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Proteomic profiling of Human Blood after Exercise Training: potential therapeutic targets?

Inger Ane Hole

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Hovedveiledar: Rudolf Schmid, IKJ
Medveiledar: Ulrik Wisløff, CERG

Noregs teknisk-naturvitskaplege universitet
Institutt for kjemi

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Contents

Abstract

Exercise capacity is the single best predictor of mortality in humans. Accordingly, regular physical activity has beneficial effects on health and is used as therapy for many diseases. Recent studies suggested that the benefits of exercise in the heart, skeletal muscle and adipose tissue are caused by systemic factors released into the bloodstream, through a hormone-like mechanism. However, the identity of such blood-borne factors is largely unknown. Identifying these molecules will provide a list of potential drug targets for a variety of diseases. In this study an unbiased screening for circulating peptides/proteins induced by 1-year exercise in humans was conducted.

Male subjects (70-75 years-old) were randomized to undergo moderate-intensity aerobic exercise training (70% of maximal heart rate, 40min/session) or into a control group. Maximal oxygen uptake (VO_2max) was measured and blood samples were collected, both at baseline and after 1-year follow-up (n=11 per group). VO_2max increase by 10.9% on average in the exercised group ($p<0.01$), while it was unchanged in the control group (0.7% between baseline and follow-up, p-value not significant).

A gel-free shotgun proteomics analysis was performed in serum samples to assess abundance of proteins/peptides. Samples were treated with ammonium bicarbonate, dithiothreitol and iodoacetic acid, trypsin digested and analyzed by LC-MS/MS. An Orbitrap Elite instrument, which combines the dual-pressure ion trap with a high field Orbitrap mass analyzer, was used for the analysis. Peptides were quantified by ion count to identify circulating proteins whose abundance in serum is altered by exercise training. It was identified seven proteins with significant increase, among these were protein z-dependent protease inhibitor, vitronectin and mannose-receptor 2, which can be associated with thrombosis, cardiovascular disease and inflammation.

Norwegian abstract

Utholdenhetskapasitet er den beste predikatoren for dødelighet blant mennesker. Regelmessig fysisk aktivitet har positive effekter på helse og er brukt som medisin for mange ulike typer sykdommer. Studier har vist at fordelene ved trening som påvirker hjerte, skjelett muskulatur og fettvev er forårsaket av hormonlignende mekanismer i blodsirkulasjonene. Likevel er disse faktorene i stor grad ukjente. Identifisering av disse faktorene vil føre til et potensielt mål for medisinsk behandling av ulike sykdommer og i denne studien blir det utført screening for å finne slike potensielle markører.

Mannlige deltagere (70-75 år) ble randomisert til å trene med moderat intensitet (70% av maksimal hjerterytme, 40 minutter to ganger pr. uke) eller til en kontrollgruppe. Maksimalt oksygenopptak (VO_{2max}) ble målt og det ble tatt blod prøve av deltagerne, både før treningen ble satt i gang og etter et år med trening (n=11 pr. gruppe). VO_{2max} økte med 10,9% i gjennomsnitt for treningsgruppen ($p<0.01$), mens gjennomsnittlig VO_{2max} i kontrollgruppen forble den samme.

For å identifisere eventuelle faktorer som blir skilt ut som et resultat av høyere oksygenopptak ble det utført en screening av alle blodprøvene ved bruk av væskekromatografi-massespektrometri. Prøvene ble behandlet med ammonium bikarbonat, dithiothretiol og iodoacetisk syre og trypsin før de ble analysert av LC-MS/MS. Et Orbitrap Elite instrument som kombinerer ionefanger med et Orbitrap massepektrometer ble brukt i analysen. Peptider ble kvantifisert ved ionetelling for å identifisere de sirkulerende proteinene med høyest tilstedeværelse i prøvene etter et år med trening. Det ble identifisert sju proteiner som hadde en signifikant økning i treningsgruppen, men ikke kontrollgruppen. Blant disse er protein z-dependent protease inhibitor, vitronectin og c-type mannosereseptor-2, som kan assosieres med blant annet trombose, hjerte- og karsykdommer og inflammasjon.

1. Introduction

1.1 Background

People of all ages and gender undergo beneficial physiologic adaptions in response to physical activity. Physical activity increases energy expenditure, and when attending physical activity several times a week the physiological systems adapt to increase the body's efficiency and capacity [1]. This capacity to perform physical activity is termed as physical fitness. Physical fitness and physical activity are correlated, but studies have shown that the association with mortality differ. Physical fitness has shown to be a stronger predictor of mortality than is physical activity [2, 3]. A significantly lower risk of premature death for fit individuals independent of their time used for physical exercise has been shown. Yet, an unfit person doing a significant amount of physical activity will eventually become more fit.
Physical fitness is often measured as maximal oxygen uptake (VO_{2max}). A high VO_{2max} is associated with a reduced risk of cardiovascular diseases [4, 5], type 2 diabetes [6, 7], colon- and breast cancer [8], musculoskeletal disorders [9] and psychological diseases [10].

1.2 Aim of this thesis

Improved aerobic exercise has been attributed to enhanced cardiac- and skeletal muscle function and thereby improving the body's ability to deliver and extract oxygen. Recent studies have suggested that biomolecules released in the blood also contribute to these adaptions and therefor for the enhanced aerobic capability [11, 12]. However, these blood borne factors are yet unknown. The goal of this project is to identify blood borne biomolecules induced by exercise training in humans who adapt well to exercise by increasing their oxygen consumption. Identifying these factors may lead to development of novel pharmacological agents who mimic the effects of exercise. This would create the possibility to deliver training effects to people who are unable to exercise (e.g. people who are permanently immobile or patients who are unable to move for a prolonged period of time), patients with aggressive diseases or to people who don't respond to exercise training.

This study will find out whether exercise can induce blood borne factors who can serve as a therapeutic target to improve health, using VO_{2max} as a cardiorespiratory fitness parameter.

The study described in this thesis is performed in cooperation with a larger clinical study conducted by Cardiac Exercise Research Group (CERG). CERG investigates the beneficial effects training has on heart, blood and skeletal muscles. The aim is to develop methods to prevent and treat cardiac diseases and help fight the most important challenges the society has in terms of inactivity, obese and type 2 diabetes. Background for the main study is the big percentage part of the population who is aging and the forthcoming problems this will bring. Science and research can help develop strategies and medicine that reduces this problem.

2. Theory

Physiological activity has numerous beneficial effects. Regardless of age, exercise training induce physiologic adaptions who are beneficial for cardiovascular and musculoskeletal systems [1]. When challenged with physical tasks several times a week, the body's efficiency and capacity will increase. The next section will look at the concept behind exercise capacity and VO_{2max}, and link this with the effects of training on molecular level.

2.1 Exercise capacity

In a study performed by Myers et al. they concluded that exercise capacity was a more powerful predictor of mortality than any other established cardiovascular diseases risk factors [3]. The study discovered that the best predictor of increased risk of death among normal healthy subjects was peak exercise capacity. This was supported by Blair et al. who revealed that higher levels of physical activity will delay all-cause mortality primarily because it lowers the risk of cancer and cardiovascular disease [5]. The correlation between exercise capacity and mortality reveals the importance of staying physical active throughout life, also for people who already have shown to have heart failure. Both studies find that the greatest reduction of mortality rates occurs between the least fit and the next-least fit individuals [3, 5]. This observation implies that a small effort in exercise training will have major importance to health and will reduce all-cause mortality for the least fit individuals.

Since the best predictor for all-cause mortality is maximal oxygen uptake (VO_{2max}) [3], the question to ask is; what will increase VO_{2max}? Maximal oxygen uptake is limited by the genetically possible maximum and is defined as the point where a person is no longer capable of increase in oxygen uptake [1]. It has been established that endurance exercise training is most likely the best way to increase VO_{2max} [13-15]. In a study conducted by Wisløff et al. they found out that exercise training increased the anaerobic threshold and VO_{2peak} (VO_{2peak} is a measured VO_2 value where the test person doesn't reach a stabilized plateau) [16]. A person's respiratory fitness and aerobic capacity are considered to be best estimated by the persons VO_{2max} [17].

The maximum oxygen uptake of a person is a function of the cardiac output (Q) and arterial-mixed venous blood difference ($A\text{-}vO_2$). Cardiac output is the total volume of blood pumped out of the left ventricle of the heart per minute, which is a product of heart rate and stroke volume. $A\text{-}vO_2$ is the difference between the oxygen content in the arterial and the mixed venous blood. Cardiac output and heart rate will increase almost proportional with the amount of work, whereas stroke volume only increases up to 40-60 % of a person's VO_{2max} before it reaches a plateau where the body is no longer capable of increasing the oxygen uptake [1]. When a person no longer is able to take up any more oxygen the respiratory system will no longer be able to produce the amount of energy (ATP) the body need to continue the work load. The respiratory system will then switch to the anaerobic energy system where lactate is the primary by-product. At lower exercise intensities lactate levels are very low, but will increase with the rise of exercise intensity and continue to increase to the point of exhaustion. Lactate threshold is the point where the lactate level are above resting level, this is an important marker for endurance performance since the lactate threshold of highly trained persons occur at a higher percentage of their VO_{2max} and are then able to work at a higher relative workload [18].

During endurance training the skeletal muscle adapts through a small increase in the cross-sectional area of slow twitch fibres. It is a direct relationship between the dominant level of fibre type and performance in certain sports. Athletes with high ratio of slow twitch fibre with high capillary density and mitochondrial content do perform better in sport challenges that requires high endurance capacity, like marathon, while people that have a higher percent of fast twitch fibres in their muscles are better in sports who require quick sprint [19]. With the connection between VO_2, endurance capacity and slow twitch fibre, it is also important to acknowledge that the percentage ratio between slow- and fast twitch fibres (moves faster) does not change significantly after prolonged exercise, and the ratio are most likely due to assigned genes [20]. But endurance training can contribute to increase the capillary density even more

[19], which will contribute to a higher blood flow. A trained muscle will allow a greater blood flow when active and thus increase the capacity of blood exchange in the muscles. This will increase the blood flow and greatly enhance the oxidative capacity of the muscle. Other long-term effects of training include increased skeletal muscle size (hypertrophy), increased stroke volume and decreased heart rate at submaximal exercise [1].

Despite prolonged exercise training it will take only weeks of detraining until maximal and submaximal performance are reduced [21]. A study where healthy young athletes were placed in bed for up to 3 weeks showed decrease in cardiorespiratory function [21], reversible glucose intolerance [22], reduced total energy expenditure and loss of muscle protein and bone mass. Also, Coyle et al. demonstrated that VO_{2max} declined with 7 % within the 21 first days of inactivity, and stabilized at a 16 % decline, which was below the initial value, after 56 days [23]. Skeletal muscle capillarization did not decline with inactivity, and the VO_{2max} reduce was linked to the decrease in stroke volume and A-vO_2-difference [23]. This supports the indication that endurance capacity does not depend on mitochondrial amount, but on the ability to transport blood in the body. The fast decrease in cardiorespiratory function also indicate that exercise has to be maintained to exploit the benefits.

2.2 Factors induced by training

Despite widespread research in the field of exercise training the effects at the molecular level are not fully understood. However, the identification of the skeletal muscle as a secretory organ that produces and secret myokines (cytokines that are produced by skeletal muscle cells) and effect organs distant from the production site has given new insight to the field [24, 25]. By influencing various factors and functions exercise training have the ability to influence exercise capacity and thereby influence VO_{2max} indirectly. This current study examines the link between VO_{2max} and circulating blood borne factors and seeks specially markers shown in people who has displayed efficacy from training.

2.2.1 Metabolic adaption

Endurance training induces adaptions in the skeletal muscle. The size and number of mitochondria are directly affected by training frequency and the mitochondrial content of red skeletal muscle is increased as the training intensity goes up. Oxidative enzymes activity are increased, as is the myoglobin content which increase the amount of oxygen stored in the muscle fibre. These adaptions, in addition to increased capillary density and blood flow, will increase the endurance capacity. Endurance training does also increase the muscle ability of storing glycogen and trained muscles can to a greater extent use fat as energy source and spare the glycogen stores [26]. The patterns that induce these adaptions will be described in the next sections.

2.2.2 Secretory factors

Myokines work in a hormone-like manner as they are secreted into the circulation and exert specific endocrine effect on distant organs such as adipose tissue, liver, pancreas, bone and brain [25]. Among the most examined myokines are myostatin, interleukin 6 (IL-6), interleukin 7 (IL-7) and irisin.

Myostatin, also known as growth/differation factor 8, was the first protein to be classified as a myokine and is a meber of the TGF-β superfamily [27]. The protein is a negative regulator of muscle cell number (hyperplasia) and hypertrophy, i.e. the deletion of myostatin leads to increased skeletal muscle growth [27, 28]. Myostatin expression is reduced by aerobic and exercise training, and the beneficial effects of exercise training is potentiated by the inactivation [29]. It has been demonstrated that increased myostatin levels are highly correlated with obesity and the severity of insulin resistant [30]. Follistatin is an inhibitor of myostatin and is found to increase in healthy individuals with a peak increase of seven-fold after acute bicycle exercise. The inhibitor, which is another member of the TGF-β-family, is not released from the exercising limb, rather, an increase was recognized in both plasma and protein expression in the liver. These findings insinuate crosstalk between muscle and liver during and following exercise [31].

Another well-known myokine is IL-6, which is a cytokine that shows a proportional increase in response to muscle contraction and was the first myokine found secreted to the bloodstream [25]. Until a decade ago it was thought that the increase in IL-6 was due to a muscle damage immune response from macrophages during exercise. However, it was recently demonstrated that the IL-6 mRNA monocytes, who are responsible for the increase in plasma IL-6 during sepsis, are not the source of the exercise-induced levels of IL-6 during exercise [32, 33]. Instead, there is now several evidence that IL-6 is produced upon muscle contraction [24, 34, 35]. The data demonstrate that transcription of IL-6 is activated in working skeletal muscles and the response is enhanced when glycogen levels are critically low, which implies that IL-6 acts as an energy-sensor as it signals to the liver to increase the glucose production [36]. This was confirmed by a study done by Febbraio et al. who infused recombinant IL-6 into healthy individuals during low-intensity exercise, to mimic the high-intensity exercise concentration of IL-6. The study showed that the glucose output was equally high and displayed a direct muscle-liver crosstalk [37]. Infusion of recombinant IL-6 did also cause the effect of increased lipolysis and fatty acid oxidation, which indicates IL-6 as a lipolytic factor [38]. Given these findings of circulating IL-6 during exercise it has been established that IL-6 has several beneficial effects and that the myokine plays a role in the association between the increased risk of chronic diseases and a physically inactive lifestyle [25].

Several studies have focused on the basal IL-6-level and found that physical activity correlates negative with the amount of basal IL-6 level [39]. Same as high levels of basal IL-6 is shown for inactive people [40]. It is also worth noting that while the plasma IL-6 level is decreasing for trained people, IL-6 receptors expression are increasing during muscular work, which can indicate an increased sensitivity to IL-6 [39]. Further, it is proven that IL-6 concentration correlate with chronic morbidity [41] and increase with age [42, 43]. The age-related increase is believed to be affected by the excessive production or reduced reduction of oxygen free radicals which stimulate IL-6 expression [44]. Summed up, circulating IL-6 concentration show elevated levels during acute exercise in proportion to intensity, duration and level of fitness and influence several biological processes, such as glucose and fat metabolism. Simultaneously, regularly physical active people tend to have lower levels of IL-6 and fewer inflammatory markers [45].

PGC1-α is another exercise induced myokine that regulates energy metabolism such as mitochondrial biogenesis and oxidative metabolism [46]. Elevated expression of PGC1-α shows a resistant to age-related obesity [47]. A study done with mice show that increased PGC1-α levels in elderly prevent sarcopenia (loss of skeletal muscle mass and strength) by

reducing apoptosis, autophagy and proteasome degradation. The loss of bone mineral density and normal increase in chronic inflammation normal for aging people, were prevented by this treatment [47]. The basal level of PGC1-α correlate with the change of muscle plasticity induced by chronic endurance exercise training, such as fibre type switching towards type I and type IIA muscle fibres. These fibres are more oxidative and show greater endurance capacity than do the type IIX and type IIB muscle fibres. This can be linked to long-term adaption of skeletal muscle form endurance training which show an increased level of PGC1-α, even between two exercise bouts [48].

PGC1-α is specifically stimulating the expression of FNDC5, which is a type I membrane protein and is further cleaved to the newly discovered hormone irisin [11]. Irisin display a powerful effect on browning of certain white adipose tissue (WAT) and data presented by Boström et al. illustrate that moderately increased level of irisin potently affect whole body expenditure, reduced body weight and show improved diet-induced insulin response [11]. Increased level of brown adipose tissue (BAT) has shown to correlate with anti-obesity and it is thought that BAT can play a role in the struggle against obesity-related health problems by increasing the whole body energy expenditure [49]. Most of the energy of oxidation is dissipated as heat rather than ATP in these brown fat cells. The specific cells contain a transport protein (uncoupling protein) which allows protons to move down an electrochemical gradient without passing through ATP synthase and is switched on when heat generation is required leading the cell to oxidize their fat stores at a higher rate than for regular energy oxidation [50]. In a study comparison between men with normal body mass index and obese men, they found BAT in 96 % of the subjects, but the activity of BAT was 4-fold higher in the lean group [51].

2.2.3 Potential target-pathways to mimic exercise

PPARδ is identified to play a key role in regulation of skeletal muscle metabolism. Running endurance increased almost 100 % when PPARδ are overexpressed in skeletal muscles and pre-program an increase in oxidative muscle fibres of transgenic untrained mice. It has also been observed that PPARδ-agonist, in combination with exercise, induces formation of type I fiber muscle cells and mitochondrial biogenesis and further enhance physical performance. Type I fibres are more resistant to fatigue by expressing enzymes that oxidize fatty acid, as opposed to type II fibres who metabolize glucose. Adaption to training are linked to higher expression of genes involved in the slow-titch apparatus, mitochondrial respiration and fatty-acid metabolism. It has been demonstrated that AMP-activated protein kinase (AMPK) can be seen in correlation with PPARδ as it regulates the transcriptional activity and has a profound effect on skeletal muscle gene expression and oxidative respiration. This was proven in a study conducted by Narkar et al. as they treated mice for 4 weeks with AICAR, a AMPK-agonist which increase phosphorylation of the AMPKα subunit, which in turn stimulates PPARδ-dependent oxidative genes, and showed that the drug treatment decreased the epididymal fat mass/body weight ratio and increased oxygen consumption [52]. The study result demonstrate that the AMPK-PPARδ pathway can be targeted by orally active drugs to increase training adaption and even to increase endurance without exercise [52].

A study conducted by Hakimi et al. tested the metabolic and physiological consequences of overexpressing the PEPCK-C gene in skeletal muscles in mice. The results indicated that the treated mice had an enhanced level of physical activity which extended into old age. Part of this

was due to the increased number of mitochondria and high level of triglycerides in skeletal muscle, as well as decreased body fat. The mice with overexpressed PEPCK-C showed a 40% increase in VO_{2max} compared to the control. The severe increase in oxygen uptake was most likely due to the genetic manipulation and the high activity level, which combined also led to increased mitochondrial biogenesis and increased level of triglyceride. The overexpression of PEPCK-C can seem to provide a mechanism for the removal of the citric acid cycle intermediates during and after training and will further lead to a more complete oxidation of glucose/glycogen. The result of these mechanism will be an increase in oxidative capacity [53].

2.2.4 Other metabolites in response to exercise

A large set of other metabolic are also identified in response to exercise training [54]. Metabolites that are correlated with acute exercise training, such as increased levels of glycolysis product (lactate and pyruvate), glycogenolysis intermediates (3-phosphoglycerate and glucose-6-phosphate), lipolysis product (glycerol) and amino acid (alanine and glutamine) was identified ~10 after training. Finding of metabolic changes in pathways not associated with exercise were also made in the study by Lewis et al. Niacin amide, which plays a role in insulin release and improve glycemic control, and increased with 79 %. Notable, the level of glycerol increased to a greater extent for those who achieved higher VO_{2max} than for those who achieved lower VO_{2max} at peak exercise. This suggest that the capacity of lipolysis is greater for people who are more fit [54] and thus they will have greater benefit from training. The impaired levels of lipid utilization and reduced level of glycerol in the less fit individuals may indicate a maladaptive response to exercise [54] and thus not have the same benefits as the more trained individuals. It was performed an experiment in cultured myotubes with five of the identified exercise-induced metabolites who modulated a cellular pathway relevant to respiration. A mixture of glycerol, niacinamide, glucose-6-phophate, pantothenate and succinate gave a rapid up-regulation of nr4a1c (nur77). Nur77 is a transcriptional regulator of glucose utilization and lipid metabolism genes in skeletal muscles. The study concludes that the exercise-induced metabolic differences between fit- and less fit individuals represent a possible target for salutary metabolic effect of exercise training [54].

2.3 Exercise impact on non-exercising muscles and other organs

As noted, a large number of studies have shown that exercise induced gene expression has a severe impact on a variety of processes including both metabolism and signalling. Largely it has been focus on the impact on the exercising muscle, but there has also been completed studies focusing on to what extent the exercising gene influences the non-exercising muscles and other organs. Catoire et al. [55] discovered that one hour of one-legged cycling changed the expression of several genes in both exercising- and non-exercising leg, with an overlap of 85 genes. This indicate that acute exercise does not only induce gene expression only in the exercising muscle, but also in the resting muscle. Among the genes that revealed equally increased expression in the exercising and non-exercising muscle is ZNF750, PDK4, KLF10 and SLC22A5 [55]. The mentioned genes are known as target gene of PPAR transcription factors and play a key role in regulation of lipid metabolism [56]. As example SLC22A5-gene has a role in controlling free fatty acid import into mitochondria and PDK4 is a gene utilizing glucose by limiting the carbon flux through glycolysis via induction of pyruvate dehydrogenase

[56]. The ANGPTL4 gene showed a higher expression in the non-exercising muscles than in exercising. The gene works as an inhibitor of LPL activity by converting active lipoprotein lipase (LPL) into inactive monomers. LPL is involved in promoting the cellular uptake including cholesterol-rich lipoproteins and free fatty acid. Thus, increased activity of ANGPTL4 in non-exercising muscles leads to decreased local uptake of plasma triglyceride-derivate fatty acid, sparing the use for exercising muscles. In the active muscles it is likely that ANGPTL4 are counteracted by AMPK-mediated down regulation, promoting the use of plasma triglycerides as energy [57].

2.4 Variability in training adaptions

Even though many beneficial adaptions to training has been found, "nonresponders" are readily observed in different training studies. The individual ability to adapt the training effects can influence the risk of long term cardiovascular health [58]. Enhanced aerobic capacity and greater insulin sensitivity are improved by classical alterations in skeletal muscle phenotype following training [59, 60]. The difference in response to training is demonstrated in a study where 60- to 71 year old women and men performed endurance training for 9 and 12 months. The result improvement in VO_{2max} varied from 0 to 43 %, the mean increase was 24 % [61].

It is important to mention that the term "nonresponders" does not mean that these people do not respond to any form of physical activity. A study investigating the effect of both endurance and resistance training showed individual differences in the response of the two different training modalities. In fact, those who displayed the lowest increase in VO_{2peak} after endurance training, showed the highest increase in VO_{2peak} after the resistance training intervention [62]. Yet, it is argued that it exist a hierarchal order on the health benefits from exercise training, whereby improved aerobic fitness should have a greater influence on future health outcome, than, for example, regulating metabolism [3, 5, 63].

It is a wide network of genes and pathways that regulate the response and adaption to training, and the study of specific gene will only partly modulate the variations between high- and low responders. There has been done several efforts to link proteins to exercise induced adaptions. For example, PGC1-α [48] and AMP-activated protein kinase (AMPK) [64, 65] have been extensively studied and are considered to contribute in the regulation of skeletal muscle phenotype response. As noted earlier in the text, overexpression of PGC1-α can increase the amount of slow twitch fibres, in addition to influence insulin sensitivity and determine overall muscle endurance performance [48]. Activation of AMPK during exercise will activate a series of skeletal muscle contraction response processes, like glucose uptake, glycogen synthesis, post-exercise insulin sensitivity, fatty-acid uptake and intramuscular triacylglycerid hydrolysis [64]. However, although these factors seems to have an effect on adaption to training, they do not appear critical for exercise-induced endurance training adaption in terms of muscle biochemistry [46, 65]. Also, no evidence of correlation between muscle adaption and subject-to-subject variability in either PGC1-α or AMPK activation has been presented [66].

The network of interacting genes affecting the VO_2 may provide a relationship between the aerobic capacity and developmental processes and it is reasonable to believe that these genes are pre-set before birth. A study conducted by Timmons et al. found 29 genes who demonstrated training-induced increase in VO_2max following both high-intensity and moderate intensity training [67]. The 29 genes could be predicted by the pre-exercise resting muscle expression

and, notable, RNA abundance of the predictor genes remained unchanged during the exercise training intervention and supports the idea that the expression was determined by genetic variation [67]. Based on these findings it is likely to think that their predictive power is explained by their ability to influence other connecting genes. In addition, it was demonstrated that there were no greater mitochondrial gene expression in high responders compared to low responders and there was no relationship between muscle energy metabolism during training and VO_{2max} improvement [68]. These findings indicate that factors regulating the acute exercise expression do not regulate the aerobic capacity adaptive process. Yet, it is important to state that for untrained individuals, aerobic capacity and aerobic performance factors were tightly linked at baseline, but these factors were not coupled the adaptive process that occurs during exercise training.

There has also been investigated the use of mRNA as biomarkers and in a study conducted by Baggish et al. they found an association between changes in specific mRNA and peak oxygen consumption. It was observed a linear relationship between absolute peak exercise mRNA-146a level and the VO_{2max}. This suggest that mRNA-146a (involved in regulation of inflammation) may represent a biomarker for peak cardiorespiratory capacity. There was also found a similar linear relationship between mRNA-20a resting level and change in VO_{2max} after the training intervention. These may point out the role of mRNA-20a (a factor involved in cancer regulation) as a marker for trainability of VO_{2max} [69]. There has also been observed an inverse correlation between mRNA-21 and mRNA-210 and VO_{2max} [70]. Bioinformatics was used to provide insight in different pathways targeted by these mRNAs. The MAPK pathway, TGF-β pathway and B-cell receptor pathway was found to significantly segregate individuals with high and low VO_{2max}.

The training adaptions mentioned here are affecting widely different mechanism and pathways in the body and there are still many questions to be answered. This study will hopefully contribute to identify possible molecular factors that are induced by training and especially can be associated with enhanced oxygen consumption after a training intervention.

2.5 Liquid Chromatography – Mass Spectrometry-based Proteomics

Considering the complex mixture of substances in the mammalian blood, some limitations had to be made for the blood analysis in the current study. Blood consists of many components such as hormones, proteins and peptides, blood cells, genetic material, ions, nutrients and metabolites. The best fit component for blood analysis, considering long half-life, hormone-like behaviour affecting distant organs and quantifiable by mass-spectrometry, are proteins. Proteins have the ability to exert direct effect on target cells, factors that can bind membrane receptors or penetrate into cells distant from the synthetic site and are detectable by high-throughput screening.

Proteins are the molecular products of gene expression. The expression depends on environmental and cellular conditions and therefore expressed at different times and under different conditions based on the circumstances for the organism the proteins is present. Proteins, in contrast to mRNA, give the functional state of the cell, rather than the potential state. To identify and understand the level of expression of a large number of proteins in different physiological states has been the focus for proteomics research for a long time [71].

The expectation is that the research will help understand biological function and also bring forth molecular health.

2.5.1 Liquid chromatography-mass spectrometry

To provide quantitative information of the expressed proteins, recent studies show that mass-spectrometry-based proteomics has revealed good results [72, 73]. LC-MS has been the method of choice in large protein studies due to its superior throughput and sensitivity [71, 74, 75].

Protein information, such as identity and quantity, is deduced from the peptides. Through proteolysis the peptides are released from the proteins. This approach is called bottom-up proteomics and is referred to as shot-gun-proteomics. The method provides an indirect measurement of protein through peptides derived from proteolytic digestion of complete proteins. The peptide mixture is fractionated and subjected to MS analysis. Identification of the peptides is achieved by comparing the mass spectra obtained from the analysis with a theoretical tandem mass spectra generated from an in silico digestion of a protein database. By assigning peptide sequences to proteins, the complete protein is specified. Since a peptide can either be uniquely assigned to one protein or fits to more than one, the protein may be further grouped and scored based on their peptides [76]. The concept of the LC-MS method will be further explained in the next sections.

In contrast to bottom-up proteomics, top-down proteomics are used to characterize intact proteins. Measurements up to 200 kDa have been done and up to 1000 intact proteins have been identified by multidimensional separation from complex mixtures. However, the disadvantages of the method, such as difficulties with protein fractionation, ionization and fragmentation in gas phase, limits the use [76]. Analysis of peptides, which are more easily fractionated, ionized and fragmented, are more universally adopted for protein analysis and shot-gun proteomics is preferred for clinical studies [77], which are the reason the method is chosen for this current study.

2.5.2 Digestion and separation

Extraction and isolation of protein has become a critical step of the analysis, considering the complex network of interacting biomolecules that regulate the localization and function of the cell. Physical and chemical interactions with other biomolecules can sometimes inhibit the separation or analysis of the protein of interest by LC-MS. Another large challenge for proteomics technology development is protein dynamic range since all steps in the LC-MS pipeline are protein-abundance dependent. To improve the analysis of low-abundant proteins it has been developed approaches to adjust protein concentrations. The two main approaches are selective depletion of high-abundance protein, which are used in this study, and selective equalization of dynamic range using combinatorial ligand library. These methods has proven to be useful in analysis of plasma and other clinical samples [76].

As earlier mentioned, protein is digested to peptides in bottom-up analysis. The mixture of proteins are often highly heterogenic and selective protease digestion create a less heterogeneous mixture as it normalize and characterize the proteins in the samples as peptides. The probability of sampling and identifying a peptide associated with a low-abundant protein is increased with multiple representations of the protein as peptide. Proteolytic enzymes differs

largely based on their specificity to cleave the amide bond by hydrolysis between individual residues in a protein. In shot-gun proteomics, trypsin has become the preferred choice for protein digestion. Trypsin, a serine protease, cleave the carboxyl-amide site between arginine and lysine. The identified peptides are filtered through the sequence-specific information the digestion gives [76]. Digestion is also used to improve the quality of the analysis. Improvement in number of protein identification and quantification of low-abundant proteins were observed with the depletion of abundant peptides in a study done by Fonslow et al. The method uses a protease already used in shot-gun pipelines which selectively digests and deplete abundant peptides with a molecular weight cut-off. The remaining peptides are then digested to completion and analysed [78].

In a general analysis, the digested proteins are separated using liquid chromatography prior to the mass-spectrometer. Liquid chromatography is advantageous because more compounds can be resolved and because of the ability to analyse thermally labile compounds at room temperature [75]. The peptides are eluted from the chromatographic column in order of their hydrophobicity, based on a solvent gradient. Very hydrophilic peptides will be poorly retained and elute immediately, while hydrophobic peptides will have longer retention time [79]. The peptides are separated in the chromatographic column using a gradient and are continuously transferred into the mass spectrometer [75].

2.5.3 The mass spectrometer

A mass spectrometer consists of an ion source, a mass analyser that measures the mass-to-charge ratio (m/z) of the ionized sample and a detector that records the number of ion at every m/z value. The most common ionization source in LC-MS are electrospray ionization (ESI) which ionize the liquid sample and create an aerosol. ESI can be coupled directly to a liquid-chromatograph set-up and are therefore preferred in MS-based proteomics [74, 77]. In the ionization process the trypsin digested proteins who are separated by chromatography flows through a needle and the peptides are subsequently ionized. The peptides are usually double protonated and designated the value $(M+2H)^{2+}$, where M is the mass of the peptide an H is the mass of a proton. As an example, a peptide with mass 1232.55 will have the m/z-value (1232.55 + (2*1.0073))/2 = 617.28 and would be seen at this value in the mass spectrum. The charge state can differ but are easy to determine since each peptide actually consist of an isotope cluster of peaks. If the different between the two isotopic peaks are 1 unit in the m/z-scale (617.28 vs. 618.28), then the charge state of the peptide ion that produce the cluster will be 1. However, if the difference between peak one and two differ with 0.5 units (617.28 vs. 617.78), then the charge must be 2. The quality of separation of the peptide ion peaks reveals the resolution power of the mass spectrometer [79]. The ionization and quantification are followed by selective ion isolation and fragmentation for sequence identification [80]. After the quantification-step done by the orbitrap, the most abundant peaks from the spectrum are selected to further fragmentation by the linear ion trap. The particular peptide is isolated, energy is transferred with the collision with an inert gas (such as nitrogen molecules or argon- or helium atoms) and the energy causes the peptide to break apart. This is called tandem MS since it couples two stages of MS and the fragments generates a tandem mass spectrum. The fragmented ions are called “precursor ions”, while the ions in the tandem mass spectrum are called “product ions” [79].

The mass analyser is important to the technology and the central parameter are sensitivity, resolution, mass accuracy and the ability to generate information-rich mass spectra from the fragments. Recent years there has been a significant increase in use of mass spectrometers

instruments such as ion trap, time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR) and Orbitrap. Orbitrap and FTICR are highly sensitive and outperform any other mass spectrometer when it comes to mass accuracy and resolution power even for small numbers of ions.

Mass accuracy is the ratio of the m/z error measurement to the true value. High accuracy in the measurement of a compounds mass can directly determine the elements composition. Correct elemental composition is one of the first steps to the structural elucidation of molecules. Elements have their mass based on the definition of carbon ^{12}C (= 12.000u) and have mass either slightly above or below their integer value of atomic number (e.g. ^{1}H = 1.007825u, ^{16}O = 15.994910). The ability to measure a compounds mass with sufficiently high accuracy will directly eliminate most other possibilities and directly determine the elements composition in combination with restriction given by Lewis and Senior chemical rules and isotopic patterns, among others [81]. Thus, accurate mass determination act as a filter useful for confirming the identity [82]. Incorrect determination of mass can either lead to wrong identification statistics if the mass accuracy window is set too wide, or to missed identification (false negative) if the window is set to narrow [83]. Accurate mass determination rely on the resolving power [79, 82]. If the constituent components are not resolved sufficiently there is a possibility of co-eluting analytics which can overlap with the compound of interest and skew the peak shape and the peak centroid will no longer correspond to the accurate mass [82]. If the extracting window is set too wide to compensate for inadequate resolving power the hidden interference will contribute to the integrated peak area detected and mass area will be compromised. This will lead to a risk over quantitation and having a false positive discovery.

The Orbitrap and FTICR mass spectrometers also has in common the current image detection system and application of Fourier transform mathematical operation for obtaining frequency mass spectra from time domain spectra [82]. Mass analysers can be combined to allow analysis of both analytics and their fragment and the combination of linear ion trap with a Fourier transform mass spectrometer has become popular because it combine the sensitivity, speed and robustness of ion trap with the high resolution of FT instruments.

For the analysis in this study, the Orbitrap Elite Instrument, showed in figure 1 was used. This instrument combine linear ion trap with a high-field Orbitrap mass analyser. The linear ion trap contribute with sensitivity and reliability, as well as greater dynamic range for better precursor protein detection and power of MS^n detection. The Orbitrap provide resolution >240.000 which increase analytical certainty by improving molecular weight determination [84]. The analytical performance (mass accuracy and resolution) combined with the ease of use makes the Orbitrap mass analyser desired for use in proteomics, metabolomics and lipidomics analysing complex mixtures and low abundant analytics.

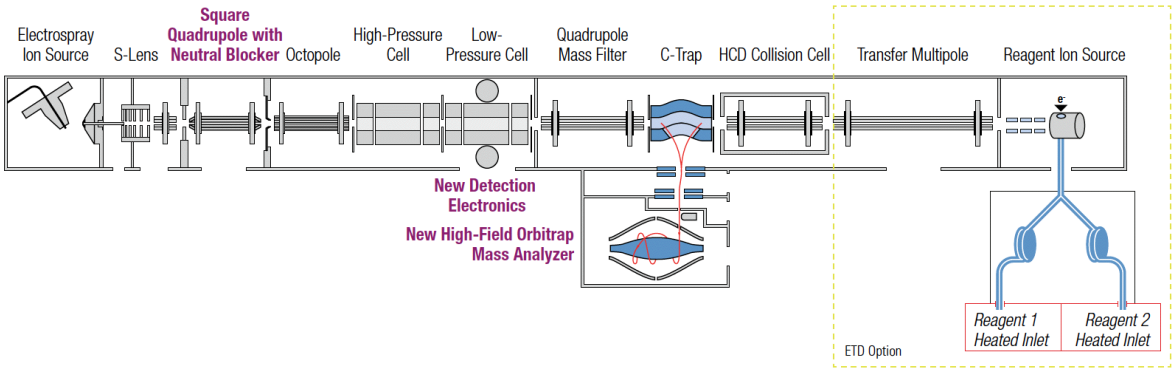

Figure 1: An illustration of the Orbitrap Elite Instrument, the instrument used in the analysis, obtained from Planetorbitrap.com [84].

When the tandem mass spectra are obtained the amino acid sequence is identified in a protein database by matching the molecular weight of the peptide and predicts the fragmented ion expected for each sequence. The goal for the mass spectral database is to identify the best match to the experimentally obtained spectrum [85]. There are computer programs designed for this purpose and algorithms developed [86] to search the database for a matching mass spectrum. When masses are within the selected mass tolerance and the selected weight window input of each other, a match occur. Only those mass matches which occur within the window will be detected [87]. Spectra with poor quality, often from low-abundant proteins, present the greatest challenge of determination. Spectra can then be searched with two or more algorithms to take the advantage of the different selective algorithms [85].

3. Method

3.1 Study population and testing procedures

Participants to the present study were selected from a larger clinical training study which took place at the Norwegian University of Science and Technology in Trondheim, Norway.[1] In total 1567 participants were included in the main study. Inclusion criteria were: born between 1936 and 1942, and able to complete the physical exercise. Exclusion criteria were cardiovascular disease where training were contradictive, infectious disease or any other illness that impede exercise. Twenty-two men were selected to the present study based on the following inclusion criteria: (i) more than 8 hours fast (ii) had completed a VO_{2max}-test. The participants were randomized into either the control group or the training group. The control group was instructed to follow the recommended level of physical exercise in Norway by them self, while the training group was offered instructed exercise performed as 50 min of continuous exercise at moderate intensity (≈ 70 % of peak heart rate) twice a week.

Baseline testing was conducted before the randomization and the follow-up testing was conducted 12 months after initiation of training. The 11 participants in the training group, with the highest increase in VO_{2max} after one year of exercise was chosen for the present study. In addition, 11 participants from the control group, with no increase in VO_{2max} at one-year follow-up, were chosen as control. It was ensured that the control group was not significantly different in weight, BMI, vascular fat, and resting heart rate from the training group. Blood samples were collected using the same protocol in the baseline test and follow-up. The tests was completed on two separate examination days both at baseline testing and at one-year follow up. The first day the participants did physical measurement, while they performed a physical endurance test on day two.

In this study there are two kind of experimental control. The first is the baseline samples, to make sure that the differences we find between pre and post are due to the intervention (exercise), and not due to random variations observed between people. Experimental control 2 is the control group. This is to avoid evaluating the effect of confounding factors, things that might have changed between pre and post independently of exercise.

Test day 1

One the first day blood pressure, height, weight and body screening was performed in addition to collection of blood sample. The participants were asked to refrain from alcohol, nicotine and caffeine the last 12 hours before testing. Weight, BMI, visceral fat, resting heart rate and blood pressure (sBP/dBP) was measured at resting state after fasting time >8 hours.

Height: the height was measured to the nearest millimetre with the participants standing with their feet shoulder width apart, against the wall.

Weight and body-consumption: the Inbody 720 (BIOSPACE Seoul, Korea) was used to measure body consumption. Four electrodes, integrated in the handles, and floor scale was used

[1] Approved by the Regional Committee for Medical Research Ethics (REK 2012/381B) and registered in clinicaltrials.gov/ct2/show/NCT01666340

to carry out the analysis. Once a week during the study the scale is auto-calibrated to ensure the quality.

The test was not conducted if the participants had pacemaker. Demographic characteristics such as gender, height and age are plotted on the display of the scale. Participants are stagnant on the scale for two minutes before weight (kg), body mass index (BMI), muscle mass (kg), visceral fat (cm^2) and fat (%) are reported.

Blood pressure: blood-pressure was measured with a Philips IntelliVue MP50 (Philips medizin systeme, Boeblingen, Germany). After resting for 5 minute blood-pressure is measured twice in the left arm, with one minute break between each measurement. If the systolic blood pressure differed ≥ 10 mmHg and/or diastolic blood pressure differed ≥ 7 mmHg, a third measurement is done. Controls are performed frequently since the device is under the quality control system at St.Olavs University Hospital, Trondheim.

Blood: using standard procedures at St. Olavs University Hospital, Trondheim, serum triglycerides (TG), glucose, HDL-, total cholesterol, C-reactive protein (CRP), glycosylated hemoglobin (HbA_{1c}), and c-peptide levels are measured immediately. Serum was centrifuged at 3000 rpm for 10 minutes at 20°C and aliquots was stored at -80°C for later analysis. Quality assurances are performed frequently by the Lab quality system at the hospital. The serum was thawed up 30 min before analysis and the further analysis procedure are described in section 3.2.

Test day 2

The physical test involved cardiopulmonary exercise testing (CPET) and the participants were instructed to refrain from alcohol, caffeine and nicotine before testing. The gas analyzer Cortex MetaMax II (Leipzig, Germany) was used for the exercise testing. All the gas-analysers were calibrated against a standardized mechanical lung (Motorized Syringe with Metabolic Calibration Kit; VacuMed, AkuMed AS, Oslo). Volume calibration was performed before every test, while gas calibration was performed every fifth test. Heart rate was monitored by Polar Electro Oy (Kempele, Finland). The participants warmed up for 10 minutes on the treadmill at their individual submaximal level which is selected based on the feedback from the participant regarding the experienced intensity and their self-reported physical activity level, as well as monitoring of the heart rate. After the 10 minute warm-up, an individualized protocol was started to measure the VO_{2max}. A face mask connected to the gas-analyzer was attached to the participants. Oxygen uptake, heart rate, perceived exertion (Borg-scale), ventilation, inclination, respiratory exchange rate (RER) and speed (km/h) were reported during the test. After 5 minutes at a submaximal work load, the speed is increased by 1 km/h (or 2% incline-increase if the participant is walking) every 90 second or if the oxygen uptake stabilize. Until true VO_{2max} is reached, this procedure is maintained. A maximal test was considered achieved when, combined with respiratory exchange ratio ≥ 1,05, the participant continued until exhaustion and oxygen uptake did not increase more than 2 ml/kg/min between two 30 second interval (a leveling-of of VO_2 despite workload-increase). Variables from the gas-analyzer was reported every 10 second, and is the last 30 second average. VO_{2max} was the average of the three highest consecutive values reported. Maximal ventilation are the mean calculated among the

three highest ventilation values corresponding to the three highest VO_{2max} values. Peak RER-value are the highest value among the same three VO_{2max} values.

3.2 Chemicals

It was performed a gel-free shotgun proteomics analysis in serum to identify the protein/peptides.

The 14 most abundant proteins was removed from the samples using MARS14 kit (Agilent Technologies) to facilitate the discovery of low-abundant proteins. The MARS14 kit protocol was followed as described in "Agilent Human 14 Multiple Affinity Removal System Spin Cartridges for the Depletion of High-Abundant Proteins from Human Proteomics Samples". The method is based on the concept of chromatography, where the serum is eluted through a column. The relevant proteins had the lowest affinity to the column and elutes first. These proteins was collected and used in the further analysis.

The obtained proteins was concentrated on a spin column down to 400 µl and further processed with NH_4HCO_3 (0,1M, 400 µl), DTT (dithiothreitiol, 200 mM, 40 µl) and IAA (iodoacetic acid, 200 mM, 160 µl) to stabilize pH, reduce S-S bridges and alkylate free SH-groups in the amide sequences, respectively. The samples was then treated with trypsin (15 µl) over night. The digested protein sample was dried for 3 hours on SpeedVac before they were eluted on a C18-column to desalt. The C18 column was loaded with methanol (50 µl) and calibrated with formic acid (50 µl) before the sample was added. Formic acid (0,1%, 11 µl) was added to the eluted peptide samples before they were applied to the Orbitrap Elite instrument. The analysis was set up using the protocol given by Thermo Fischer Orbitrap Elite Manual and done in collaboration with the Proteomics Core Facility at NTNU, Trondheim.

3.3 Statistical analysis

Raw data files from the mass spectrometer were analyzed using Max Quant v 1.5 [88] with the June 2014 version of the human protein database from UniProt [89]. The following search parameters were used: enzyme specified as trypsin with maximum two missed cleavages allowed; precursor mass tolerance was 10 ppm and fragment mass tolerance was 0.5 Da. N-terminal acetylation, carbamidomethylation of cysteine and methionine oxidation were set as dynamic modifications. False discovery rate was set to 0.01, thus peptides with high confidence only were used for final protein identification. Label free quantification algorithm [90] was used to estimate the protein amounts in the sample. The values were log transformed with base 2 and replicates were collapsed to a median representative value. Independent t-test was carried out over these values to assess the differentially expressed proteins.

4. Results

Table 1: Demographic and clinical characteristics of the two groups at baseline- and one-year follow-up testing. RER-max was measured during the VO_{2max}-test, while the other values are collected at resting state. Blood values at baseline testing are also displayed.

	Baseline			**One-year follow up**		
	Control group n = 11	**TG n = 11**	**p-value**	**Control group n = 11**	**TG n = 11**	**p-value**
Age (year)	72.2 ± 1.8	71.6 ± 1.7				
Height (cm)	177.7 ± 7.6	178.1 ± 5.4				
Weight (kg)	80.0 ± 8.6	83.9 ± 8.6	0,382	79.5 ± 8.3	82.7 ± 7.6	0,491
BMI (kg/m^2)	25.3 ± 1.7	26.5 ± 2.2	0,156	25.2 ± 1.4	26.1 ± 2.3	0,291
Fasting time (h)	12.4 ± 1.7	11.3 ± 1.4	0,166	14.3 ± 2.2	14.1 ± 2.0	1,00
Visceral fat (cm^2)	104.1 ± 11.0	110.5 ± 16.7	0,225	103.6 ± 12.6	109.5 ± 20.9	0.433
Resting heart rate (bpm)	65.8 ± 13.6	58.1 ± 9.9	0.144	62.1 ± 11.6	55.7 ± 7.1	0.141
RER-max	1,17 ± 0.1	1.15 ± 0.1	0.488	1.13 ± 0.1	1.10 ± 0.1	0.339
sBP (mm Hg)	137.9 ± 16.2	138.4 ±15.7	0.937	136.5 ± 12.1	135.8 ± 16.3	0.915
dBP (mm Hg)	77.7 ± 9.0	77.7 ± 7.4	0.990	76.6 ± 8.4	76.2 ± 8.2	0.903
CrP (mg/l)	1.45 ± 1.37	2.63 ± 3.40	0.195			
Glucose (mmol/l)	5.89 ± 0.69	5.66 ± 0.40	0.355			
Hemoglobine (g/100ml)	14.82 ± 0.74	15.45 ± 1.25	0.161			
Hemoglobine A (g/100ml)	5.62 ± 0.28	5.46 ± 0.25	0.180			
Total cholesterol (mmol/l)	5.49 ± 1.18	6.15 ± 1.36	0.242			
HDL (mmol/l)	1.69 ± 0.45	1.54 ± 1.39	0.409			
LDL (mmol/l)	3.42 ± 1.22	4.04 ± 1.29	0.256			
Triglyseride (mmol/l)	0.85 ± 0.34	1.26 ± 0.84	0.157			

Values are mean ± SD, TG: training group.

4.1 Background data

No baseline demographic or physiological differences was observed between the controls and training group (table 1). The participants in the training group did not display any significant changes in weight, BMI or visceral fat after one year of training, and the one-year follow up test did not demonstrate any significant differences in physiological characteristics between the two groups. Blood characteristics obtained at baseline are presented in table 5.An outlier had a CrP-value of 31.89 and are omitted from the mean calculation of CrP. LDL-values for both groups lies above the upper limit of normal value range.

4.2 VO_{2max} test

The participants was chosen consciously based on their VO_{2max} increase after the one year-training intervention, because of the design of the experiment. The mean relative increase in VO_{2max} for the training group was 4.4 ± 2.2 ml/min/kg (ranging from 1.39-8.83 ml/min/kg), while for the control group is 0.2 ± 0.5 ml/min/kg (ranging from -0.01-0.86 ml/min/kg). This make a percentage difference of, respectively, 10.9 ± 4.6 % and 0.7 ± 1.2 % (p-value < 0.05) (table 2) for training and control group.

Table 2: Change in VO_{2max} after one year of intervention in control and training group (TG).

	Control **n = 11**	**TG** **n = 11**	**p-value**
Δ VO_{2max} relative (ml/kg/min)	0.2 ± 0.5	4.4 ± 2.2	< 0.05
ΔVO_{2max} (%) (ml/kg/min)	0.7 ± 1.2	10.9 ± 4.6	< 0.05

Values are mean ± SD, TG: training group, VO_{2max}: maximal oxygen uptake, Δ: delta change..

4.3 Analysis

A total of 433 proteins was identified with high confidence. A significant increase ($p < 0.05$) was observed for seven proteins in the training group, but not in the control group. Figure 2 present the protein expression value for the proteins detected by LC-MS It is important to note that an observed increase of the protein expression level in the control group was not significant and was consequently less than was for the training group. The different levels suggest that there are factors that differ the participants that showed an enhanced level of oxygen uptake after a year of training from those who showed no substantial different in VO_{2max}. . A paired t-test showed an even lower p-value for two of the proteins. These are protein z-dependent protease inhibitor and peptidoglycan recognition protein 1. Table 3 present the identified proteins, the assigned gene and their main function in the body. The seven proteins will be further explained in the next section.

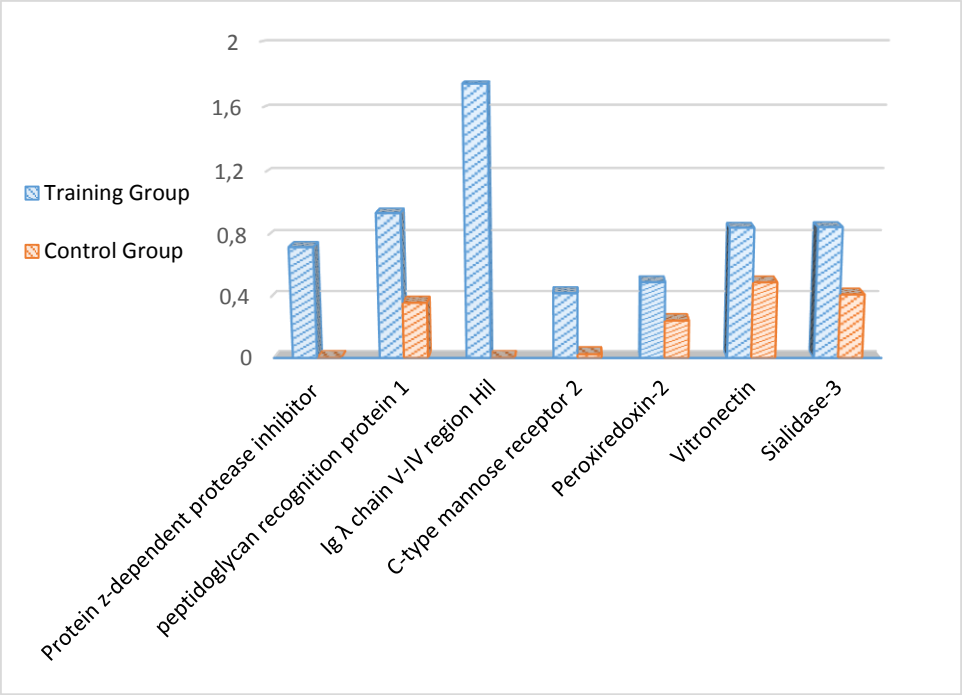

Figure 2: Relative protein expression between the training group end the control group. Protein expression are given in relative values. Values in the control group was not significant.

Table 3: A presentation of the seven significant increased proteins in the study.

Protein	**Gene**	**Main function**	**Fold-change**	**P-value**
Protein z-dependent protease inhibitor	SERPINA10	Regulates blood coagulation	1.62	<0.05
Peptidoglycan recognition protein 1	PGLYRP1	Inflammatory effects	1.87	<0.05
Ig λ chain V-IV region Hil	*N/A*	Participate in the immune system	3.25	
C-type mannose receptor 2	MRC2	Participate in glycoprotein clearance	1,31	<0.05
Peroxiredoxin-2	PRDX2	May play an antioxidant protective role	1.41	<0.05
Vitronectin	VTN	Signal transduction	1.74	<0.05
Sialidase-3	NEU3	Reduces sialic-glycoproteins	1.87	<0.05

It was also performed a complimentary analysis, comparing control group and training group, both at baseline and after one year of intervention. Interestingly, it was found that the protein dermokine showed higher abundance in the exercised group than control only at follow-up (1year intervention), but not at baseline (table 4). Abundance of dermokine was more than 2-fold superior in moderate than control at follow-up, and the low p-value (crossing multiple comparison corrections), indicates that dermokine might also be induced by training.

Table 4: p-value and fold-change of dermokine between control and training group, at baseline and one-year follow-up.

	Baseline		One-year follow-up	
	p-value	**fold-change**	**p-value**	**fold-change**
	0.37	1.8	5.24E-05	4.2

5. Description of the proteins

In the next section the seven different proteins who was detected with a significant increase in the analysis are presented. It is given a description of where and how the proteins affect different processes in the body and circulation and, if identified, which mechanism they influence.

5.1 Protein z-dependent protease inhibitor

Protein z-dependent protease inhibitor (ZPI) is a protein expressed by the gene SERPINA10 belonging to the SERPIN-superfamily which are a group of proteins able to inhibit protease. Protease is an enzyme performing proteolysis, a breakdown of proteins into smaller parts. Protein z-dependent protease inhibitor works in the extracellular matrix and bind protein z to inhibit factor Xa and factor XIa. Factor Xa and factor Xia is an enzyme functioning in the blood coagulation cascade. The blood coagulation cascade system requires bot inhibitors and activators in its dynamic process to maintain normal haemostasis, which is the process that causes bleeding to stop and is the first step in wound healing. Disturbance of this the balance in the dynamic or deficiency of one of the inhibitors can predispose to thrombosis [91]. It is suggested that ZPI plays a physiological role in inhibiting thrombosis. In the blood coagulation system factor X is activated by factor IX and factor VII to factor Xa. In the presence of Ca^{2+} and cofactor protein Z factor, ZPI will inhibit factor Xa and prevent coagulation. It is suggested that ZPI and antithrombin has complementary roles in the control of coagulation, as antithrombin are located in endothelium and ZPI are located in the platelet membrane [91]. ZPI deficiency are thought to be a new form of thrombophilia. But in this context, the question is what the increased values do, and an increased value of ZPI will help prevent blood clotting.

5.2 Vitronectin

Vitronectin (subunit somatodemin B) has shown to play a part in cell adhesion, complement regulation and cell differentiation, as well as having a functioning role in blood coagulation. The well-studied protein was identified with a significant increase in this study. The protein promotes cell adhesion and contributes in the maintaining of cell structure and signal transduction between cells. Vitronectin are divided into several subunits and the N-terminal somatomedin-B domain [92] was one of the proteins detected with highest abundance in the samples. Somatomedin-B binds to plasminogen activator inhibitor (PAI-1) [92] which is a key regulator of fibrinolysis and involved in the regulation atherosclerosis [93]. All active parts of PAI-1 circulating in the blood are known to be in complex with vitronectin [94]. Vitronectin/PAI-1 may regulate the intimal hyperplasmia by inhibiting thrombin, an important participant in the blood coagulation cascade where it activates factor XI to factor XIa. This mechanism can be linked to the formation of thrombosis. It has been discovered that decreased vitronection promotor activity is associated with a 1.5-fold risk of cardiovascular disease [95]. Patients with severe liver failure have also been reported to have reduced levels of vitronectin

[96]. In contrast to these findings, there has also been studies suggesting that PAI-1 is associated with higher risk of atherosclerosis [97]. It was not detected high level of PAI-1 in the participants of the training group, who showed elevated levels of vitronectin, even though these are expected to be observed in a complex. As vitronectin is an inhibition factor of coagulation, it will reduced the risk of venous clot.

5.3 Sialidase-3

Sialidase-3 is a protein encoded by the gene NEU3. The protein is an enzyme associated with sialic acid reduction from glycoproteins and glycolipids. Sialic acid-rich glycoproteins has high affinity to selectin, which is a cell adhesion molecule important in the inflammation response. A study demonstrated that overexpression of NEU3 in the liver lead to increased insulin-sensitivity and glucose-tolerance. This was due to the increased tyrosin phosphorylation of IRS1 (the insulin receptor) in the basal state leading to enhanced insulin sensitivity. There has been suggested that the NEU3 expression level is regulated by tyrosine phosphorylation in skeletal muscle in response to insulin. The same study also identified that NEU3 overexpression increased PPARγ activity in the liver. This is somewhat surprising considering that PPARγ activity has found to be associated with increased triglyceride accumulation and induced hyperlipidaemia which is related to metabolic syndrome. Enhanced PPARγ expression is also enhanced in a number of obesity models.

5.4 Peroxiredoxin-II

The ability of the blood to flow through the vein is important for maintenance of cardiovascular health and endurance health. It has been reported that world-class endurance athletes has greater red blood cell deformability, meaning that the blood cells has a higher ability to move through narrow capillaries [98]. A protein that can be linked to this occurrence is peroxiredoxin-II. This is a protein belonging to the ubiquitous family of antioxidant enzyme. These proteins use their antioxidant capability through their peroxidase activity, whereby hydrogen peroxide and a wide range of organic hydroperoxides are reduced and detoxified ($ROOH + 2e^- \rightarrow ROH + H_2O$) [99]. Peroxides like H_2O_2 are produced as a normal cellular response, but the ability to convert to toxic radicals and damage cellular components provide a risk to the cell after long-term presence of peroxidases and it is suggested that peroxiredoxin-II is responsible in protecting red blood cells from oxidative stress [100]. Loss of peroxiredoxin-II-activity lead to reduced red blood cell formability and increased methememoglobin and heme degradation products [101]. Heme degradation products are detected during cellular aging and in various diseases. Peroxiredoxin-II $^{-/-}$ mice had elevated levels of structural abnormal cells in the dense blood cell fraction, which parallel contains remarkably higher levels of reactive oxygen species (ROS) [100]. In our study peroxiredoxin-II had a fold-change of 0.5 ($p<0.05$) in the training group, indicating that peroxiredoxin-II may be an indicator of enhanced physical capacity or a feature of the participants who had increased the aerobic capacity.

Peroxiredoxin-II contribute in the regulation of heme degradation and increase red blood cell deformation ability. High aerobic capacity also show increased red blood cell deformability, but they also display a higher turnover, meaning they have a higher rate of young blood cells. The reason for this is the high oxygen flux during vigorous exercise which causes damage to susceptible blood cells, which the activity of peroxiredoxin-II cannot prevent.

Peroxiredoxin-II has also shown to have an important role in eliminating hydrogen peroxide which has been involved in the pathogenesis of several disease [99]. It has been reported that

patients with Alzheimer- and Parkinson disease has an increased expression level of peroxiredoxin-II in the frontal cortex. The increased level is suggested play a role in the ability to protect the cortical neuron in the presence of toxic concentration of beta-amyloid-β peptide (Aβ), yet the mechanism is unknown. Amyloid-β binding alcohol dehydrogenase binds Aβ, and high level of the complex lead to mitochondrial dysfunction and death of neuron, which is speculated to be caused by oxidative stress [102]. The elevated level of peroxiredoxin-II may be a cellular response to protect the brain from further oxidative damage in various neurodegenerative diseased brains.

5.5 Peptidoglycan recognition protein 1

Peptidoglycan recognition protein 1 (PGRP-1) is in the family of recognition receptors that hydrolyse peptidoglycans to inactivate its processes. Peptidoglycan (PGN) is the polymer forming the plasma membrane/cell wall in all bacteria. PGN activates macrophages through two pattern recognition receptors, CD14 and TLR-2, which causes the production of cytokines and chemokines. Overproduction leads to infection, fever, inflammation, leucocytosis, sleepiness, deceased appetite and decreased peripheral perfusion [103]. PGRP-1 is a peptidoglycan recognition proteins and is especially coupled to Gram-positive bacteria where peptidoglycans is an important component of the cell wall. In Gram-positive bacteria PGRP-1 activates the CssR-CssP two-component system. The activation of the system will lead to membrane depolarisation, cessation of RNA, DNA and protein synthesis, and to production of hydroxyl radicals which are responsible for the bacterial death [104]. It has been shown high expression of PGRP-1 in mammalian bone narrow and lower expression in lymphoid tissues, which suggest a role for PGRP-1 in the immune system. The binding specificity of PGRP-1 is preferential for unmodified PGNs and bind poorly to more pathogenic bacteria. The non-pathogenic bacteria are easily recognized by PGRP and effectively removed in the innate stadium of the immune system [103]. A study done in the well-known fruit fly Drosophila, the PGRP-1 is coupled to the Toll pathway by the hydrolysis of peptidoglycans. It is thought that the hydrolysis will activate the Toll pathway, especially TLR-2, which then leads to the production of antimicrobial peptides [105]. TLR-2 is a membrane protein recognising many bacterial and viral substances. Usually this result in endosome/phagosome-uptake of bound molecules and cellular activation. In response to Gram-positive bacteria, TLR-2-deficient mice displayed an impaired production of IL-6, TNFα (tumor necrosis factor α) and NO. Also, TLR-2-deficient mice negated the PGN-induced NF-κβ DNA binding activity, indicating that TLR-2 is participating in the activation of the intracellular signalling pathway generated by PGN [106]. NF-κβ is a protein-complex that responses to stimuli such as stress, cytokines and free radicals, and play a key role in immune response to infection.

5.6 C-type mannose receptor 2

C-type mannose receptor 2 (also known as ENDO180) belongs to an underfamily of the C-lectin superfamily. This superfamily consist of transmembrane and soluble proteins such as collectins and sellectins. The mannose receptor is a transmembrane protein consisting of an extracellular N-terminus and an intracellular C-terminus. The extracellular part are composed of 8 consecutive c-type carbohydrate domain closest to the membrane, followed by a fibronectin II domain and a cysteine-rich N-terminal domain [107]. The initial interest for this receptor originate from its ability to recognise and adopt a spectrum of monosaccharaides and lysosomal enzymes. The C-type mannose receptor-2 recognizes terminal mannose on glycoproteins and contribute in the clearance of glycoproteins from the circulation. This include clearance of

sulphated glycoprotein hormones and glycoproteins released in response to pathological events. The clearance regulation helps control the level of released molecules in the circulation during inflammatory response and thus regulating the bioavailability of these important sulphated proteins. Among the sulphated proteins recognizes are the hormones lutropin and thyroid stimulating hormone [107]. The primary function of thyroid are the production of the T_3 and T_4 hormones, which are associated with energy metabolism. An underproduction of the hormones T_3 and T_4 (hypothyroidism) has shown a relationship with diabetes mellitus type I and II. The relationship is greater for type I diabetes mellitus [108]. This may be due to the shared genetic susceptibility to the two conditions. The thyroid antibody is presence in 25 % of patients of type I diabetes at the time of diagnosis, which then can be a predictor of later autoimmune thyroid dysfunction [108]. Hypothyroidism is also characterized by abnormal lipid metabolism, cardiac dysfunction, increased diastolic blood pressure resulting in atherosclerosis and ischemic heart disease [109]. C-type mannose receptor 2 are transcribed from the gene MRC2, which is highly expressed in the smooth muscle cells and cardiac myocytes. Cardiac myocytes (cardiac muscle cells) are responsible for the contractile power in the muscle. Aging is characterized by increased extracellular matrix and decreased myocyte number that will result in a rearrangement of the structure. The extracellular matrix is a fibrillar network that embeds the cardiomyocytes and the remodelling is a critical part of mortality in elderly. The myocardial structure are affected by the process of aging through increased blood pressure, atherosclerosis and decreased physical activity [110].

Mannose binding receptor (MBL) has also been connected to peptidoglycan (PGN) by binding to the polymer in the presence of Ca^{2+} [111]. Studies of MBL knock-out mice suggest that MBL is necessary for down-regulation of TNF-α and IL-6. MBL is a collectin whose function appears to recognise pattern in the first line defence. MBL may play a role to reduce the PGN-induced inflammation [111].

5.7 Immunoglobulin chain V-IV region

The study also identified a significance increase for the immunoglobulin (Ig) chain V-IV region. This is an antibody produced to by plasma cells and used in the immune system to recognise, identify and neutralize foreign viruses and bacteria. The antibody recognize the antigen, an unique part of the foreign target. The general structure of immunoglobulin is similar with two large heavy chain and two smaller light chains. It's the different tip formation that gives us the different types, termed region. The Ig chain V-IV region belongs to the primary immunoglobulin class IgG. This is the most abundant class in human serum and protects the body from infections.

6. Discussion

The study showed a significant increase for seven proteins after one year of different training modules. The participants that showed the most increased in VO_{2max} revealed beneficial effect from training. Because of the analysis of both the training group and the control group at pre-training and after one year of training, there is no risk of evaluating confounding factors, things that might have changed between pre and post independently of exercise. As noted earlier, physical fitness are associated with a decreased risk of early mortality than physical activity. Peak exercise is the best predictor of mortality, which most likely is because it lowers the risk of cardiovascular diseases and other types of lifestyle disorders.

The proteins that increased as a result of training, has not been identified to increase VO_{2max} directly. None of the identified proteins can be linked directly to higher oxygen consumption, but many of the proteins can be linked to the beneficial effect from training, like reduced risk of thrombosis, increased insulin sensitivity and glucose tolerance. Two of the proteins can be coupled to blood coagulation. ZPI was shown to inhibit factor X and factor Xa which activates thrombin to stimulate the formation of fibrin to prevent bleeding. This is a direct link between a protein and the possibility to affect the formation of thrombosis. Thrombosis is the formation of blood clots inside the blood vessels. When the inside wall of a blood vein is damaged thrombin will act to form fibrin. When the blood clot is big enough to prevent blood flow it will result in ischemic stroke. ZPI may be a factor that can prevent the formation of blood clot and the association between this protein and high VO_{2max} found in this study may be one of the factors that protect individuals with enhanced oxygen consumption from cardiovascular diseases. Vitronection is another protein that is associated with reduced risk of atherosclerosis, even though some contradictory result has been presented. Vitronectin works in complex with PAI-1, and may have an inducing effect on the PAI-1 level. In this study there was detected an increased level of vitronectin after one year of exercise, while the analysis does not say anything about PAI-1. However, the findings in this study suggest that vitronectin contribute to the beneficial effect from training in sense of preventing the risk of cardiovascular disease by acting in complex with PAI-1 to inhibit thrombin.

Sialidase-3 is a well-known protein located in the membrane and responsible for a wide range of biological processes, including possible molecular mechanism associated with metabolism. It is shown that overexpression of NEU3, the gene transcribing sialidase-3, correlate with increased insulin sensitivity and glucose tolerance. This can be utilized considering people with type II diabetes, where insulin-resistance is one of the causes of the disease. Another protein that revealed an increase after one year of exercise is C-type mannose receptor-2. The protein is associated with regulating of sulphated glycoproteins during inflammation response. Among these sulphated proteins are thyroid hormones, where the most known are T_3 and T_4. These hormones are known to increase metabolism and underproduction is associated with diabetes. It is, however, not clear how the MRC-2-T_3/T_4-mechanism acts. It is interesting that MRC-2, which is increased among the trained individuals, are associated with the the clearance of glycoproteins and will then lead to a decreased level of thyroid hormone. As stated above, a decreased level are associated with lower metabolic function and diabetes, something that is not associated with well-trained persons.

The role of MRC-2 for people with higher VO_{2max}, as found in this study, may be associated with the expression in smooth muscle cells and cardiac myocytes. As people gets older, the amount of cardiac myocytes will decrease, which is effected by high blood pressure, atherosclerosis and less physical activity. The results from this study suggest that increased mannose receptor 2 expression associated with physical exercise and high VO_{2max} may inhibit some of the features of aging.

Inflammation is the body's protection from unknown pathogenic invaders. Acute inflammation is the first line of defence and will increase blood flow to the place of initiation and induce a number of biological processes. A regular acute inflammation can become chronic inflammation and lead to replacements of cell types in the place of action and is associated with diseases like atherosclerosis, obesity, cancer, diabetes and dementia. Exercise is known to increase many pro-inflammatory factors. An example is cytokines that stimulates energy

expenditure and IL-6 which is a pro-inflammatory factor increasing fatty acid oxidation. You might say that exercise is a training in inflammatory response. PGRP-1 is a protein detected in this study associated with the immune system. PGRP-1 recognise peptidoglycan in the membrane of bacteria and act to eliminate them. PGRP-1 is also associated with the TLR-2, which recognise microbes in the circulation and activate an immune response. A decreased expression of TLR-2 leads to a reduced expression of IL-6, indicating that these two factors may be working together in the inflammatory response. IL-6 has been detected as a result of exercise and secreted by the muscle cells. The elevated level of PGRP-1 in the subject who had a significant increase in VO_{2max} can regulate the inflammatory response the body tend to have during exercise.

Perxiredoxin-II showed increased values in the participants with increased VO_{2max}, as well as in endurance athletes. This is a protein that protects the red blood cells from oxidative stress, which are a result from the normal metabolism of oxygen whereas reactive oxygen species are a product. Peroxiredoxin-II is detoxifying these products and in this way protect the blood cells. In this way the blood cells maintain their deformability and further enhance the ability to move through narrow capillaries. As peroxiredoxin-II is a protein that seems to directly affect the endurance capability and cell deformability it may be a therapeutic agent that can be used to prevent the oxidative stress found to be active in aging and in various disease. Peroxidase-II is also coupled to Alzheimer disease as it acts to protect the neuron cells from oxidative stress. Whether it actually prevents the degradation of the neuron cell is still a question not answered, but if peroxiredoxin-II do it would be an interesting factor to explore in the research of Alzheimer disease.

The protein dermokine was more than 2-fold higher in the training group than in the control group, and was the only protein passing the multiple correction test. Dermokine was first found to be expressed in differentiated layers of the skin and is proved to be differentially regulated in inflammatory conditions as a cytokine [112]. Gene expression is induced by exercise in skeletal muscle of dogs [113], so it is assumed that the protein is released into the circulation by the muscle and then be one of the factors responsible for the effects of training. Dermokine are present as different isoforms which may differ in their biological role, but any mechanism has not been provided yet.

Even though the proteins who displayed a significant increase after one year of exercise seems to regulate some of the mechanism that can be linked to the beneficial effects from training and an enhanced level of oxygen consumption, it cannot be concluded whether these elevated protein are the cause of increased VO_{2max} or if they are a consequence of VO_{2max}. But the proteins are found to be participating in several mechanism that is linked to beneficial effects from training. It is in this study proved that there is a connection between an increased oxygen consumption and the identified proteins. There was no proteins that was linked directly to the mechanism of VO_2, but there was several proteins that can be linked to the earlier proved beneficial outcome from exercise training. Although the mechanism of these proteins are not yet fully understood, they have no shown to correlate with high VO_{2max} and maybe a potential target for mimic the benefits of exercise.

Further work can expose the true mechanism of the proteins and if studied in the context of cardiovascular disease it can contribute as a step towards the identification of therapeutic targets.

7. Conclusion

The study showed a significant increase for seven proteins after one year of different training modules. The participants that showed the most increased in VO_{2max} revealed beneficial effect from training. There is no doubt that there is several mechanism that affect the body's ability to adapt to training and some people have an advantage because they respond well to exercise. In this study there was identified protein that can contribute in the search for therapeutic targets that can mimic exercise.

8. References

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