 activates the MAPK and PI3K/Akt pathways which both promotes angiogenesis. The PI3K/Akt pathway promotes migration of endothelial cells, and the MAPK pathway stimulates the proliferation and migration of endothelial cells [41]. Endothelial migration is supported by pericytes that release several angiogenic factors, including VEGF [91]. Both pathways also lead to an increase in HIF-1α, and thus a further upregulation of VEGF [99]. In addition, through the PI3K/Akt pathway, endothelial nitric oxide synthase (eNOS) is activated. eNOS synthesize nitric oxide, which cause relaxation of the vascular smooth muscle and increase CBF [100]. As a result, new vessels are formed and CBF is increased, enchaining the chance to rescue the penumbra [41].

Increased angiogenesis is an important neuroprotective effect, but VEGF may also have direct neuroprotective effects as VEGFR1, VEGFR2 and NP-1 are present on neurons and glial cells in addition to on the endothelium [101, 102]. One possible underlying mechanism is through the upregulation of HIF-1α and its downstream signal transduction pathways that promotes self-renewal and inhibits cell apoptosis [103].

As previously described, a part of the angiogenetic effects is to contribute to loosen the endothelial cell connections to allow endothelial sprouting. This effect is largely mediated through the action of VEGF–VEGFR2 and the Src-suppressed C kinase substrate (SSeCKS) pathway, but activation of the PI3K/Akt pathway also plays a role [41]. By these mechanism,

VEGF increase vascular permeability leading to a disrupted BBB function [89]. An increased BBB leakage in the early phase of stroke could further worsen neuronal damage and stroke outcome, in the way previously described. This gives VEGF a dual effect in stroke; in the first phase after acute ischemic stroke, increased VEGF cause cerebral edema and localized inflammation, thus damaging neurons and tissue. After this acute inflammatory phase, however, VEGF leads to multiple protective effects, including the promotion of angiogenesis and neurogenesis, leading to improved functional recovery [104].

The prevailing opinion is that serum VEGF levels in humans increase after stroke compared to healthy controls, as seen in several studies [105-108]. Even so, a meta-analysis of 14 case–control studies showed that serum VEGF levels were not significantly different in ischemic stroke cases as compared with healthy controls, and that serum levels after stroke are time-independent [109]. Whether these conflicting findings reflect differences in the time of sample collection after stroke, differences in the type or severity of the stroke or other factors is not known. When it comes to studies done on VEGF levels and stroke outcome, lower levels have been shown to correlate with poor outcome when measured several days after stroke [105, 108, 110].

1.5.2 Brain derived neurotrophic factor

BDNF belongs to the neurotrophin family, a group of secreted proteins that promote neuronal proliferation, survival and differentiation [8]. It is widely expressed in the adult brain, present in high amounts in axons and neuronal cell bodies, with the highest levels found in hippocampal neurons [111]. BDNF is synthesized as the precursor proBDNF before it is cleaved to mature BDNF protein. Both the syntheses of proBDNF and the formation of mature BDNF from proBDNF are increased in response to neuronal activity. The secretion of BDNF is also activity-induced, and normally triggered by membrane depolarization or by extracellular factors, including BDNF itself [112]. When secreted, a mixture of pro- and mature BDNF is released [111]. ProBDNF preferentially binds p75 neurotrophin receptors ($p75^{NTR}$) and mature BDNF binds with high affinity to tropomyosin receptor kinase B (TrkB), and with lower affinity to $p75^{NTR}$. The main effect of mature BDNF is therefore through activation of TrkB. ProBDNF and mature BDNF are associated with opposing effects on cellular function; proBDNF activation of $p75^{NTR}$ can induce apoptosis, while mature BDNF activation of TrkB can promote cell survival [113]. In the adult brain, mature BDNF is the prominent isoform, a tenfold more

abundant that proBDNF [114]. The main focus will therefore be on the effects of mature BDNF, from here on referred to as BDNF.

Like BDNF, TrkB is also widely expressed in the adult human brain [115]. Activation of TrkB leads to activation of downstream signaling pathways such as the MAPK, PLC-γ and PI3K/Akt pathway [116]. Activation of the MAPK pathway leads to an increased expression of genes involved in neuronal and synaptic plasticity, including expression of the BDNF-gene itself [117, 118]. Activation of the PLC-γ pathway generates inositol-1,4,5-triphosphate (IP_3), and diacylglycerol (DAG) which is important for survival, neurite outgrowth and synaptic plasticity [119]. While activation of the PI3K/Akt pathway promotes cell survival, neurite growth and cell proliferation [116].

Neurons are the predominant source of BDNF even in the ischemic brain, but with significant neuronal death also non-neuronal cells such as microglia, astrocytes and endothelial cells will contribute to the secretion of BDNF [120] Animal studies have shown that BDNF is upregulated in the brain following stroke. Increased levels was seen already 4 hours after stroke onset [59, 120], and the increase is reported to be sustained up to 7 days [121]. Secretion of BDNF following stroke have been shown to have multiple protective roles. All the three pathways activated by BDNF-TrkB leads to transcription of pro-survival genes, and thus protect neurons form apoptosis. The MAPK and PI3K/Akt signaling pathways also suppress apoptosis by interacting with apoptosis-regulating proteins [122]. In addition, BDNF plays a central role in neurite outgrowth and neurogenesis [123, 124].

Human studies comparing stroke patients with healthy controls have shown that circulating BDNF are significantly decreased in the stroke objects [125, 126]. Many of these studies have also established that low levels of circulating BDNF in samples taken within 24 hours after stroke onset are associated with poor stroke recovery [9, 125-127].

1.5.3 Basic fibroblast growth factor

Fibroblast growth factors (FGFs) form a large family of signaling proteins. They are potent regulators of cell proliferation and differentiation of a wide range of cells including endothelial cells [128]. The human FGF family bind to and activate a family of high affinity protein tyrosin kinase receptors (FGFRs) 1-4 [129]. One of the most studied FGFs in relation to acute ischemic

neural injury is FGF2, also known as basic FGF (bFGF) [129]. bFGF is widely distributed in the CNS. It is found in neurons and glia, with a predominant localization to astrocytes, where it also is synthesized [130, 131]. It is released as a response to cell death, wounding or other injury [132].

Once released, bFGF binds to its receptors FGFR1 (IIIb), FGFR1 (IIIc), FGFR2 (IIIc) and FGFR4, but it prefer to interact with FGFR1c [133]. In the brain, FGFR1 is expressed in neurons, glia and myelin sheets [134]. Activation of FGFRs by bFGF requires interaction between bFGF and cell surface heparan sulfate proteoglycans (HSPG). HSPG acts as a binding partner and stabilize the FGF-FGFR interaction. Activation triggers the MAPK, PI3K/Akt and PLC-γ pathways, where the MAPK and PI3K/Akt pathways are the best understood ones. The MAPK pathway leads to cell growth and differentiation, and the PI3K/Akt pathway is implicated in cell survival and cell fate determination [133].

After an ischemic injury both bFGF and FGFR1 have been shown to be upregulated in the brain, especially in the penumbra. Glial cells are the predominant sources of bFGF [135, 136]. bFGF signaling is involved in neuronal protection and repair [137] and has been shown to promote NSCs growth, survival, and differentiation into mature neurons within the infarct region hence contributing to neurogenesis [138]. bFGF is also a potent inducer of angiogenesis by enhancing mitogenesis of endothelial cells, and indirectly by stimulating secretion of other GFs such as VEGF [139]. In addition, bFGF has been shown to stimulate astrocyte proliferation. This findings suggest that bFGF is an important contributor to the upregulation of astrocytes observed in injured brains, which is beneficial for BBB integrity and neuronal function [140].

Previous studies have found that serum bFGF levels in human stroke patients are significantly higher than those in control groups, and that higher bFGF serum levels are correlated to improved clinical outcome [81, 141, 142].

1.5.4 Epidermal growth factor

The EGF family consist of 12 GFs classified into five groups, where EGF belongs to the group that primarily interact with the EGF receptor (EGFR, also known as ERBB1) [143]. EGFR is a receptor tyrosin kinase, which after ligand binding primarily dimerizes with itself but also with

its homologs ERBB2,-3 and -4 [144]. Dimerization and the subsequent autophosphorylation activate downstream components of signaling pathways including MAPK, PLC-γ, PI3K/Akt, and signal transducers and activators of transcription (STAT) pathways [145, 146]. The MAPK pathway stimulate cell proliferation, the PI3K/Akt pathway is important for cell survival, PLC-γ pathway activates MAPK and thus also regulate cell proliferation, while STAT pathway regulate gene transcription of specific target genes [145].

In the CNS, EGF is produced by neurons, astrocytes, oligodendrocytes and microglia, and it is also taken up from the peripheral circulation [85, 147]. The same cells express EGFR, but normally at low levels. The NSCs/progenitor cells on the other hand, express EGFR at high densities [148]. EGF contribute to multiple protective effects in the CNS, mainly by stimulating proliferation and differentiation of NSCs into neurons and glia [60], but also by stimulating angiogenesis and reduce apoptosis [144] [146].

After brain injury, EGF stimulates NSCs proliferation, differentiation and migration toward the injured area to replenish the injured neurons and facilitate axon regeneration [148]. In addition is EGFR upregulated in astrocytes, where increased EGF activation stimulates mitosis and thus enhanced astrocyte proliferation, which is important for reestablishing the BBB [58, 149]. Activation of EGFR is also important for astrocyte morphology so the astrocytes can surround neurons and sustain neuronal function [150, 151]. Another contributor to sustained neuronal function is the myelin sheets produced by oligodendrocytes. If demyelinating insult occurs, EGFR signaling will stimulate the production of oligodendrocyte progenitors, and their migration and maturation to myelinating cells [147]. EGF has also been shown to induce angiogenesis via activation of PI3K, MAPK and eNOS [152]. There are very few studies, if any at all, on EGF plasma or serum levels after stroke in humans.

1.6 Physical activity and stroke

The Norwegian Institute of Public Health (NIPH) define PA as "*any bodily movement performed by skeletal muscles, which results in a significant increase in energy consumption compared to a resting state*" [153]. Exercise is a subset of PA that is planned, structured, and repetitive and is performed with the intention of improving physical fitness [154]. PA is often divided into light, moderate and hard PA, depending on how much energy turnover the activity requires [153].

Physical inactivity is listed as one of the five main risk factors for ischemic stroke [66]. PA could be beneficial in three ways regarding stroke: 1) Pre-stroke PA can prevent stroke incidence and post-stroke PA can prevent recurrent strokes [155], 2) Pre-stroke PA may make the brain better suited to cope with a stroke, reducing adverse effects if stroke should occur [16], 3) Post-stroke PA can promote recovery and neuronal plasticity [156].

Regular PA is known to reduce the risk of both primary and secondary stroke. A meta-analysis of 21 studies (n= 650 000) concluded that high level of PA performed outside working-hours reduced the overall risk of stroke incident by 20-30 %, in both sexes [155]. This preventive effect is mainly thought to be due to positive modifications of risk factors for stroke, such as high blood pressure, obesity, high cholesterol and diabetes [157]. But PA and exercise could also have a direct effect on the cerebral tissue through improved neuronal plasticity and anti-oxidant and anti-inflammatory effects [158, 159].

Exercise can be seen as a stress factor resulting in upregulated antioxidant enzyme activity. As long as the stress do not exceed toxic levels, i.e. the pauses between each exercise session in sufficiently long to counteract the stress, trained individuals may have an enhanced endogenous antioxidant capacity compared to inactive people. Theoretically, this may result in an increased ability to handle acute oxidative stress such an acute ischemic stroke [158]. Also an anti-inflammatory effect is observed in response to regular PA, with decreased proliferation of microglia and other immune factors [160]. This could be beneficial for the ischemic brain, but it has not yet been proven.

Regular PA stimulates neural plasticity mechanisms, resulting in for instance angiogenesis and neurogenesis [99, 161]. This applies to both pre- and post-ischemic PA, and may contribute to reduce the infarct volume and neurological deficits following stroke [16, 46, 162]. An important role in promoting exercise-related plasticity is played by GFs, mainly neurotrophins including some of those mentioned above. The underlying mechanism by which GFs are induced in response to exercise, remains to be completely understood. PA-induced release of BDNF for instance has neuroprotective effects and enhances neurogenesis, such as previously described. In addition, BDNF may improve mitochondrial function, thereby reducing oxidative stress after ischemia [163]. Other neurogenetic factors such as bFGF is reported to be upregulated in response to PA as well, but the upregulation may be transient and less robust than that of BDNF [164].

PA-induced increase in VEGF has been reported [165]. One way that exercise can induce cerebral VEGF is through lactate accumulation and activation of the lactate receptor hydroxycarboxylic acid receptor 1 (HCAR1). Activation of HCAR1 leads to enhanced VEGF secretion, possibly through the PI3K/Akt pathway in fibroblasts, causing increased angiogenesis in the brain [99].

Another factor that is released in response to exercise and which affects brain health is irisin. Irisin is a hormone-like myokine that is secreted from muscle cells in response to PA and exercise. It is cleaved and released from its precursor fibronectin type III domain containing protein 5 (FNDC5) [166]. In the brain, irisin is found in neurons, astrocytes and microglia [167], but it is not yet known if irisin is cleaved and released from these cells [168]. However, what is known is that irisin can cross the BBB [169]. Thus, an elevation of peripheral irisin will be mirrored by elevated levels of irisin in the CNS. In response to exercise, the FNDC5 gene expression is increased in skeletal muscle, leading to subsequent increment in circulating irisin [170]. Peripheral overexpression of FNDC5 have been shown to increase BDNF expression in the brain [171], which may explain some of the neuroprotective effects of irisin: irisin inhibits neuronal apoptosis and induces NSC differentiation after cerebral ischemia. Other beneficial effects of irisin are inhibited post-ischemic inflammation, reduced oxidative stress and improved mitochondrial dysfunction [172, 173]. These are effects that can better stroke outcome. In fact, decreased concentrations of irisin are associated with poor functional outcome in ischemic stroke [174]. In addition, PA-induced irisin can prevent obesity and improve glucose homeostasis, thereby lowering key risk factors for stroke [170].

The optimal mode, frequency and duration of exercise for achieving the neuroprotective of PA remain a constant source of debate. A mode of exercise that has received attention lately, is high intensity interval training (HIIT). HIIT is defined as periods with high exertion separated by intervals of recovery either at low-intensity exercise or complete rest [175]. HIIT produces many of the same health benefits as other forms of exercise, but in a shorter amount of time. These benefits include lower body fat, blood sugar, heart rate (HR) and blood pressure [176], all of which represent risk factors for stroke [66]. To what extent HIIT can potentiate neuroprotection remains largely unexplored [175], but HIIT appears to be promising at enhancing neuroplasticity markers such as BDNF, VEGF and irisin [177-179].

HIIT as an exercise mode post-stroke have also shown to better stoke outcome. A report form 2018 analyzing 10 studies done on the effects of post-stroke HIIT, suggested that HIIT might improve functional, cardiovascular, and neuroplastic rehabilitation outcome [180]. In particular PA-induced BDNF was associated with motor recovery improvements [181, 182].

1.7 Determination of cognitive and functional performance after stroke

There have been developed a series of standardized tests to screen for cognitive and functional deficits after stroke. These tests are usually done at or before hospital discharge. The Mini-Mental State Examination (MMSE) is the most widely applied test to screen for cognitive impairment and dementia after stroke [183]. Other tests to measure cognitive decline are the Trail Making Test A and B (TMTA and B), and the clock drawing test (CDT). To measure functional outcome after stroke the most used tests are the Modified Rankin Scale (mRS) and the Barthel activities of daily living (ADL) index. In addition, the Hospital Anxiety and Depression Scale (HADS) is widely used to measure anxiety and depression in stroke patients, as post-stroke depression and -anxiety are commonly seen.

All the tests are measured by a point score, except the TMTs and OCD which are measured in seconds it takes for the subject to complete the task [184, 185]. MMSE comprises thirty items providing information about orientation, attention, learning, calculation, delayed recall, and construction, with a total possible score of 30, indicating normal cognitive function [186]. The mRS is a 6-point assessment that includes reference to both limitations in activity and changes in lifestyle. A grade of 5 is set when severe disability is seen, and a grade of 0 is set when the individual shows no symptoms at all [187]. The Barthel ADL index rate the independency of 10 common ADL activities, with a total score of 20. A higher number indicates a greater ability to function independently following hospital discharge [188]. The HADS is based on self-reported symptoms and consists of 14 questions. The score range from 0 to 42, where 42 indicates the highest level of anxiety and depression [189].

2. Aims of the study

During rehabilitation after an acute stroke, differences in cognitive and motor function recovery is seen in patients even with the same type (size and location) of brain injury presented in the acute phase. To this day, the cause of this variation in recovery is not known, and hence it is difficult to predict recovery. During a stroke, a number of different substances such as GFs,

neurotransmitters, inflammatory mediators etc. can be detected in the blood. A hypothesis is that the combination/balance of these factors can be used to indicate prognosis. Exercise may be protective in stroke by affecting the same GFs.

The aim of the present study is to compare the levels of GFs and irisin in patients with acute stroke and the basal levels found in healthy control persons, as well to investigate whether some of the same alterations are induced by exercise. By screening different factors, we can detect which of them are changed after a stroke, and possibly identify factors that potentially affect the brain during the rehabilitation phase. This may lay the foundation for the identification of biomarkers (or combinations of biomarkers) that can be used to predict progression in cognitive and/or functional performance after stroke. The specific research questions of this master thesis are

Question 1: Which neuroactive factors are secreted from brain to blood during a stroke?
Question 2: Which neuroactive factors are secreted from brain to blood during high-intensity interval exercise in people >50 years of age?

3. Materials and methods

The following section will describe the chosen methodology, from the exercise regime to the sample collection and analysis. Exercise, cognitive testing and sample collection was performed by Linda Thøring Øverberg (PhD candidate) or employees at the Akershus University hospital (Ahus). The immunoassay of irisin were done in collaboration with Linda Thøring Øverberg.

3.1 Ethics

The project was approved by REK (REK ID 2018/2555) and NSD (NSD ID 539270) and has been registered in the quality assurance system for health and medical research, at UiO, Helseforsk. All permits and cooperation agreements have been obtained.

Informed consent was obtained from the control group before inclusion. The stroke patients had signed informed consent to participation in the biobank. Admission to the samples from the biobank was granted for this project through an agreement with the owner of the Biobank. All data material was processed at group level and is thus unidentifiable when used in

publications and presentations, including this master thesis. The data material was, and still is, kept out of reach of anyone other than the researchers who are affiliated with the project. The work was carried out according to the Helsinki Declaration of 1964.

3.2 Study subjects

The study included 48 patients with ischemic stroke and 48 age- and gender-matched healthy controls.

3.2.1 The stroke patients

The patients included through the biobank consisted of stroke patients (n= 48; 18 males; 30 females) enrolled in the biobank at Ahus (REK ID: 2011-1015). Inclusion criteria were; >50 years of age, ischemic stroke as the main cause for hospitalization and for whom the time between stroke onset and the hospitalization had been noted in the patient journal. Exclusion criteria were; patents with a hemorrhagic stroke and cognitive impairment that prohibited an informed consent to be obtained.

The following information were collected form the patient journal when available by associate professor/MD, PhD Ole Morten Rønning at Ahus; 1) time from symptom onset to admission, 2) size of lesion, 3) Lacunar vs cortical stroke or TIA 4) the scores obtained by the patient on the mRS, MMSE, TMTA and TMTB. Their score on the Barthel ADL index was also included.

3.2.2 The control group

In total, 63 healthy elderly participants were enrolled in this study. Of these, 48 (n= 18 males; 30 females) were matched based on the sex and age with stroke patients from the Biobank group. The inclusion criteria were: elderly (>50 years), healthy (defined by self-reported experience of their own health), cognitive abilities that allow for an informed consent, and able to run/walk on a treadmill for the duration of the test. The exclusion criteria were: participants with previous stroke or a TIA.

3.2.2.1 Investigational test

Prior to the examination, all participants underwent different investigational test with focus on attention and cognitive function, as well as depression and/or anxiety. The first test was the

Norwegian Revised MMSE (MMSE-NR3). The second test was the CDT followed by the Norwegian Revised TMT (TMT-NR3). Finally, a HADS test was conducted after the interval exercise.

3.2.2.2 Measurements and habits

Resting HR and blood pressure were measured, as well as weight and hight to calculate body mass index (BMI). Maximal HR was calculated using a max pulse calculator (HRmax estimate 211-0,64 x age) [190]. All participants answered questions about their exercise habits; type of activity and a rough approximation of number of hours, whether they had a familial predisposition to stroke and/or heart disease, if they were using blood-thinning drugs or whether they were on other prescribed drugs regarding heart and lung conditions, and whether they smoked or used other nicotine stimulants.

3.2.2.3 Training procedure

A HIIT procedure were completed after the cognitive tests were performed. Prior to the session, the subjects were asked to retain from physical activity and caffeine, but this was not always complied to. There was no claim of overnight fast. The training regime consisted of a 10-minute warm-up at 60 % max pulse, followed by 4 x 4-minute intervals on a treadmill either running or walking up hill. Between the intervals there was a period of three minutes of active rest, defined as 70 % of max pulse.

The workload was increased based the participants HR, the perceived exertion using Borg´s scale (Figure 2) [191] and the observations made by the observer. The ultimate goal during the intervals was to reach a HR between 80-95 % of their maximum HR.

Level	Percived exerton
6	Rest
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somwhat hard
14	Hard
15	
16	Very hard
17	
18	Extremely hard
19	
20	Maximal exertion

Figure 2 Borgs scale. *A Norwegian Borg´s scale was used in this study but has been translated into English for the purpose of this thesis. The scale was used to rate the perceived exertion at the beginning and the end of each of the four intervals. The scale ranges from 6 to 20, where 6 means "rest" and 20 means "maximal exertion".*

3.3 Sample collection

Blood samples were taken of the stroke patients between 12 and 1 pm, the day after admission. The samples were centrifuged, and the plasma was aliquoted and stored at -80°C until further analysis.

Two sets of blood samples were taken from the control group: one small sample of capillary blood from the fingertip (for immediate lactate measurements) and one 6 mL sample of venous blood for analysis of GFs etc. The capillary samples for lactate measurements were taken at four time points; before training, immediately after the second interval and again after the fourth interval, and finally at 30 minutes after the end of the HIIT. The participants washed their hands prior the exercise regime to avoid incorrect measurements. Blood was taken from the side of the fingertips and filled directly into capillary tubes. The capillary tubes were then put in a pre-filled micro test tube with haemolysing solution (glucose / lactate – haemolysing solution 20ul end to end capillaries). The micro test tube was mixed end-over-end 10 times and analysed by the Biosen C - Line Glucose and lactate analyser.

Blood samples for analysis of GFs were collected from one of the veins at the inside of the elbow, at three different times: immediately before the warm-up (baseline), immediately after HIIT and 30 minutes after HIIT. Blood samples taken before warm-up were taken at rest (after

at least 30 min of sitting still). From each subject, a total of three 6 mL whole blood was drawn into tubes containing K2 ethylendiaminetetraacetic (K2EDTA) at each time point. The tubes containing blood samples were gently inverted about 10 times before centrifugated by using the Eppendorf centrifuge 5810 R at 1000 rcfg for five minutes. The supernatant (plasma) was then gently pipetted and immediately aliquoted in 500 µL pcr tubes. Each tube contained 190-200 µL plasma, and were marked with date, participant number and blood sample number (1, 2 or 3) before placed in a 30 mL tube for each patient/timepoint. The plasma samples were then frozen and stored at -80 °C degrees.

3.4 ELISA

Plasma levels of the factors were analyzed using a sandwich enzyme-linked immunosorbent Assay (ELISA) technique, following the instructions of the manufacturers (R&D Systems, MN, USA). ELISA is a type of immunoassay that is commonly used to quantify levels of a specific target within a sample. In sandwich ELISA, two matching antibodies are used to detect the antigen in the sample. These are denoted the capture antibody (CA) and the detection antibody (DA). The two antibodies binds highly selective to different, non-overlapping parts (epitopes) of the antigen molecule [192].

The wells of a 96-well microplate were coated with the CA overnight, washed and then blocked using Reagent Diluent (RD) buffer (Provided as 10X in the kit, and diluted to 1X in milliQ water). The RD buffer contained 1 % bovine serum albumin (BSA) which blocks unspecific binding surfaces, reducing background interference. Sample were then added and incubated for 2 hours, allowing the protein of interest to bind to the CA on the plate, and then washed to remove unbound antigens. A biotin-labeled DA was added; this antibody binds to a separate epitope on the target protein and incubated for two-hours. The plate was then washed before enzyme-conjugated streptavidin was added. Streptavidin interacts strongly with the biotin making the complex detectable. Then the enzyme substrate was added resulting in a color signal that was proportional to the amount of analyte present in the original sample. Color intensity was measured using a plate reader and the concentration was calculated by comparison to a standard curve of known concentrations, which was obtained for each plate [192, 193]. An overview of the sandwich ELIAS method is given in Figure 3.

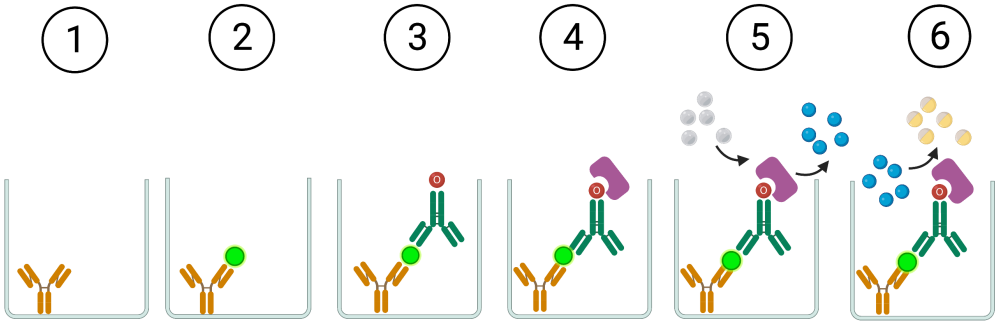

Figure 3 Sandwich ELISA. *1. A capture antibody (orange) binds to the surface of the well, 2. The analyte (green sphere) then binds to the detection antibody; The percentage of detection antibodies that binds the analyte is proportional to the concentration of the analyte in the sample. 3. A biotin-labeled (red sphere) detection antibody (dark green), then binds to the antigen. 4. Enzyme-conjugated streptavidin (purple rectangle) interact strongly with the biotin. 5. Enzyme substrate (grey sphere) is added and converted into a colored product (blue sphere) 6. Finally, the stop solution is added to terminate the enzyme substrate reaction, turning the color from blue to yellow. The intensity of the yellow signal can be detected in the plate reader as a measure of the concentration of the analyte in the sample (Figure created with BioRender).*

3.4.1 Materials and chemicals

All chemicals were supplied by R&D Systems. Table of materials and chemical used are listed in Table 1.

Table 1 Materials and chemicals used in ELISA. *A DuoSet Ancillary Reagent Kit and a DuoSet ELISA for the specific antigens, were used in the study.*

Kit	Kit contents	Antigen	Catalogue #
DuoSet Ancillary Reagent Kit			DY008
	96 well microplates		
	Substrate solution		
	Stop solution		
	Plate coating buffer		
	Wash buffer		
	Reagent Diluent Concentrate		
DuoSet ELISA			
	Capture Antibody		
	Detection Antibody		
	Recombinant Standard		
	Streptavidin-HRP		
		VEGF-A: Human VEGF	DY293B
		BDNF: Human/Mouse BDNF	DY248
		FGF2: Human FGF basic/FGF2/bFGF	DY233
		Human EGF	DY236-05
		Human Irisin/FNDC5	DY9420-05

3.4.2 Reconstitution and working diluents

Before analyzing each new factor, the reagents in the DuoSet ELISA-kit had to be reconstituted. The standard and DA were reconstituted in RD, and CA was reconstituted in ELISA plate Coating Buffer which consist of sterile-filtered 1X Phosphate buffered saline (PBS). After reconstitution, the components were to sit for a minimum of 15 minutes with gentle agitation, before they were distributed in Eppendorf tubes and stored at -80 °C degrees according to the lot-specific Certificate of Analysis for storage conditions.

Working dilutions of the reagents were prepared to the concentrations specified in the package insert or on the vial and used immediately after preparation. CA was diluted in ELISA plate Coating Buffer, while DA, Standard and streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP) were diluted in RD. Concentrated wash buffer and RD was diluted in purified water.

All reagents used in both reconstitution and working dilutions was brought to room temperature before use.

3.4.3 Sample dilution

To find the optimal dilution factor for each specific factor analyzed, plasma samples from a person who was not included in the study were used. The person underwent the same exercise regime as the control group, and blood samples were taken at the same time points and prepared as described for the control group. These plasma samples were diluted with RD to four different concentrations: 1:5, 1:10, 1:20 and 1:100. Before each transfer new pipette tips were used, and the solution was mixed on a vortex mixer. Next, the ELISA assay was performed on the four different dilutions from each of the three samples to find the dilution factor that was within the standard curve. This dilution factor was then further used in the samples from the control subjects and the stroke patients. An illustration of the described serial sample dilution in found in Figure 4.

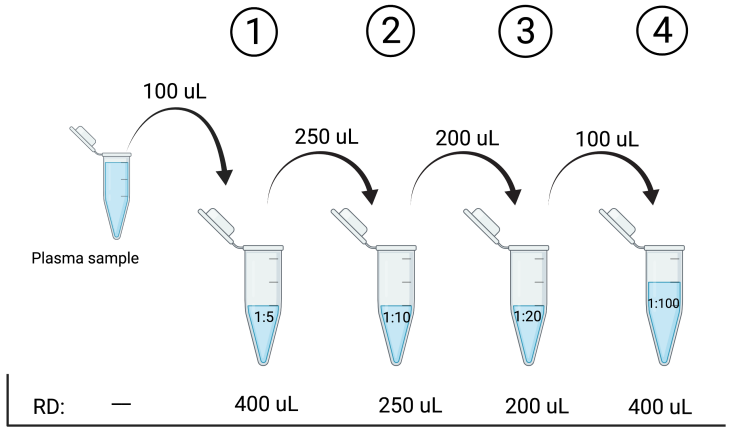

Figure 4 Serial dilution of plasma samples. *Four different concentrations were made: 1:5, 1:10, 1:20 and 1:100. Before each transfer the plasma and/or diluents were mixed on a vortex mixer. Step 1: 100 µL plasma was added to 400 µL reagent diluent (RD) to make a 1:5 dilution, Step 2: 250 µL of the 1:5 dilution was added to 250 µL RD to make a 1:10 dilution, Step 3: 200 µL of the 1:10 dilution was added to 200 µL RD to make a 1:20 dilution, Step 4. 100 µL of the 1:20 dilution was added to 400 µL RD to make a 1:100 dilution (Figure created with BioRender).*

All plasma samples were diluted immediately before applicated to the plate. The day before, RD was added to Eppendorf tubes and stored in the fridge overnight. On the day of analysis, the Eppendorf tubes containing RD were brought to room temperature before plasma was added. The plasma samples were mixed on a vortex mixer before transfer and stored on ice prior to and after transfer so they could be used in later experiments.

3.4.4 Standards

All standards were made according to the manufacturer's protocol. A 7 or 8 point standard curve was made. Eppendorf tubes of 1.5 mL were placed in a rack and numbered S1-S8, where S1was a negative control containing only RD, and S8 was the highest standard concentration. S8 was the undiluted reconstituted standard, and the rest of the standards were made by using 2-fold serial dilutions of this in RD (Figure 5). Before each transfer new pipette tips were used, and the solution was mixed on a vortex mixer.

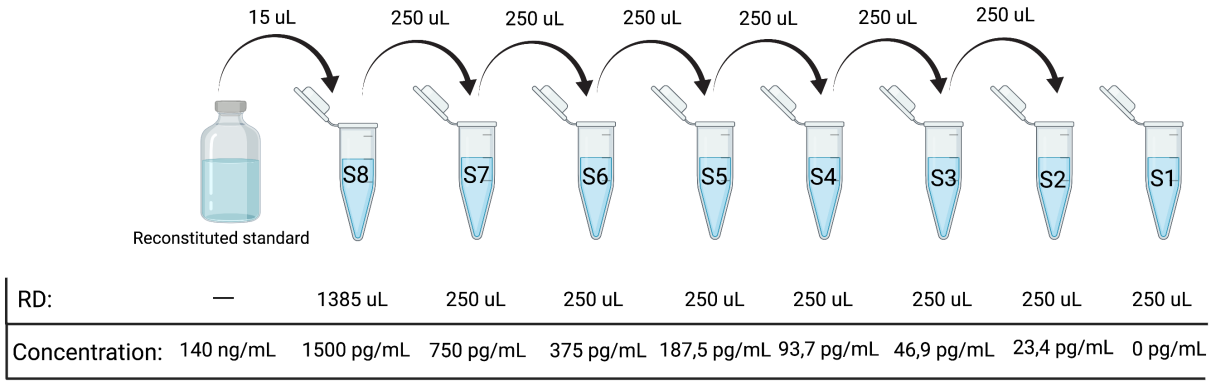

Figure 5 Example of a serial dilution of the ELISA BDNF-standard*. An example of a serial standard dilution from a stock solution to generate a standard curve of 23.4 –1,500 pg/mL. Before each transfer the finished standards were mixed on a vortex mixer. A negative control (S1) with RD only, was also included (Figure created with BioRender).*

3.4.5 Procedure

3.4.5.1 Plate Preparation

1. CA was diluted to the working concentration in ELISA plate Coating Buffer. A 96-well microplate was immediately coated with 100 µL per well of the diluted CA. The plate was sealed and incubated overnight at room temperature.
2. The next day, each well was aspirated and then washed with Wash Buffer for a total of three washes using Inteliwasher 3D-IW8 Microplate washer (BioSan, Latvia). After the last wash, any remaining Wash Buffer was removed by inverting the plate and blotting it against clean paper towels.
3. RD (300 µL) was added to each well to block the unspecific binding sites, and then incubated at room temperature for a minimum of 1 hour.
4. The aspiration/wash was repeated as in step 2.

3.4.5.2 Assay Procedure

1. Diluted samples or standards (100 µL) was added per well and ran in duplicates to provide enough data for statistical validation of the results. To avoid systematic errors, samples from the control group and the stroke patients were ran together (Figure 6). The plate was covered with an adhesive strip and incubated for 2 hours at room temperature.
2. The aspiration/wash was repeated as in step 2. of the Plate Preparation.

3. DA was diluted to the working concentration in RD and 100 µL of working concentration of DA was added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature

4. The aspiration/wash was repeated as in step 2. of the Plate Preparation.

5. Streptavidin-HRP B was diluted to the working concentration specified on the vial label using RD and 100 µL of this dilution was added to each well. The plate was covered and incubated for 20 minutes at room temperature, protected from direct light.

6. The aspiration/wash was repeated as in step 2. of the Plate Preparation.

7. Substrate Solution (100 µL) was added to each well. The plate was then incubated for 20 minutes at room temperature, protected from direct light. A blue color started to appear in the wells as the substrate was converted into a blue end product by the streptavidin-HRP enzyme. The intensity of the color was proportional to the amount of HRP in the sample, which in turn was proportional to the concentration of antigen in the original sample.

8. Stop Solution (50 µL) was added to each well. The blue color turned to yellow. To ensure thorough mixing, the plate was gently tapped until all wells were completely yellow- and not green.

9. The optical density of each well was immediately determined by using Victor X4 Multilabel Plate Reader (Perkin Elmer, USA) at 450 nm.

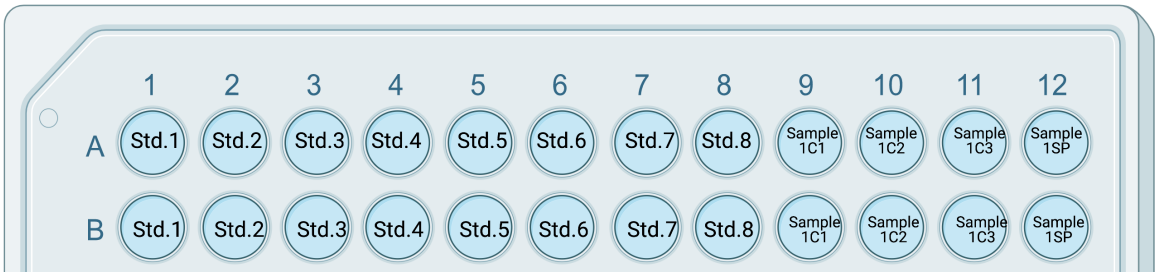

Figure 6 Example of ELISA plate chart. *100 µL of standards and samples were added to each well in vertical duplicates. Standards were added in ascending order (Std.1-8). The control group and the stroke patients were run together, starting with the sample taken before training (1C1), followed by right after training (1C2), 30 minutes after training (1C3) and sample form matching stroke patient (1SP). The remaining plate was filled with series of eight samples (duplicates of each the three time points for the control person and duplicates of the sample from the corresponding stroke patients) as examplified for paired control person 1 (C1) and the corresponding stroke patient (SP1) (Figure created with BioRender).*

3.5 Data analysis

All samples were assayed in duplicate on each plate, in order to test the intra-assay variation. Precision was measured as a coefficient of variation (CV) from the mean value of the two duplicates to expose any human error or uniformities. A CV below 20 % was accepted, but care was taken to keep the CV as low as possible. In most cases it was below 10 %. Duplicates exceeding a CV of 20 % was reanalyzed.

A standard curve was made by using a four parameter logistic (4-PL) curve-fit. The standard curve fit was used to calculate the concentrations from the standards. The concentration read from the standard curve were multiplied by the dilution factor in the diluted samples to find the concentration of antigen in the original plasma sample.

3.6 Statistics

Since the samples obtained at three different time points from each control person were dependent on each other, while the sample obtained from the stroke patient was independent, a standard one-way ANOVA test could not be performed. Instead, the statistical analysis was performed by a series of Student's *t*-tests, paired when samples from the control persons were compared to each other and unpaired when samples from the stroke patients were compared to the basal levels in the controls. To compensate for multiple comparisons, a Bonferroni correction was performed manually by multiplying the p-value form the Student's *t*-test with the number of comparisons. A corrected p-value of <0.05 was considered statistically significant.

4. Results

In this section, the results from the analyses conducted using the methodology described in section 3 are presented. Tables, figures and discussions are presented along with the findings for all factors investigated in this thesis; BDNF, bFGF, EGF, irisin and VEGF.

4.1 Background characteristics of the study group

The term "study group" will from here on refer to the matched pairs of one control person and the corresponding stroke patient. Table 2 presents an overview of the 96 subjects in the study group (48 stroke patients and 48 controls). For the control group, blood samples were taken at

three time points (before, right after, and 30 minutes after training). For the stroke patients, one sample was taken the day after admission to the hospital. The time between stroke onset and admission to the hospital varied greatly, from 1 hour to >240 hours. The average age of the subjects studied was 70.2 years, and 62.5 % (30 out of 48 in each group) were female. Of the stroke patients, 32 subjects were categorized with a small stroke, 5 with a medium stroke, 4 with a large stroke, and 7 subjects were categorized with TIA.

Cognitive abilities were tested with MMSE and TMTA and TMTB. For the control group, this was performed prior to the exercise regime. For the stroke patients, these tests were performed between day 3 after admission and the time discharge from the hospital (the patients were normally hospitalized for 4-10 days). The degree of disability or dependence among the stroke patients was measured by mRS, and found to be 1.91±1.37, with a median of 2. The ADL skills of the stroke patients were also tested, using the Barthel ADL index; the average Barthel ADL score was 17.5 (of 20). Of the stroke patients, 13/48 had a score below 20 and of these, 6 were below 10. The control group had a significantly higher MMSE score than the stroke patients (p-value < 0.001; unpaired student's t-test) with a mean of 29.1 (of maximal 30) in the control group vs. 25.5 in the group of stroke patients. Of the stroke patients 13/40 had a MMSE score blow 25, of these 3 had a score below 20. For comparison, none of the control persons had a score below 25. Time spent to complete the TMTA for the control group was 37.1 seconds (average), while the stroke patients used significantly longer times; 60 seconds (p=0.001; unpaired student's t-test). TMTB scores were also significantly higher for the stroke patients with 131 seconds vs. 88 seconds for the control group (p=0.001; unpaired student's t-test). See Table 2 for summary characteristics for the full dataset.

Table 2 Summary characteristics for the full dataset*. The study group consisted of 48 stroke patients and 48 age- and gender-matched subjects in the control group. The average age of both groups was 70.2 years. There was an overweight of female subjects (30 out of 48) in both groups. *Stroke patients performed statistically significantly from the control group; p < 0.05.*

Description	**Control group**	**Stroke patients**
Number of participants (n)	48	48
Age, mean ±SD	70.2 ± 7.7	70.2 ± 7.7
Female, n (%)	30 (62.5 %)	30 (62.5 %)
Exercise regime	4 x 4 min HIIT	-
Stroke size, n (%) Small (S); Medium (M); Large (L); TIA (TIA).	-	S: 32 (67 %); M: 5 (10.4 %) L: 4 (8.3 %); TIA: 7 (14.6 %)
Time between stroke onset and hospitalization, median (min-max)	-	21.5 hours (1 - >240 hours)
Number of blood samples from each person; time of blood sample(s)	3; before, immediately after, and 30 min after exercise	1; between 12:00 and 13:00 the day after admission to hospital
mRS average ± SD; median (n)	-	1.9±1.4; 2 (48)
MMSE average ± SD; median (n) (30= maximal score)	29.1 ± 0.9; 29 (48)	25.5 ± 4.1; 26.5 (40)*
TMTA; average time spent (min) ± SD; median (n)	37.1 ± 14.0; 22.3 (48)	60.0 ± 34.5; 49 (27)*
TMTB; average time spent (min) ± SD; median (n)	88.4 ± 45.9; 77 (48)	131 ± 64.5; 114 (25)*
Barthel ADL; average ± SD; median (n) (20= self-sufficient)	-	17.5 ± 4.9; 20 (48)

We also measured the blood lactate in the control group at four different stages: before exercise, after two intervals, after four intervals and 30 minutes after training. The lactate level before exercise had a mean of 1.52±0.08 mmol/L. After 2 intervals the mean was significantly higher (5.1±0.37) than the baseline level ($p=1.08x \times 10^{-14}$; paired student's t-test). After 4 intervals, the lactate level was even higher (7.2±0.4) than after two intervals (p=0.0003; paired student's t-test). 30 minutes after exercise, the lactate level was significantly lower than after exercise

(2.5±0.2), both after 2 and 4 intervals ($p=2.1 \times 10^{-8}$, $p=1.9 \times 10^{-17}$; paired student's t-test). See Figure 7.

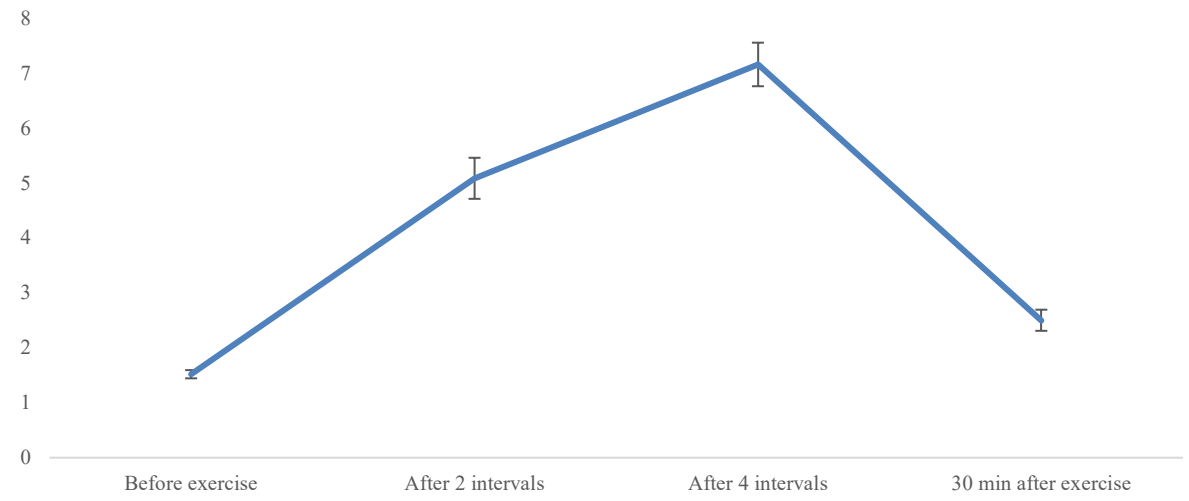

Figure 7 Mean concentration of blood lactate level. *The figure illustrates the development in lactate level (mmol/L) with corresponding standard error measures at four different stages; before exercise, after 2 intervals, after 4 intervals and 30 minutes after training. The increase after 2 and 4 intervals was significant compared to the baseline level. 30 minutes after exercise, the lactate level was significantly lower than right after exercise.*

4.2 Growth factor levels in plasma

When looking at the distribution for each factor for the control group, there was one clear outlier across all analyses (indicated by the red dotted box in Figure 8), who had plasma levels of most GFs that was several folds above the rest of the control group. For the purposes of the statistical testing, it was chosen to exclude the outlier, as the subject was not representative and would disturb the results. To keep the age- and gender of the stroke patients and the controls equal, we also removed the stroke patient who was matched with this control person, reducing the number of observations in both groups in this section (4.2) from 48 to 47. The exception is when looking at stroke sizes, as this are a separate measure which only applies for the stroke patients. Here the number of observations will still be 48.

The data was somewhat right skewed, but the normality assumption was considered to hold for the statistical testing. Since we had 47 observations left after removal of the outlying subject,

the sample size was considered large enough not to normalize the dataset further or deploy non-parametric tests on the data.

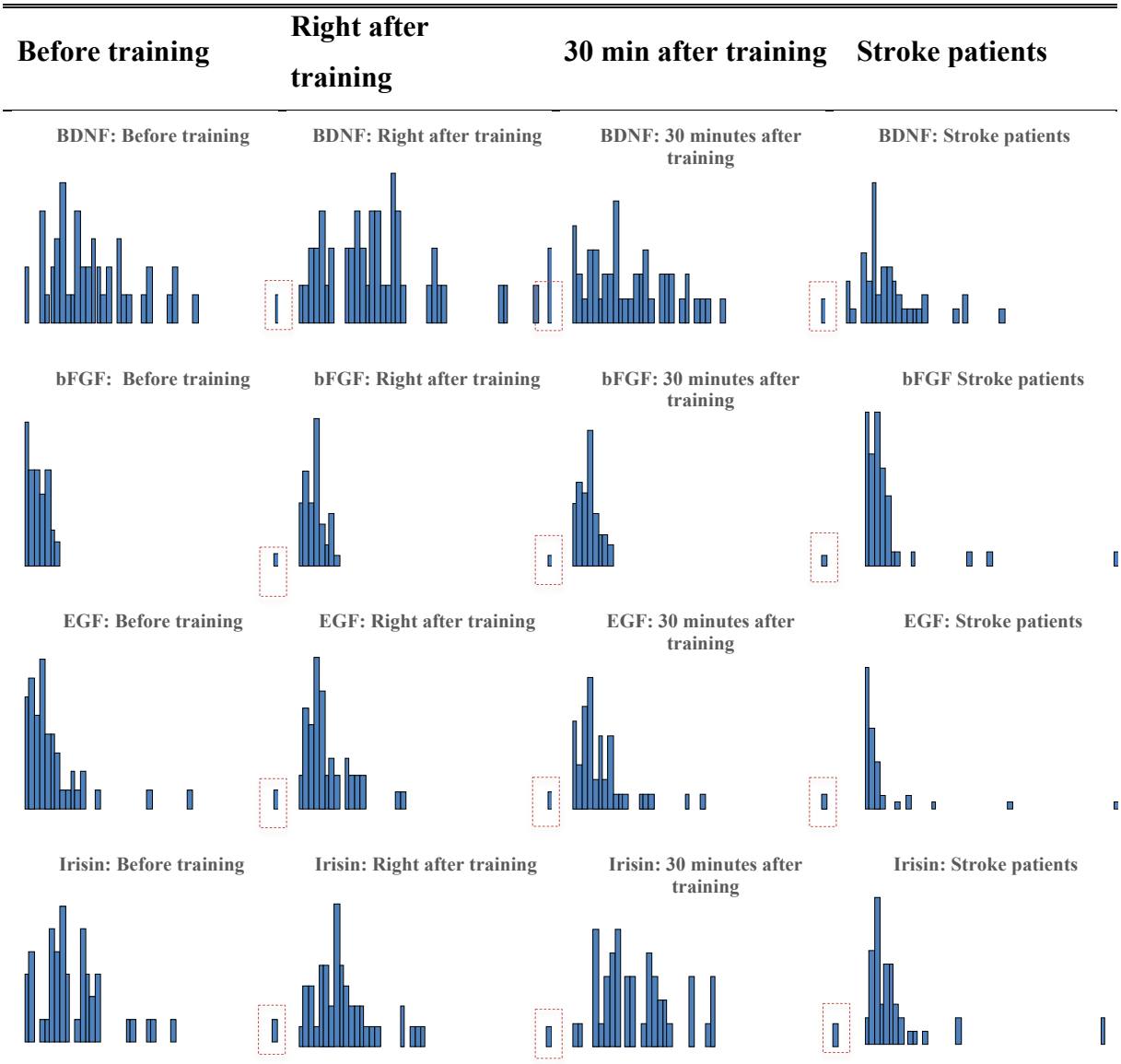

Figure 8 Concentration distributions for growth factors and irisin. *The figures illustrate the distribution of concentrations for BDNF, bFGF, EGF and irisin in the control group before training, right after training, 30 minutes after training and in the group of stroke patients. Please note the outlier (indicated by the red dotted box); this participant was excluded from the analysis.*

In the following sections, a consistent approach to data presentation is used. First, the mean concentrations for the full dataset are presented. Then, the study group is further segregated by sex, and by different age groups. Finally, the impact of stroke size is explored. Since the three samples obtained from the control group (before training (hereafter referred to as "baseline"), right after training, 30 minutes after training) are from the same persons, these measurements

are related. The samples from the stroke patients, although matched with the control persons, are independent. Hence, a paired student's t-test was used to examine the relationship within the control group (before training, right after training, 30 minutes after training). When testing the control group against the stroke patients, we deployed an unpaired student's t-test. Both tests were two-tailed and subjected to Bonferroni corrections when the results showed significance (p-values below 0.05 multiplied with the number of comparisons).

4.3 BDNF

4.3.1 Full dataset BDNF

The BDNF concentrations had a large variation, with the minimum concentration before training at 5,231 pg/mL and the maximum 35,057 pg/mL. Still, as displayed in Figure 9, most of the data points were distributed around the mean values. Table 3 present summary statistics for the full BDNF dataset.

Table 3 Summary statistics for BDNF dataset. *The concentration of mean plasma BDNF measured in the control group at three time points (before, right after, and 30 minutes after the end of the exercise) is shown along with the concentration of BDNF in plasma from the stroke patients The table also show the median BDNF and the lowest (Min) and highest (Max) observed concentration value, SEM = Standard error of the mean.*

	Before training	**Right after training**	**30 min after training**	**Stroke patients**
Mean	16,702	18,393	15,921	6,690
Median	15,398	17,142	14,367	5,375
Min	5,231	5,178	5,365	1,428
Max	35,057	46,904	32,295	29,613
SEM	1,038	1,375	1,065	709

In the control group, the BDNF concentration before training was 16,702±1,038 pg/mL (mean±SEM). No difference was observed in BDNF levels in response to exercise: BDNF levels measured after exercise were 18,393±1,376 pg/mL and 15,921±1,065 pg/mL immediately after and 30 minutes after training, respectively. There was no clear pattern in the mean of BDNF concentration before and after training (p=0.11 and p=0.28; paired student's t-test). However, the mean concentration was significantly lower in the stroke patients, where

BDNF was measured to 6,690±710 pg/mL compared to the baseline levels in the control group ($p= 2.6 \times 10^{-11}$; unpaired student's t-test; Bonferroni corrected). The mean concentration±SEM for each timepoint/group are displayed in Figure 9.

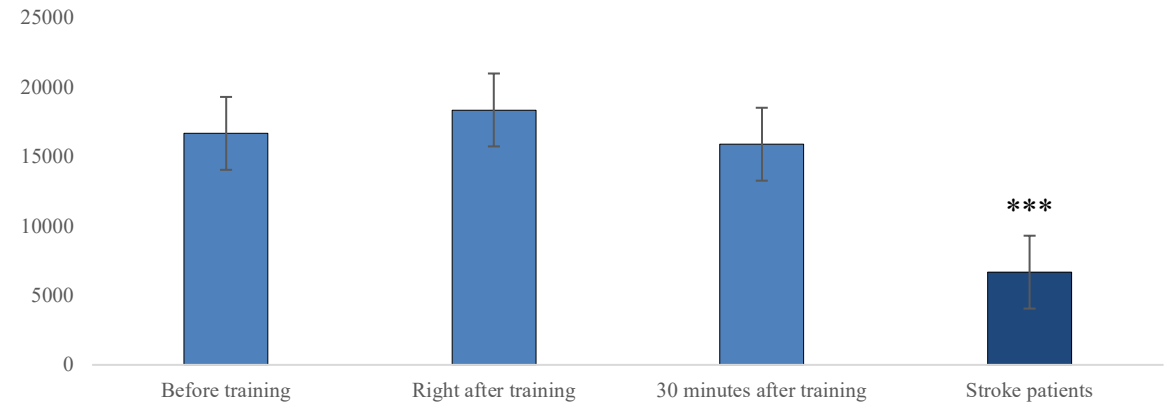

Figure 9 Mean concentration of BDNF. *The mean concentration of BDNF levels is displayed in this figure for each of the different timepoints: before training, right after training, 30 minutes after training and in the stroke patients. The concentration of BDNF (pg/mL) was significantly lower in the stroke patients than in the control persons before training, but did not change significantly within the control group in response to exercise. ***p<0.001.*

4.3.2 Effects of gender on the levels of BDNF

When the study group was divided based on gender (each group consisted of 30 females and 17 males), we found that the pattern was similar for females and males (Figure 10). The males had a slightly lower baseline level of BDNF than the females (17,900±1,378 in female vs. 14,590±1,383 in males), but this did not reach statistical significance (p=0.11; unpaired student's t-test). The BDNF levels right after exercise reached statistical significance in the males (p=0.05; paired student's t-test; Bonferroni corrected) but not in the females (p=0.35; paired student's t-test). The observed mean BDNF concentration was lower in the stroke patients than at baseline for both genders (p <0.001; unpaired student's t-test; Bonferroni corrected).

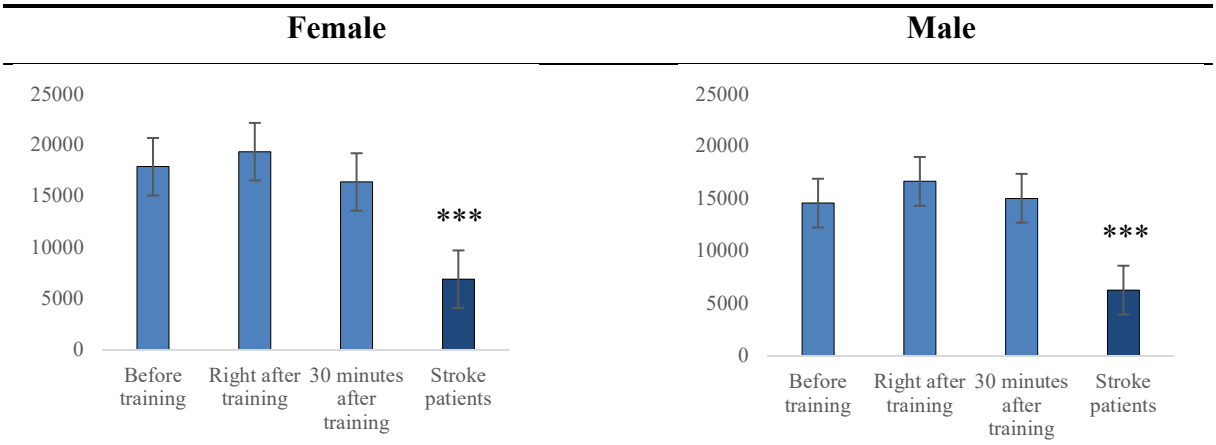

Figure 10 Mean concentration BDNF per gender. *The mean concentration of BDNF is displayed in this figure for each of the timepoints: before training, right after training, 30 minutes after training and in stroke patients. The concentration of BDNF (pg/mL) was clearly lower in the stroke patients of both genders compared to the baseline levels (before training) in the respective gender. There was no clear pattern in the measurements before training. ***p<0.001.*

4.3.3 Effects of age on the levels of BDNF

To test whether there were any differences in the results based on age, the subjects were categorised into four age groups: 1) Below 65 years old (n=11), 2) between 65 and 69 years old (n=10), 3) between 70 and 75 years old (n=14) and 4) above 75 years old (n=12). The results obtained in the different age groups did not differ from each other, or from the general trend reported in Figure 8. In all four age groups, the levels of BDNF were lower in the stroke patients compared to baseline levels in the control groups (Figure 11). For the age group > 65 p=0.04, 65-69 p= 0.02, 70-75 p=7.4 x 10^{-5}, >75 p= 0.001 (unpaired student's *t*-test; Bonferroni corrected).

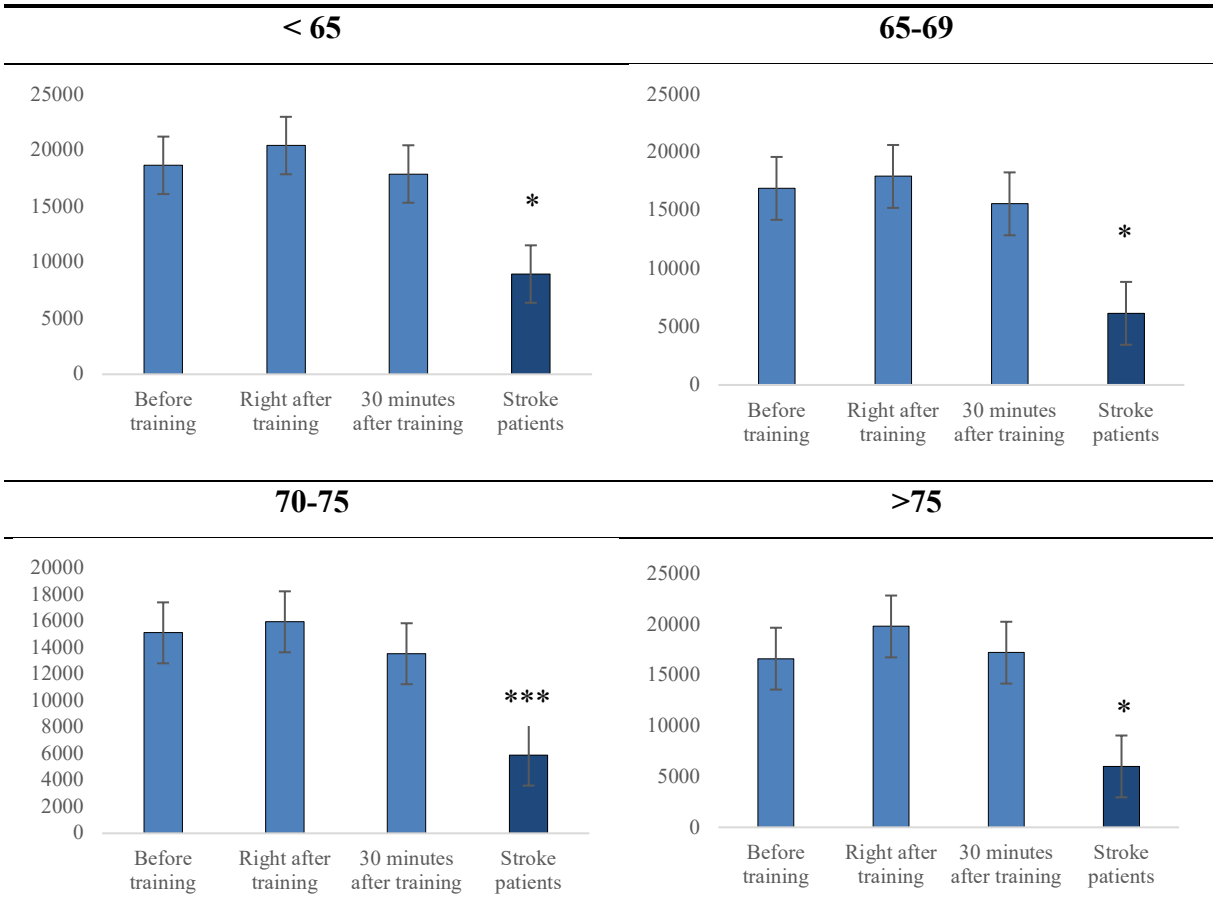

Figure 11 Mean concentration BDNF for the different age groups. *The figure display an overview of the chosen age groups analysed in this section. The subjects were divided into four groups based on age and analysed separately. Numbers of observations (n) across the groups were quite similar. *Statistically significant difference from baseline level with a p-value < 0.05 (Bonferroni corrected), ***p<0.001.*

4.3.4 Effects of stroke size on the levels of BDNF

We then went on to determine if the difference in BDNF concentrations in the stroke patients was dependent on the stroke size. The strokes were classified based on the information provided in the patient journal into either small, medium or large strokes or TIA. Of the 47 patients, 32 had a small lesion, 5 had a medium lesion, 4 had a large lesion and 7 were classified as TIA. Figure 12 illustrate the mean BDNF concentration and SEM for the different stroke sizes. There was no statistical significance when testing the difference between small stroke and medium stroke (p=0.07; unpaired student's t-test), small and large stroke (p=0.23; unpaired student's t-test) or small stroke and TIA (p=0.20; unpaired student's t-test).

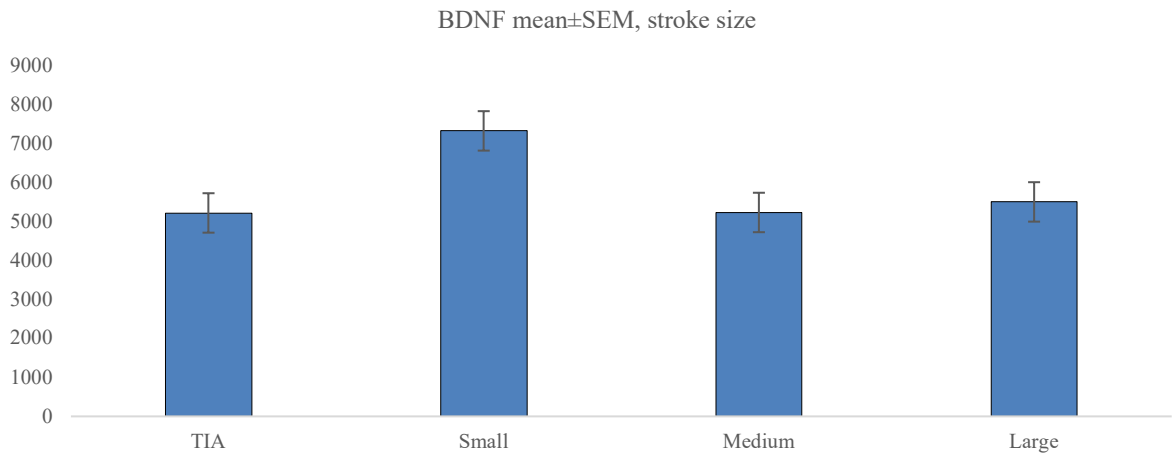

Figure 12 Mean concentration of BDNF in stroke patients with different stroke sizes. *The mean concentration of BDNF (pg/mL) after stroke are displayed in this figure, divided based on the stroke size. The concentration of BDNF was marginally higher in patients with small lesion sizes compared to in patients with medium stroke sizes (p=0.07), but there was no difference in BDNF levels between patients with small and large lesions, between patients with small and medium lesions sizes or between patients with TIA and small lesions.*

4.4 bFGF

4.4.1 Full dataset bFGF

The summary statistics for the full bFGF dataset, including mean and median levels along with the lowest (Min) and highest (Max) measured levels at each time point (before, immediately after and 30 minutes after exercise) in the control group and levels in the stroke patients, are presented in Table 4, along with the corresponding SEM values.

Table 4 Summary statistics for bFGF dataset. *The concentration of plasma bFGF (mean) measured in the control group at three time points (before, right after, and 30 minutes after the end of the exercise) is shown along with the concentration of bFGF in plasma from the stroke patients. The table also show the median bFGF and the, lowest (Min) and highest (Max) observed concentration value, SEM = Standard error of the mean*

	Before training	**Right after training**	**30 min after training**	**Stroke patients**
Mean	683	685	673	588
Median	668	685	668	558
Min	413	397	383	315
Max	1,092	1,070	1,067	1,417
SEM	26	25	24	30

When plotting the mean concentration for the subjects before training, right after training, 30 minutes after training and in stroke patients, there was not a clear pattern in the data: The concentration of bFGF (pg/mL) was significantly lower in the stroke patients (588±30 pg/mL) than in the control persons at baseline (683±26 pg/mL) when only doing an unpaired Student's t-test (p-value= 0.027), but with Bonferroni correction the result was no longer significant (p-value= 0.107). bFGF levels did not change in response to training in the control group for the comparison between bFGF right after training and 30 minutes after training with the baseline level of bFGF in the same patients (p=0.81 and p=0.25; paired student's t-test). See Figure 13.

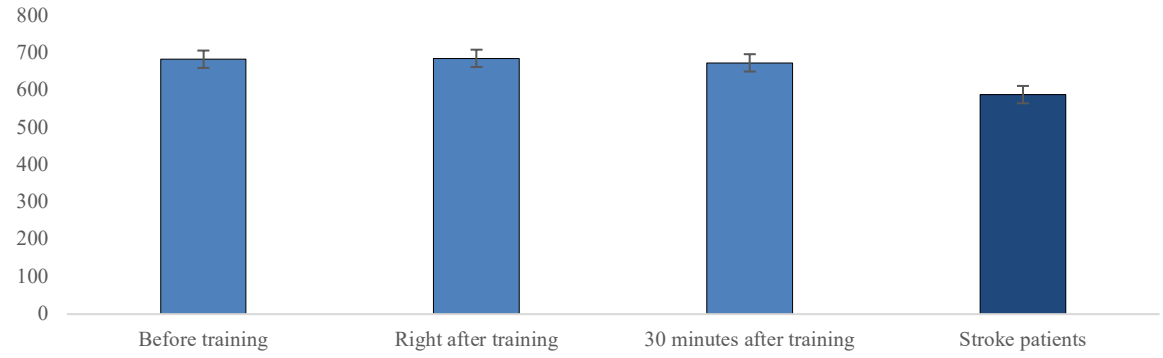

Figure 13 Mean concentration of bFGF. *The mean concentrations of bFGF are displayed in this figure for each of the measured stages: before training, right after training, 30 minutes after training and in stroke patients. There was a tendency towards a lower concentration of bFGF (pg/mL) in the stroke patients compared to the baseline level of bFGF in the control group, but this was not statistically significant after Bonferroni correction. Exercise did not affect the bFGF concentrations.*

4.4.2 Effects of gender on the levels of bFGF

When the study group was divided based on gender (each group consisted of 30 females and 17 males), we found that the pattern was quite similar for females and males (Figure 14). The males had a slightly higher baseline level of bFGF than the females (653±35 pg/mL in female vs. 736±31 pg/mL in males), but this did not reach statistical significance (p= 0.14; unpaired student's t-test). The bFGF levels after exercise did not reach statistical significance in the males (p= 0.44; paired student's t-test) or in the females (p= 0.82; paired student's t-test). The observed mean bFGF concentration was lower in the stroke patients than at baseline for males (p=0.03), however not significantly after Bonferroni correction (p= 0.12; unpaired student's t-test). For females the difference in the observed concentration for stroke patients to the baseline did not reach statistical significance (p= 0.21; unpaired student's t-test).

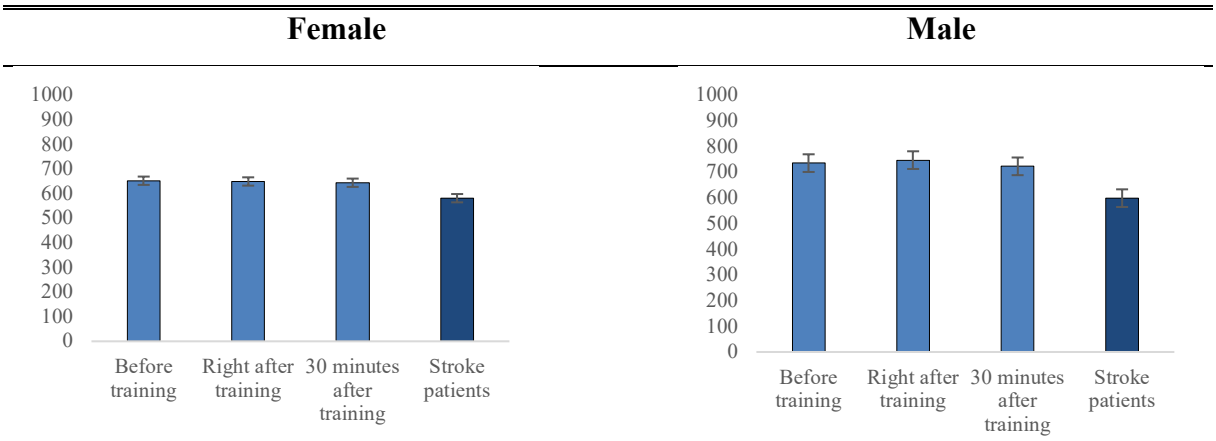

Figure 14 Mean concentration of bFGF per gender. *The mean concentrations of bFGF are displayed in this figure for each of the timepoints: before training, right after training, 30 minutes after training and in stroke patients. For the males, the observed concentration of bFGF (pg/mL) was slightly lower in the stroke patients compared to the baseline levels of the control group (not significant after Bonferroni correction), while this was not seen in the females. There was no effect on bFGF levels in response to training.*

4.4.3 Effects of age on the levels of bFGF

To test whether there were any differences in the results based on age, the subjects were categorized into the age groups described under section 4.3.3.

The results obtained in the different age groups did not differ from each other and did not deviate from the trend in Figure 15. In all four age groups, the levels of bFGF tended to be

lower in the stroke patients compared to baseline levels in the control groups, however this did not reach statistical significance for any of the age groups (<65 p= 0.48, 65-79 p= 0.28, 70-75 p= 0.40, >75 p= 0.06; unpaired student's t-test).

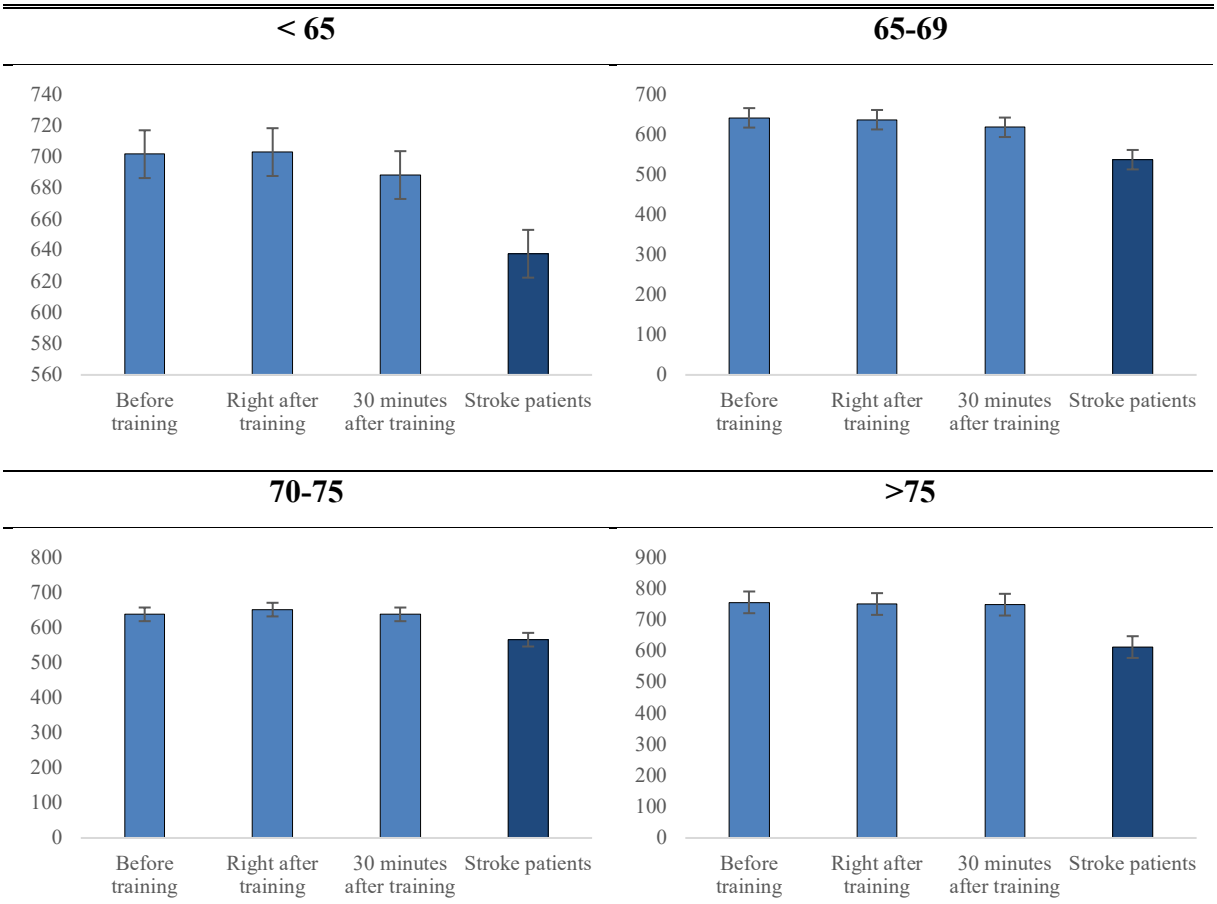

Figure 15 Mean concentration of bFGF for the different age groups. *The figure display an overview of the chosen age groups analysed in this section. The subjects were divided into four groups based on age and analysed separately. Numbers of observations (n) across the groups were quite similar. There were no significant differences from the baseline level of bFGF.*

4.4.4 Effects of stroke size on the levels of bFGF

To determine if the difference in bFGF concentrations in the stroke patients was dependent on the stroke size, a separate analysis was performed on the 32 patients who had a small lesion, the 5 who had a medium lesion, the 4 patients who had a large lesion and the 7 patients who were classified as having a TIA. Figure 16 illustrate the mean bFGF concentration for the different stroke sizes. There was no statistical significance when testing the difference between small stroke and medium stroke (p=0.55; unpaired student's t-test), small and large stroke (p=0.89; unpaired student's t-test), small stroke and TIA (p=0.12; unpaired student's t-test).

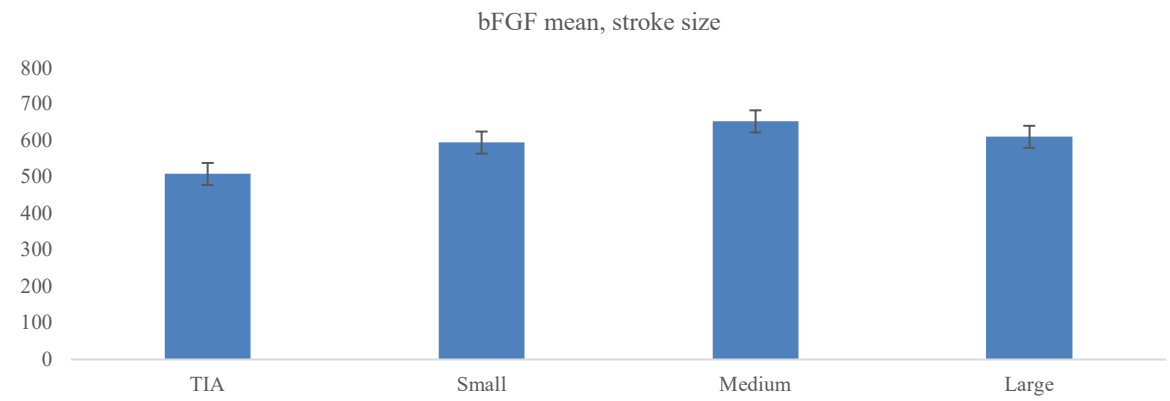

Figure 16 Mean concentration of bFGF depending on the stroke size. *The mean concentration of bFGF after strokeare displayed in this figure, based on the stroke size. Although the figure may give the impression that bFGF levels increase with the severity of the stroke (from TIA to small to medium lesion sizes), this did not reach statistical significance.*

4.5 EGF

4.5.1 Full dataset EGF

The FGF concentrations had a large variation between the individuals. For instance, the FGF baseline levels varied almost 7-fold with the minimum concentration before training at 540 pg/mL and the maximum 3,766 pg/mL (Table 5). In stroke patients, plasma EGF varied between 16 pg/mL and 2,427 pg/mL, a 151-fold difference.

Table 5 Summary statistics for the EGF dataset*. The concentration of plasma EGF (mean) measured in the control group at three time points (before, right after, and 30 minutes after the end of the exercise) is shown along with the concentration of EGF in plasma from the stroke patients. The table also show the median EGF and the, lowest (Min) and highest (Max) observed concentration value, SEM = Standard error of the mean.*

	Before training	**Right after training**	**30 min after training**	**Stroke patients**
Mean	1,080	1,235	1046	184
Median	913	1086	862	67
Min	540	566	479	16
Max	3,766	3,011	3,014	2,427
SEM	89	78	78	59

In the control group, the EGF concentration before training was 1,080±89 pg/mL (mean±SEM). In response to exercise, the EGF levels increased to 1,236±79 pg/mL immediately after exercise (p=0.05; paired student's t-test; Bonferroni corrected). 30 minutes after exercise, there was no statistical difference to the baseline levels (p=0.45; paired student's t-test). The EGF concentration was almost 6 times lower in the stroke patients, where EGF was measured to 184±60 pg/mL, compared to the baseline levels, ($p= 3.0 \times 10^{-12}$; unpaired student's t-test; Bonferroni corrected). The mean concentration±SEM is displayed in Figure 17.

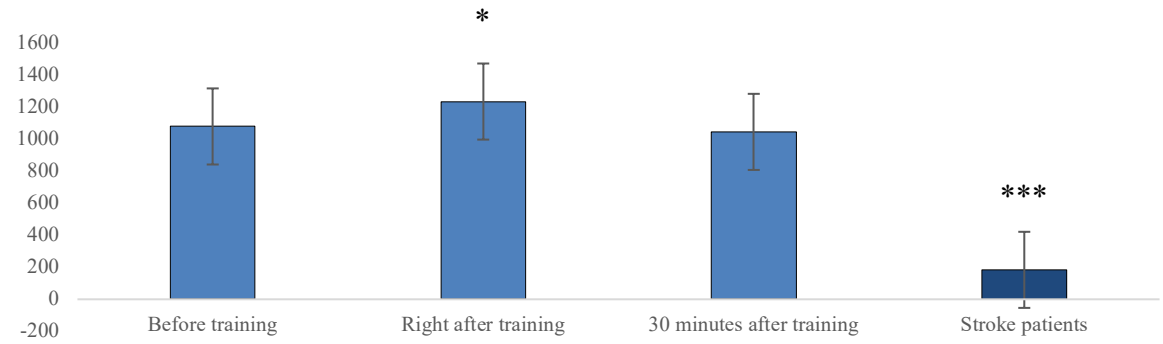

Figure 17 Mean concentration of EGF. *The mean concentration of EGF are displayed in this figure for each of the different time points for the control group: before training, right after training, 30 minutes after training and for the stroke patients. The concentration of EGF (pg/mL) in the stroke patients was clearly lower than in the control group. The concentration also increased right after training, before decreasing 30 minutes after training. *p<0.05; ***p<0.001 (Bonferroni corrected).*

4.5.2 Effects of gender on the levels of EGF

In this section, differences in EGF means across the female and male subjects were investigated. Figure 18 illustrate the mean concentration before training, right after training, 30 minutes after training and in stroke patients, for both females and males separately.

When the study group was divided based on gender (each group consisted of 30 females and 17 males), we found that the pattern was similar for females and males (Figure 18). The males had a slightly lower baseline level of BDNF than the females (1,133±101 in female vs. 942±50 in males), but this did not reach statistical significance (p=0.35; unpaired student's t-test). The EGF levels after exercise were elevated compared to the baseline in the males (p=0.04; paired student's t-test; Bonferroni corrected). Although the same tendency was observed in the females, this did not reach statistical significance (p=0.07; paired student's t-test). The observed

mean EGF concentration was significantly lower in the stroke patients than at baseline for both genders ($p<0.001$; unpaired student's t-test; Bonferroni corrected).

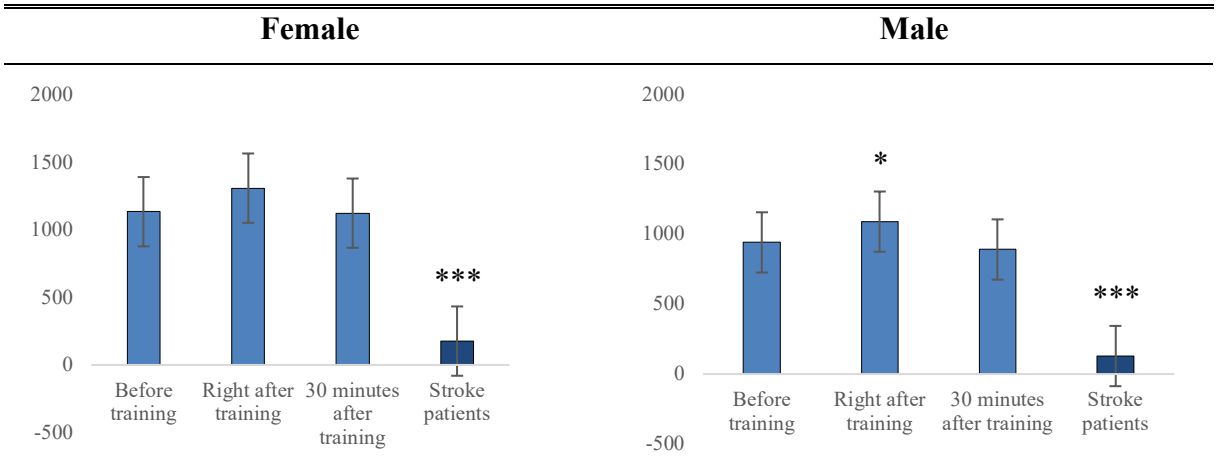

Figure 18 Mean concentration of EGF per gender. *The mean concentration of EGF are displayed in this figure for each of the different time points: before training, right after training, 30 minutes after training for the control persons, as well as for the stroke patients. The concentration of EGF (pg/mL) was significantly lower in the stroke patients for both genders compared to the baseline levels. Although EGF tended to increase after training in both genders, this was statistically significant only in the males. *p<0.05; ***p<0.001 (Bonferroni corrected).*

4.5.3 Effects of age on the levels of EGF

To test whether there were any differences in the results based on age, the subjects were categorized into the age groups described under section 4.3.3.

The results obtained in the different age groups did not differ from each other and did not deviate from the trend in Figure 19. In three out of four age groups, the levels of EGF were lower in the stroke patients compared to baseline levels in the control groups ($p<0.001$ for all comparisons; unpaired student's t-test; Bonferroni corrected). For the age group below 65 years old, the difference in EGF levels in the stroke patients compared to the baseline before training reached statistical significance before the Bonferroni correction, but not after ($p=0.07$; unpaired student's t-test; Bonferroni corrected).

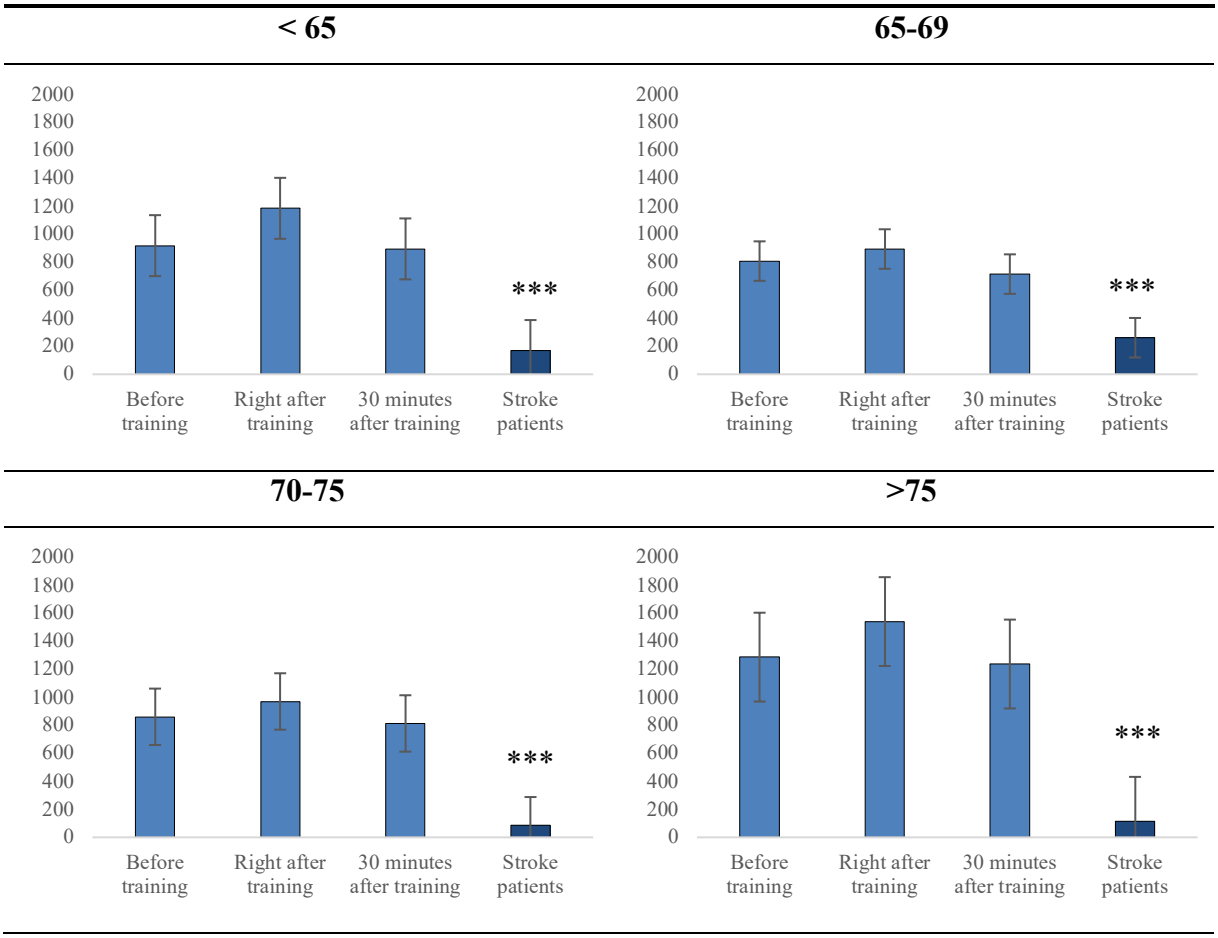

Figure 19 Mean concentration EGF for age groups. *The figure display an overview of the chosen age groups analysed in this section. The subjects were divided into four groups based on age and analysed separately. Numbers of observations (n) across the groups were quite similar. ***statistically significant difference from baseline level, p<0.001 (Bonferroni corrected).*

4.5.4 Effects of stroke size on the levels of EGF

We then went on to determine if the difference in EGF concentrations in the stroke patients was dependent on the stroke size. Figure 20 illustrate the mean EGF concentration for the different stroke sizes. Although a string tendency was observed towards reduced EGF levels in the patients with medium stroke size (n=5) compared to those with small lesions (n=32), there was no statistical significance between these groups (p=0.06; unpaired student's t-test). These same was true for the comparison between small and large stroke (n=4; p=0.63; unpaired student's t-test). When comparing small stroke to TIA, the p-value were significant before Bonferroni (p=0.04; unpaired student's t-test), but not with Bonferroni correction (p=0.16: unpaired student's t-test; Bonferroni corrected).

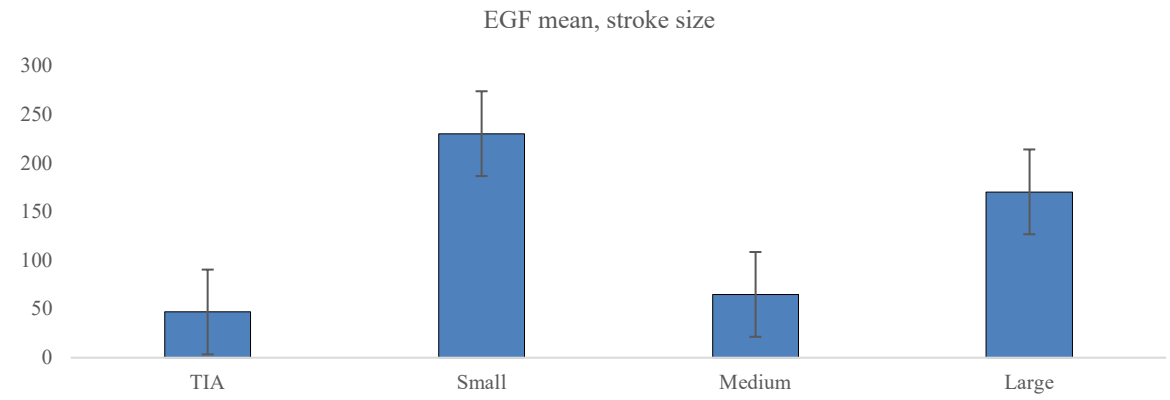

Figure 20 Mean concentration of EGF divided based on the stroke size. *The mean concentrations of EGF after stroke are displayed in this figure, based on the stroke size. The concentration of EGF (pg/mL) did not show a clear pattern based on the stroke size, and the differences between small lesions and TIA, medium and large did not reach statistical significance.*

4.6 Irisin

4.6.1 Full dataset

Table 6 present summary statistics for the full irisin dataset. Mean and median values are given along with the lowest (Min) and highest (Max) concentrations measured and the corresponding SEM for each time point (before, immediately after and 30 minutes after exercise) for the control group, as well as for the stroke patients.

Table 6 Summary statistics for irisin dataset. *The concentration of plasma irisin (Mean) measured in the control group at three time points (before, right after, and 30 minutes after the end of the exercise) is shown along with the concentration of irisin in plasma from the stroke patients The table also show the median irisin and the smallest (Min) and largest (Max) observed concentration value, SEM = Standard error of the mean.*

	Before training	**Right after training**	**30 min after training**	**Stroke patients**
Mean	5,269	5,194	5,250	6,742
Median	4,470	4,342	4,526	4,100
Min	1,854	1,197	659	0
Max	18,473	21,746	18,205	50,899
SE	425	499	446	1,475

In the control group, the irisin concentration before training was 5269±425 pg/mL (mean±SEM). No difference was observed in irisin levels in response to exercise: irisin levels measured immediately after exercise was 5,194±499 pg/mL and 30 minutes after exercise 5,250±446 pg/mL (p=0.67, and p=0.91 for the two comparisons, respectively; paired student's t-test). The irisin concentration was higher in the stroke patients, where irisin was measured to 6,742±1,475 pg/mL. However, this difference did not reach statistical significance (p=0.35; unpaired student's t-test). The mean concentrations with corresponding SEM is displayed in Figure 21.

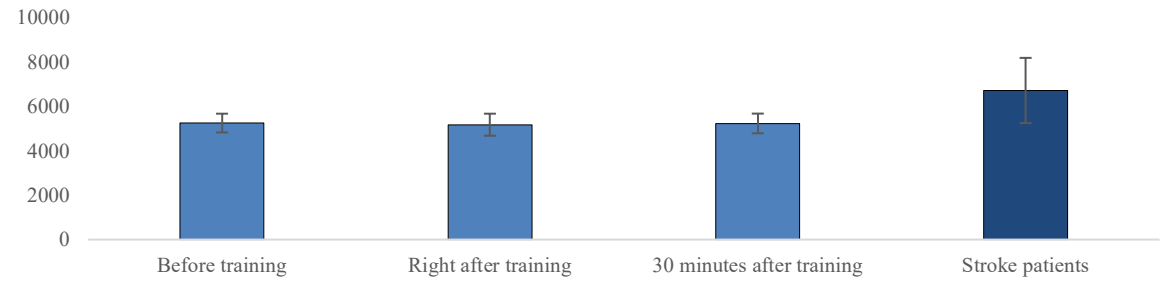

Figure 21 Mean concentration of irisin. *The mean concentration of irisin are displayed in this figure for each of the different time points for the control persons: before training, right after training, 30 minutes after training as well as in the stroke patients. The concentration of irisin (pg/mL) appeared higher in the stroke patients compared to baseline, but this was not statistically significant.*

When we segregated the irisin data further into gender and age, all results were still highly insignificant. We therefor do not include further analyses in this section for irisin.

4.7 VEGF

Optimization: It was decided to dilute the samples a 5-fold when measuring VEGF, as this dilution factor fell within the linear area of the standard curve when tested a test-person who was not included in the study. A graph of the standard curve for VEGF is given in Figure 22.

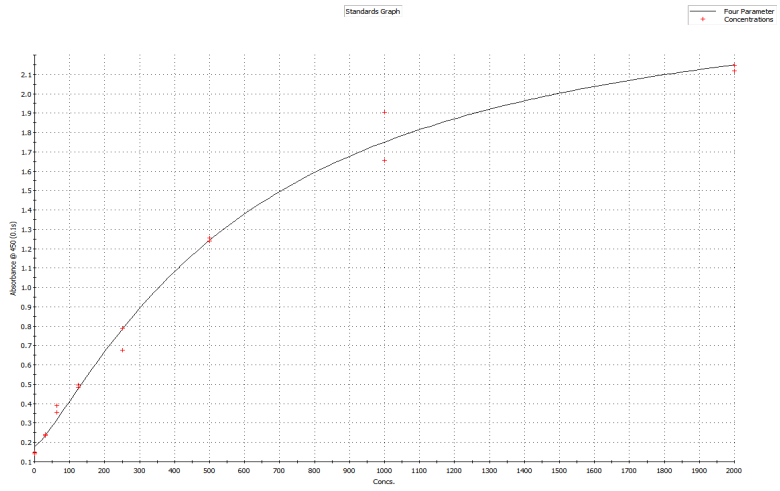

Figure 22 VEGF Standard curve. *Victor X4 Multilabel Plate Reader (Perkin Elmer, USA) was used to generate a four-parameter logistic (4-PL) curve-fit for the standards. The VEGF standards had the following concentrations: 2,000 pg/mL (highest), 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL (lowest) and a negative control containing only reagent diluent (0 pg/mL).*

With this dilution factor, 19 of 40 samples were below the standard curve, while one was above the standard curve with a concentration of 2,902.85 pg/mL. The rest of the samples were distributed over the lower parts of the curve, with concentrations between 309.9 pg/mL and 1.70 pg/mL. Since all except one were so low on the standard curve, it was decided to try a 2-fold dilution.

When trying a 2-fold dilution on 40 new samples, 22 of 40 samples were below the standard curve. The remaining samples had a concentration in the lower parts of the curve, with concentrations between 9.6 pg/mL and 354 pg/mL. Even if it was not specified for this ELISA-kit, the manufacturer (R&D Systems) often recommend to dilute the samples 2-5 fold in sample dilution buffer when using complex matrices such as plasma. The dilution buffer contains components that block unspecific binding sites and, hence, an undiluted sample may be encumbered with a higher unspecific signal. In addition, undiluted plasma would require a bigger sample volume, which in our case was limited. Due to this, it was decided not to precede with experiments on VEGF.

5. Discussion

5.1 Methodological considerations

5.1.1 Sandwich ELISA as immunoassay

In this thesis, the presented levels of different GF and irisin were measured by using sandwich ELISA. Sandwich ELISA is a well validated immunoassay for the detection of high molecular-weight antigens. The advantages of ELISA in general, compared to other immune assays, are the use of surface bound reactants, making it easy to get rid of unbound materials that could other ways result in un-specific binding. The advantage of sandwich ELISA compared to other ELISAs, is the use of two antibodies recognizing different non-overlapping epitopes of the protein of interest, increasing the overall selectivity.

The ELISA method is based on the use of antibodies to detect the antigen of interest. The quality of the result, therefore, is highly dependent of the quality of the antibodies used. Antibody quality depends on several factors, including the specificity/selectivity (the ability of the antibody to bind the epitope of interest without binding other epitopes; this includes low-affinity binding to several epitopes as well as high-affinity selective cross-reactivity to an on, or a small number of, epitope(s) that resembles the epitope of interest). Specificity is defined as 100 % selectivity, which is only possible in theory. Therefore, in this discussion, the term “selectivity” is used when talking about a graded ability to bind one epitope over others. The term specificity and selectivity are used interchangeable when referring to the concept in general. Another important factor is the affinity, which refers to the strength of the binding between the antibody and the antigen. The affinity and the specificity both affect the sensitivity (the ability to detect true positives), which in turn decides the lower concentration of antigen that can be detected with a high enough degree of certainty (the lower detection limit). The upper detection limit is dependent in the specificity of the antibody as well as the number of binding sites available (as these may be saturated at higher concentration of the antigen). The antibodies used in the present study were all purchased as part of validated ELISA kits from R&D Systems. These are immunoglobulin (Ig)-Gs, which are the most commonly used Igs for immunohistochemistry and immunoassays. The properties of the antibodies (Abs) largely depend on whether they are monoclonal (mAbs) or polyclonal (pAbs). mAbs only bind one single epitope on the antigen, resulting in a lower sensitivity and a somewhat higher selectivity compared to pAbs that contain a heterologous mixture of IgGs against different epitopes on the antigen. pAbs are also

more tolerant to structural changes of the epitope such as denaturation [194]. In our study, the samples were kept at -80°C until immediately before analysis and were kept on ice though the whole procedure. Repeated freeze-though cycles were kept at a minimum (max 3-4 times per sample). Hence, denaturation of the proteins of interest is believed to have a low impact on the data. Denaturation may represent a larger source of uncertainty when tissue samples are exposed to cross-linking fixation and/or detergents. mAbs, are often more selective than pAbs as they are less likely to bind unspecifically to multiple epitopes [194]. Sandwich ELISA often utilize the beneficial attributes of both pAbs and mAbs by using them together. Often pAbs are used as capture antibodies, to take advantage of their higher overall affinity and hence the high sensitivity. mAbs are often used as detection antibodies to enhance the selectivity of the detection. However, in the case of the ELISA kits used in this thesis, the manufacturer did not provide this information about the antibodies, making it difficult to assess the degree to which this may have affected the results. There is also a slight chance for cross-reactivity between the two antibodies. By using antibody pair combined in a kit, such as the DuoSets used in this study, the matched antibodies are optimized by the manufacturer, reducing the likelihood for such cross-reactivity. Furthermore, the kits used in the present study were validated for plasma samples, which was also the medium used in the present study. Using validated kits for other biological samples than what they are meant for, increases the probability for cross-reactivity; different biological material may contain different proteins, some of which may have epitopes that resembles the epitope of interest and hence may produce cross-reactivity. The DuoSets used are well validated, and much used. All antibody pairs have been assayed for cross-reactivity and interference by the manufacturer. Formulation of the diluents, the concentrations of the antibodies and general assay condition may also affect the specificity and sensitivity. To optimize this, we used the recommended DuoSet Ancillary Reagent Kit 2 for all additional reagents.

One example of specific cross-reactivity that is worth mentioning, is the possible detection of both proBNDF and mature BDNF with the BDNF antibodies. As previously described, pro- and mature BDNF have opposite effects in the brain and are released in concert from the same BDNF secreting cells. As epitopes used in the Human/Mouse BDNF DuoSet ELISA-kit are not unique for mature-BDNF, there is a chance for antibody binding to proBDNF as well. Information about the affinity for mature BDNF compared to for proBDNF was not available for the kit used in the present study. This may give false positive results and must be taken into account when interoperating the results. The ration between mature BDNF and proBDNF,

however, is normally quite high. Therefore, BDNF detected in this study can be expected to mostly represent mature BDNF.

The point of concern when working with sandwich ELISA is the many steps involving pipetting of small volumes. Precise volume transfers in each step is critical for accurate and reproduceable results. In the present study, care was taken to keep the accuracy and reproducibility as high as possible in each step, but during the assay there are limited options for verifying this. The only way to measure precision is by applying each sample in duplicates and then calculate the CV. This is not done before the assay is completed, making it impossible to know in which step a possible error could have occurred. Once a deviant CV-value is exposed, one cannot know for sure whether this is due to human errors, the reagents used or due to technical issues. To avoid introducing systematic errors due to the steps prior to application to the ELISA plate, all samples and standards were mixed thoroughly immediately before use and all analysis were performed by the same operator and as standardized as possible. Nevertheless, there will always be some uncertainty in the final results. Samples from all groups were run on the same plates, reducing the likelihood that systematic errors would affect one group specifically. Hence, the uncertainties mentioned above may have introduced variation in our analysis but likely did it not alter the overall results.

5.1.2 Plasma as a medium

Since we are mainly looking for the effects of GFs on the brain, the most ideal medium for analysis would be cerebrospinal fluid (CFS) as CFS is in direct contact with the CNS and thus gives a more direct reflection of the milieu of the CNS. To obtain CSF, spinal puncture is needed, and this is not done unless specifically required in the treatment and/or diagnosis of the patient. The procedure is painful and involves a certain risk, and therefore collection of CFS for this project was not considered ethically acceptable. Instead, it was decided to use plasma. The GFs and irisin analyzed in this study are all known to pass the BBB, and therefore changes in their concentration within the CNS may be reflected in the blood.

It was decided to use plasma over serum. Plasma is easier and less time consuming to separate, as the blood need to cloth in order to make serum. In addition, the percentage volume of plasma is higher in comparison to serum, leaving more volume available for the assay. One disadvantage of using plasma is the content of anticoagulant. Especially when measuring BDNF, as K2EDTA (which was used as an anticoagulant in our samples) can activate platelets

which store BDNF and can secrete it upon degranulation [195]. The major problem with this is that the BDNF released from platelets *in vitro* may mask differences in BDNF released from the brain in response to exercise and/or stroke. Furthermore, the release of BDNF from platelets can be influenced by age, specific disease conditions, or pharmacological treatments [196], which gives a heterogenous influence on the measured levels. Serum have thus shown to be more suitable when measuring of BDNF. Even so, plasma as the often the chosen medium for measuring BDNF is widely used for this purpose.

Another disadvantage of use of plasma is lower concentrations. For instance, serum have shown significantly higher values of VEGF compared to what is found in plasma [197]. As much as 6-fold higher values of VEGF in serum compared to plasma have been reported [198]. By use of serum instead of plasma, we might have gotten higher concentrations of VEGF, reaching above the lower detection limit. But this remains speculative. Importantly, all our samples were treated identically, and hence the choice of plasma over serum or CSF is not likely to underlie the differences in concentrations detected between the stroke patients and the control group.

5.1.3 The control group and the measurement of exercise intensity

To monitor the exercise intensity during HIIT, HR and Borg´s scale were used, in addition to measuring blood lactate at four time points (before training, immediately after the second and the fourth interval, and 30 minutes after the end of the HIIT).

Even if direct measurement of maximal oxygen uptake (VO_2max) is the optimal method of assessing cardiorespiratory fitness and maximal capacity, the use of Borg´s scale have proven to be a valid tool for monitoring and prescribing exercise intensity and achieve an accurate prediction of O_2max [199, 200]. In addition to perceived exertion, the workload was based on the subjects HR and observations made by the observer, to make sure a high intensity was obtained throughout all four intervals. All participants increased their blood lactate in response to the exercise (Figure 7), demonstrating that exercise was above the anaerobic threshold.

All subjects in the control group voluntary enrolled the study, well knowing that the study would involve an exercise session at high intensity. Most of the subjects were even recruited from a gym class. These subjects, hence, do not necessarily reflect the average elderly population, and especially not the average stroke patient. Stroke patients often present a combination of risk factors such as overweight, type 2 diabetes, high blood pressure etc, most

of which can be reduced by regular exercise. Recruiting among a physically active group of elderly resulted in a control group where most of the participants probably lack the main risk factors of stroke. The two different groups in this study (control group and stroke patients) may not be comparable for these reasons, which can be criticized. However, this study depended on the control persons to be competent to consent to participation, and to be able to train with high intensity. The upside by using a healthy control group without any known brain-diseases, is that they are representative for a basal level of the different factors in a healthy elderly brain. Hence, the basal level will not be disrupted by external factors; only a very few in the control group used medications or nicotine on a regular basis.

5.1.4 Statistics

The statistical analysis was performed by a series of Student's *t*-tests: paired when samples from the control persons were compared to each other and unpaired when samples from the stroke patients were compared to the basal levels in the controls. We used a Bonferroni correction to compensate for the multiple comparisons by manually multiplying the p-value from the Student's *t*-test with the number of comparisons. This correction was performed to counteract the chance of observing rare events, which increase with multiple comparisons. This is a strict approach which reduce the chance of a type 1 error (false positive), but it could on the other hand lead to more type 2 errors (excepting a false negative). In fact, some of our findings within the control group were statistically different when compared with the student's *t*-test alone, but not when subjected to the Bonferroni correction. In this thesis, such findings have been presented as "tendencies", and both the corrected and the uncorrected p-value have been shown to allow the reader to judge these data themselves. When differences between the stroke patients and the control group were found, however, these were generally large (see BDNF and EGF), reaching statistical significance even after being Bonferroni corrected.

5.2 Discussion of study results

5.2.1 Did stroke affect the outcome on cognitive tests?

Our study revealed significant differences in cognitive performance when comparing the stroke patients with a healthy age- and gender-matched control group. In the MMSE, the stroke patients showed a significantly lower score than the control group ($p<0.001$). In both TMTs the stroke patients used a significantly longer time to finish ($p=0.001$ and $p=0.001$ for TMTA and

TMTB, respectively). Even so, it is noteworthy that the mean MMSE score for the stroke patients were above 25, which is an indicator for normal cognition. When looking at the scores separately, none in the control group obtained a score lower than 25, while 13 of 40 stroke patients did. Of these 13, a score below 20 was reported for 3 subjects. Scores below 25 indicate mild dementia, and scores below 20 indicate moderate dementia [186].

Although there was not performed an official Barthel ADL test on the control group, it is conceivable that these subjects would have had a high score (probably around the maximal score for all participants) indicating low dependency in daily activities. The average Barthel ADL score for the stroke patients was 17.5 of 20 possible points. Of the stroke patients 7 of 48 had a score below 20 indicating moderate dependency, and 6 of 48 had a score below 10 indicating severe dependency [188].

Stroke is known to be a major contributor to both physical and cognitive decline [3-5]. Based on our results, we cannot conclude whether the differences seen in cognitive abilities between the stroke patients and the control group were caused by the stroke *per se*, since the control group and the stroke patients are two separate groups of subjects. It is unknown if the cognitive declines seen in some of the stroke patients are due to stroke or other factors present prior to the stroke. Also, it is not known whether the low cognitive level of some of the stroke patients is temporary and will improve during rehabilitation.

5.2.2 Discussion of BDNF results

For BDNF, we found significant lower levels in the stroke patients compared to the baseline levels of the control group ($p<0.001$). Whether these differences are due to stroke, cannot be known for certain, as there is a chance that the stroke patients had lower BDNF levels independently of the stroke. The differences are, however, quite large, making it more plausible that they are caused by a major event (like stroke) than by natural variation. Furthermore, our findings are in line with previous studies on BDNF, when comparing stroke patients with a control group [125, 126].

In our study, we did not detect a significant increase in BDNF levels in response to exercise. Although the absolute values (mean and median) for BDNF were ~10 % higher after exercise, this did not reach statistical significance. This is not in line with the well-established opinion

that acute exercise increases circulating levels of BDNF [164, 201-203]. Changes in serum BDNF in response to acute exercise have been reported to have a significant correlation with changes in blood lactate, indicating that the BDNF increase in response to exercise is intensity-dependent [204]. However, the control persons in this study did show significant increase in blood lactate in response to HIIT ($p<0.001$), without a statistically significant increase in BDNF. This excludes the possibility that the intensity during HIIT was the reason we did not see increased BDNF levels following exercise. As discussed above, however, this may have been affected by the use of plasma instead of serum. Most studies done on PA-induced effects of circulating BDNF are done by measuring the levels in serum, and not in plasma. In fact, there are studies done where serum levels were increased while plasma levels were not, in response to HIIT [195, 205]. The study done by Pareja-Galeano et.al [195], stated that BDNF concentrations in plasma and serum respond differently to HIIT, and that changes in BDNF in response to HIIT is unlikely to be revealed when measured in plasma.

Even if our studies did not demonstrate a significant increase of exercised-induced BDNF when looking at the full sample, we found significant changes in the males when comparing the baseline levels (before exercise) to the levels right after exercise ($p=0.05$). These finding are interesting, as a meta-analysis from 2015 demonstrated that the magnitude of BDNF in response to exercise is weaker in females compared to males [202].

5.2.3 Discussion of bFGF results

When compared with a control group, previous studies have shown higher levels of bFGF in stroke patients [81, 141, 142]. In our study, the opposite was seen with a tendency towards lower mean bFGF level in stroke patients compared to baseline levels in the control group, however this did not reach statistical significance with the Bonferroni correction. Lower levels, though not significant, of bFGF in the stroke patients may be due to other factors than stroke, as we cannot exclude that these patients also had lower levels prior to the stroke as well.

In the study done by Golab-Janowska [141], the contol subjects and the stroke patients were mached not only by gender and age, but also by known risk factors for stroke. This is in contrast to our control group, which probably lacked many of these risk factors and hence was a poorer match in this respect than the ones used in Golab-Janowskas study. Two other factors are also worth considering: The size of the stroke lesion and the time of the blood samples after stroke

onset: Guo and co-workers [142] found that the increase in bFGF levels depended on the severity of the stroke, where patients with larger strokes showed larger increases in bFGF. In the present study, we recruited patients with a mRS score of 1,91±1,37 (median score: 2), and with cognitive abilities to provide an informed consent. This means that the patients included in the present study were not among the most sever stroke patients. In the study by Issa and colleagues [81], very little information is given regarding the patients from which the serum bFGF were measured, but they state that all patients were admitted to the hospital within 24h after stroke onset. This implies that the stroke may -on average- have been more severe than for the patients recruited for the present study, which were admitted to the hospital an average of 30 h (varying from 1 h to <240 h) after stroke onset. The increase in brain bFGF in the paper by Issa et al., are from stroke patients who died within 2 months after stroke (except for one patient, who died after 110 days). According to Guo and colleagues [142], serum bFGF peaked at 3 day after stroke and remained elevated for 14 days. In our study, the time between stroke onset and blood sampling varied greatly, as blood was routinely drawn between 12:00 and 13:00 the day after admission. Most of our samples, however, would probably have been obtained either before or after the peak in bFGF could be expected, reducing the likelihood of finding a large increase. The lower severity of the stroke and the early blood sampling after stroke onset, combined with the difference in risk factors between the control group and the stroke patients in the present study may therefore underlie the decrease seen in our study.

Just as it is known that exercise increase BDNF in the brain, the same is also known for bFGF [206]. The lack of studies makes it difficult to know if these changes in cerebral bFGF is reflected in the levels of bFGF in the circulation. In our study, we found no clear pattern in the mean of bFGF concentration before and after training, in either genders. Another study, looking at bFGF levels after a one-year exercise intervention in postmenopausal women also did not find any difference in bFGF levels [207]. In a third study, more similar to our, middle-aged men (<50-60) were exposed to an “all-out” anaerobic exercise. This study reported that bFGF decreased in response to this exhaustive exercise, reaching undetectable levels; the levels of bFGF were shown to decrease immediately after exercise, and stay low 50 min into recovery, when compared to the levels detected before exercise [208].

5.2.4 Discussion of EGF results

To our knowledge there are to this day no published studies done on the circulating concentrations of EGF in regard to stroke. Thus, we have very little to compare our finding

with. However, our studies showed significantly lower concentrations of EFG in the stroke patients compared to the baseline levels of the control group ($p<0.001$). With our conservative approach (Bonferroni correction), this did not reach significance for the age group >65 years, but was evident in the full dataset as well as in all the other age-segregated groups. Once again is it important to remember that the differences seen in the stroke patients and the control group don't necessarily represent alterations of GFs in response to stroke. The stroke patients may have had lower EGF levels compared to the control group also prior to the stroke.

In response to exercise, we found a significant increase of EGF immediately after accomplished HIIT, before it decreased to lower levels within 30 minutes (the latter was not significant). However, when we looked at the genders separately, we saw that this was statistically significant only for the males ($p=0.04$), and not the females. A lack of studies looking at acute effects on EGF levels in response to training, makes our findings difficult to discuss. However, at least one study by Accattato and co-workers [209] is quite similar to ours, and did also find decreased circulating EGF levels in response to acute exercise. Another study stated that also regular training led to a decrease in circulating EGF [210]. Since the latter study only measured EGF at the end of the training period, and at least 5 days after the last training session, it does not give information about how long after each exercise session the levels started to decrease. A third study has shown increased plasma values of EGF in response to prolonged exercise (2h run) [211], which is in contrast to our findings. Interestingly, this study showed increased EGF values in plasma, while no difference was detected in the EGF serum levels, which substantiate the difficulties in choosing between plasma and serum as the medium in which to measure GFs.

5.2.5 Discussion of Irisin results

When analyzing irisin, we did not find any significant differences either between the stroke patients and the control group, or in response to HIIT. There was not seen any clear pattern in the mean that could indicate an increase or decrease of irisin after training. For the stroke patients, however, the mean irisin level was 28 % higher than the baseline level. Still, when conducting an unpaired student's *t*-test, the difference did not reach statistical significance.

The lack of differences found in our study could be due to the large variation in the data for the stroke patients. The minimum value was below the detection limit (set to 0 pg/mL) and the maximum value was 50,900 pg/mL (the mean irisin level for stroke patients was 6741 pg/mL).

There were quite a few extreme values in the data for the stroke patients, causing the SEM to be much higher than in the control group. This could indicate that the sample is not representative for the population, as they are not closely distributed around the mean. The large SEM makes it more difficult to obtain significant results from the student's t-test. Nevertheless, the large variation in irisin levels may be of interest in itself, as they may represent a measure for later stroke outcome.

Previous human studies on circulating irisin levels after stroke havemainly been done to estimate the functional outcome of the stroke patients. The levels are therefore compared to other stroke patients, and not to a control group. At least two studies have established that low serum irisin levels are related to poor functional outcome in ischemic stroke patients [212, 213]. In both studies, the levels of irisin were measured on the same day or the day after admission, and then later compared to the patients mRS score 6 or 3 months after. Poor outcome was defined as mRs score >2, and was significantly associated with low levels of irisin. In the present study only 14/48 were classified with mRs> 2, and this was at hospital discharge. This may be the reason for why the mean irisin level of our stoke patients did not differ from the control group.

Post-exercise changes in irisin levels are not easy to predict, as studies have come to different conclusions [214, 215]. Lagzdina and co-workers [215], found quite divisive changes, with 56 % of the participants showing no significant change in irisin concentration after exercise, while 23 % showed a decrease, and 19 % showed an increase in irisin levels in response to exercise. Even so, the prevailing opinion is still that exercise increase the levels of circulating irisin immediately after acute exercise [216-219].

6. Conclusion and future perspectives

The purpose of this thesis was to investigate how circulating GFs and irisin change after stroke, and whether the same alterations were induced by exercise. Through our research we found significantly lower levels of BDNF and EGF in the stroke patients compared to the baseline levels in the control group. When measuring bFGF and irisin, we did not detect significant differences in the levels found in the stroke patients, when compared to the baseline levels in the control group. In response to exercise, the levels of BDNF and EGF significantly increased.

This tendency was observed in both genders but was more prominent in men than in women, and for BDNF it was only statistically significant for the males. For Irisin and bFGF, on the other hand, no significant changes in the levels was discovered in response to exercise.

It remains to establish whether the studied GFs and irisin are crucial for the stroke patient's progression, and whether exercise can be used to restore the balance in the same factors. The results from this study lays the foundation for further studies on alterations in GFs and other biomarkers secreted in the blood, as a response to stroke and exercise. A natural next step would be to examine a larger group of patients, measure the levels of GFs, irisin and other factors in their blood, and then follow the patients through the rehabilitation phase. By following the same patients over time, we may be able to see if any of the differences detected in the present study can predict progression in the rehabilitation phase.

A further research objective could be to include the detection of inflammation markers and perhaps screen miRNA in blood (to identify other alterations of interest), followed by ELISA for any relevant blood-born factors identified by the miRNA analysis. The effects of each alteration could also be tested in animal models, but for some of the GFs, translation from mouse to men has been challenging.

Information gained from the long-term follow-up suggested above, may be critical when aiming for more individually tailored treatment services after stroke. This may in turn contribute to lessen the major disabilities seen after stroke, hence also the major burden on the effected individuals and the societal costs.

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