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Gene expression profiling of inherited and acquired maximal oxygen uptake

Relations to the metabolic syndrome

Thesis for the degree of philosophiae doctor

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Norwegian University of
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Faculty of Medicine
Department of Circulation and Medical Imaging

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Genstudie av medfødt og opptrent kondisjonsnivå
Relasjoner til metabolsk syndrom

Kondisjonsnivå har mye å si i en aktiv hverdag, men har også vist seg å kunne forutsi risikoen for å utvikle hjerte- og karsykdommer. Kondisjonsnivået sier faktisk mer om risikoen for hjerte- og karsykdom enn de kjente risikofaktorene slik som høyt kolesterol, fedme og røyking. Selv om hjerte- og karsykdommer er blitt kraftig redusert her i landet de siste tiårene, er det fortsatt den alvorligste dødsårsaken i Norge. Siden utholdenhetstrening øker kondisjonsnivået, minker samtidig sjansen for utvikle hjerte- og karsykdommer hos de som trener regelmessig. Dette er en kjent sak, men vi vet fremdeles lite om er hva som skjer med genene våre når vi trener. Hovedmålet for prosjektet var derfor å identifisere gener og cellulære prosesser som er assosiert med medfødt og opptrent kondisjonsnivå. Tanken bak dette er at de flere av de samme genene og cellulære prosessene sannsynligvis også er relatert til risiko for hjerte- og karsykdom.

Vi sammenliknet genuttrykk i hjertet (studie I) og skjelettmuskulaturen (studie II) hos rotter med medfødt høyt- og lavt kondisjonsnivå, og studerte endringene ved trening hos begge disse gruppene. Det viste seg at de rottene med lavt kondisjonsnivå hadde en opphopning av risikofaktorer for hjerte- og karsykdom, som fedme og høyt blodtrykk, i tillegg til høyt nivå av både glukose og fett i blodet. Disse funnene tyder på at rottene med medfødt lav kondisjon har utviklet metabolsk syndrom (en diagnose for individer med opphopning av risikofaktorer for hjerte- og karsykdom). I hjertet fant vi store forskjeller i genuttrykk avhangig av om man var født med høyt- eller lavt kondisjonsnivå. Disse tydet på at energibruken var forskjellig avhengig av om de hadde høyt- eller lavt kondisjonsnivå. I tillegg hadde de med lavt kondisjonsnivå tegn på sykelig vekst av hjertet. Disse genetiske endringene kan settes i sammenheng med utvikling av hjerte- og karsykdom, og bør studeres videre for å avdekke potensielle angrepspunkter for forebygging og behandling. I skjelettmuskelen fant vi tegn på at lavt kondisjonsnivå kunne ha en sammenhang med en mutasjon som fører til dårlig energiproduksjon i cellene. Mennesker med denne mutasjonen og rottene med lavt kondisjonsnivå har flere likheter, som diabetes, og intoleranse for trening.

Flere studier viser at intervall trening (4x4 prinsippet) er den mest effektive treningsmetoden for å øke kondisjonsnivået. Vi har derfor valg å bruke dette treningsregimet i alle tre studiene. Treningen ga størst genetisk forandring i skjelettmuskel hos rottene som var født med høyt kondisjonsnivå, hvor vi fant tegn på forbedret evne til å ta opp fett og omdanne til energi. Dette skyldes sannsynligvis at hjertet hos disse rottene har god kapasitet for blodforsyning, og at muskelen derfor kan respondere normalt på trening. I studie 3 undersøkte vi genetiske endringen i blod hos pasienter med metabolsk syndrom. Etter 16 uker med trening fant vi flere endringer i genuttrykk som kanskje kan forklare den forbedringen vi fant i karfunksjon. Slik informasjon kan være nyttig for å forstå mekanismene bak forbedret helse ved trening.

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Contents

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List of papers

Paper I

Aerobic capacity-dependent changes in cardiac gene expression

Anja Bye, Mette Langaas, Morten A. Høydal, Ole J. Kemi, Garrett Heinrich, Lauren G. Koch, Steven L. Britton, Sonia M. Najjar, Øyvind Ellingsen, Ulrik Wisløff

Physiol Genomics. 2008 Mar 14;33(1):100-9.

Paper II

Gene expression profiling of skeletal muscle in exercise-trained and sedentary rats with inborn high and low VO_{2max}

Anja Bye, Morten A. Høydal, Daniele Catalucci, Mette Langaas, Ole J. Kemi, Vidar Beisvåg, Lauren G. Koch, Steven L. Britton, Øyvind Ellingsen, Ulrik Wisløff

Submitted to Physiol Genomics June 2008.

Paper III

Transcriptional changes in blood after aerobic interval training in patients with the metabolic syndrome

Anja Bye, Arnt E. Tjønna, Tomas O. Stølen, Ragnhild E. N. Røsbjørgen, Ulrik Wisløff

Submitted to EJCPR May 2008.

These papers will be referred to by their roman numerals in this thesis.

Abbreviations

AC6	Adenylate cyclase 6
ADAMTS1	A Disintegrin and Metalloproteinase with ThromboSpondin motifs1
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AUH	Enoyl CoA hydratase
Ca^{2+}	Calcium ion
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
cDNA	Cyclic deoxyribonucleic acid
CO_2	Carbon dioxide
COX	Cytochrome *c* oxidase
CPT1α	Carnitine palmitoyltransferase 1α
cRNA	Cyclic ribonucleic acid
CROT	Carnitine o-octanoyltransferase
CVD	Cardiovascular disease
DDIT4	DNA damage inducible transcript 4
DNA	Deoxyribonucleic acid
e^-	Electron
ELISA	Enzyme-linked immunosorbent assay
ERβ	Estrogen receptor β
ES	Enrichment score
FA	Fatty acid
FAO	Fatty acid oxidation
FFA	Free fatty acid
FGF1	Fibroblast growth factor 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	Growth hormone
GLUT4	Glucose transporter 4
GSEA	Gene set enrichment analysis
H^+	Hydrogen ion
H_2O	Water

HCR	High capacity runners
HDL	High-density lipoprotein
HF	Heart failure
HIF1α	Hypoxia-inducible factor 1α
HK2	Hexokinase 2
IGF	Insulin-like growth factor
IP_3	Inositol triphosphate
K^+	Potassium ion
KLF15	Kruppel-like factor 15
LARS2	Leucyl-transferRNA synthetase
LCR	Low capacity runners
LDL	Low-density lipoprotein
LV	Left ventricle
MELAS	Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, and Stroke-like episodes
MIAME	Minimum information about microarray experiment
mRNA	Messenger ribonucleic acid
NH_3	Ammonia
NO	Nitric oxide
O_2	Oxygen
PDGF1α	Platelet-derived growth factor 1α
PFKFB2	6-phosphofructo-2-kinase
RNA	Ribonucleic acid
RNAi	RNA interference
SERCA2a	Sarcoplasmic reticulum calcium ATPase 2 a
siRNA	Small interfering RNA
SR	Sarcoplasmic reticulum
SV	Stroke volume
tRNA	Transfer ribonucleic acid
UCP4	Uncoupling protein 4
VLDL	Very low-density lipoprotein
VO_{2max}	Maximal oxygen uptake
VWF	Von Willebrand factor

Definitions

Artificial selection: In a genetically isolated population, random mating is prevented and mating is limited to those individuals who exhibit desired characteristics.

Gene and protein nomenclature: Gene and protein names are abbreviated in different ways depending on species.

Species	Gene symbol	Protein symbol
Homo sapiens	*SHH*	SHH
Rattus norvegicus	*Shh*	SHH

Gene expression: The process by which inheritable information from a gene, such as the deoxyribonucleic acid (DNA) sequence, is made into a functional gene product, such as acid ribonucleic (RNA) or protein. Several steps in the gene expression process may be modulated, including the transcription step and the post-translational modification of a protein. Gene regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism. Gene regulation may also serve as a substrate for evolutionary change, since control of the timing, location, and amount of gene expression can have a profound effect on the functions of the gene in the organism.

Maximal oxygen uptake: The highest oxygen (O_2) uptake that can be achieved by an individual during exercise with dynamic use of a large muscle mass; considered as the best indication of cardiorespiratory capacity.

MELAS: A progressive neurodegenerative disorder. The features of the illness includes metabolic disorders caused by dysfunction of mitochondrial DNA, lactic acidosis, stroke-like episodes, seizures, diabetes mellitus, and exercise intolerance.

Metabolic syndrome: To be diagnosed with the metabolic syndrome according to the definition from the World Health Organisation (1999), at least one of the three following diagnoses must be present:

- type 2-diabetes
- impaired glucose tolerance
- impaired fasting glucose or insulin resistance

in addition to at least two of the following diagnoses:

- blood pressure: ≥ 140/90 mmHg
- dyslipidaemia: triglycerides ≥ 1.695 mmol/L + circulating high-density lipoprotein (HDL) ≤ 0.9 mmol/L ♂ ≤ 1.0 mmol/L ♀
- central obesity: waist/hip ratio > 0.90 ♂ > 0.85 ♀, and/or Body Mass Index > 30 kg/m^2
- microalbuminuria: urinary albumin excretion ≥ 20 mg/min or albumin/creatinine ≥ 30 mg/g

MIAME: Minimum Information About a Microarray Experiment is a standard for reporting microarray experiments. It is intended to specify all the information necessary to interpret the results, and to potentially reproduce the experiment.

Microarray: A high-throughput technology used in molecular biology and in medical research. The microarray consists of thousands of microscopic spots of DNA oligonucleotides, that each contains picomoles of a specific DNA sequence or a short section of a gene (probe). Cyclic DNA (cDNA) or cyclic RNA (cRNA) from the sample being studied hybridizes to these probes under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence to determine relative abundance of nucleic acid sequences in the sample. In standard microarrays, the probes are bound to a solid surface by covalent attachment to a chemical matrix.

Background

The Western society epidemic of lifestyle-related diseases is an impending threat to public health, and calls for effective prevention and treatment strategies[1]. Exercise training may represent such a strategy, as there is a close line between an individual's fitness level and long-term prognosis[2]. Identifying the cellular and molecular mechanisms associated with aerobic fitness is important, because it may help us develop new and better methods to prevent and treat cardiovascular disease (CVD). From the HERITAGE Family Study we are starting to get a glance of which genes that contribute to the adaptations to exercise, but currently little information is available on the combination of individual lifestyle factors, environmental influences and genetic factors in determining an individual's level of physical fitness[3, 4]. Recently, gene expression profiling using microarrays have revealed unexplored fields of biomarker discovery and gene expression profiling of disease. Microarray technology is constantly improving, and might represent an important tool for identifying the genes associated with aerobic fitness level and CVD.

The metabolic syndrome

The metabolic syndrome is defined as a cluster of conditions that may predispose for CVD (see Definitions). Although each of these factors is an independent predictor of cardiovascular mortality, they become more potent than the sum of each single parameter when occurring together. In recent years, there has been an expansion in the knowledge about how inactivity interacts with genes and forms a basis for development of chronic disease[1]. Some argue that the modern human is still genetically adapted to the hunter-gatherer lifestyle because the human genome has changed little during the past 10,000 years. Accordingly, today's genotypes could have evolved through natural selection in an environment were physical activity were obligatory for survival[5]. Booth *et al.* speculated whether humans carry a set of disease susceptibility genes that produces some relative risk which, in combination with physical inactivity, could lead to chronic disease[6]. Following this argument, the human body is thus not ideally suited for a modern Western lifestyle where we maintain only 38% of the daily energy

expenditure as compared to our ancestors[1]. Today, the metabolic syndrome is one of the most challenging threats to human health in the Western civilization, and is now present in about 25 % of the US adult population[7, 8].

The pathophysiology of the syndrome is complex and has only partially been elucidated. The most important factors for developing the metabolic syndrome seem to be aging, genetics and lifestyle (physical activity and diet)[7, 9]. There has been debate regarding whether obesity or insulin resistance is the cause of the metabolic syndrome or whether they are consequences of a more far-reaching metabolic disorder. Given the growing number of persons suffering from the metabolic syndrome, more knowledge on the genetic susceptibilities is urgently needed.

There are several more or less efficient treatment strategies for the metabolic syndrome today, as lipid, cholesterol, and blood pressure lowering drugs[10]. However, with the syndrome reaching a pandemic, these treatment strategies do not seem to be very successful. A major goal is to treat both the underlying cause of the syndrome, and the CVD risk factors if they persist. Accumulating evidence indicate that regular physical activity has profound beneficial effects on both prevention and treatment of the metabolic syndrome, although the mechanisms are still unclear[11-16]. Moreover, several studies indicate that there is an inverse relationship between the incidence of the metabolic syndrome and aerobic fitness[17-21]. Interestingly, rats selected over generations for low aerobic fitness (Low Capacity Runners; LCR rats) that were exposed to the same environmental factors as rats selected for high aerobic fitness (High Capacity Runners; HCR rats) developed characteristics that fit into the definition of the metabolic syndrome[22]. This indicates a very strong genetic factor determining the predisposition for the syndrome.

Maximal oxygen uptake

Maximal O_2 uptake (VO_{2max}) is dependent on the lung ventilatory capacity, the hearts pumping ability, the function of the endothelium, the O_2-carrying capacity of blood (i.e. hemoglobin) and the capacity of utilizing O_2 in mitochondrial respiration. The higher

VO_{2max}, the more O_2 has been transported to and used by exercising muscles, which increases the level of intensity at which the individual can exercise.

VO_{2max} is determined both by genetic and environmental factors[23]. The genetic factors contribute to the untrained fitness level, but also the potential of training-induced improvements[24, 25]. Since low VO_{2max} is a strong and independent predictor of the metabolic syndrome and cardiovascular mortality, the ability to deliver and utilize O_2 during exercise seems to represent a point of divergence for cardiovascular health[2, 17-21, 26-28]. In patients with insulin-resistance, the low VO_{2max} is associated with impaired mitochondrial function that has largely been attributable to impaired skeletal muscle glucose metabolism[29, 30]. These observations are consistent with impaired regulation of mitochondrial function as an important mechanism for low VO_{2max} and cardiovascular risk factors linked to the metabolic syndrome. Also in rats born with a low VO_{2max} (LCR rats), impaired mitochondrial function is suggested to be a leading cause of impaired O_2 uptake[22].

Endurance training is a physiologically attractive treatment strategy for the metabolic syndrome. Endurance training improves blood pressure, endothelial function, whole-body glucose disposal, insulin sensitivity, caloric expenditure, neurohormonal factors, body composition, and lipid metabolism[11, 20, 31]. Due to the inverse relationship between VO_{2max} and the metabolic syndrome, exercise that increases VO_{2max} the most, is potentially the most effective treatment strategy. Several studies now agree that high-intensity aerobic interval training is superior to moderate training in improving VO_{2max} in healthy individuals, healthy rats, patients with coronary artery disease, and patients with post-infarction heart failure (HF)[32-36].

Recently, the HERITAGE Family Study investigated the role of genetic contribution to the individual response to endurance training[24]. A significant genetic component in the trainability of VO_{2max} was reported, which means that the degree to which VO_{2max} can be improved by exercise varies widely in the human population. Some families were characterized by a high trainability pattern, whereas others were characterized by low responsiveness with little or no benefits of training. The heritability was determined to

be 47 %, and a significant maternal effect was observed. This raised the possibility that mitochondrial DNA is involved in determining the individual training response. Since inborn and acquired VO_{2max} has a strong genetic component, it should be feasible to identify the genes or mutations responsible for determining inborn VO_{2max} and the response to exercise training.

Exercise-induced improvements in VO_{2max} depend mainly upon adaptations in the skeletal muscle, blood vessels and in the heart, as lung function is hard to improve by means of exercise.

The heart

The metabolic syndrome has damaging effects on the heart, and triggers several maladaptive responses in the myocardium. Due to insulin resistance, the metabolic syndrome heart is susceptible to energy deprivation, cardiomyopathy, diastolic and systolic dysfunction, impaired contractility, pathological left ventricle (LV) hypertrophy, and fibrosis[37, 38]. However, most of these conditions can be reversed by performing regular endurance training. After long-term endurance training, growth mechanisms are activated that causes an increase in LV chamber size, wall thickness and total mass[39]. Consequently, stroke volume (SV) and O_2 delivered to working muscles increases. In addition, sympathetic activity and myocardial O_2 demand decreases, whereas vagal tone is enhanced[40, 41]. For metabolic syndrome patients, exercise-induced improvements of insulin sensitivity and are therefore particularly important, to progressively reduces the risk of cardiovascular events.

Cardiac hypertrophy

Cardiac hypertrophy is an adaptive response of the heart to preserve LV function in physiological or pathological states[42-44].

Physiological cardiac hypertrophy, induced by long-term endurance training, normal body growth, and pregnancy, triggers functional and morphological changes in the heart, to meet increased demands while maintaining normal LV function[39].

Physiological LV hypertrophy includes different hypertrophy patterns that are induced by different forms of exercise training. Strength training increases peripheral resistance, or afterload, thereby stimulating concentric hypertrophy. Long-term endurance training increases venous return and blood volume, and hence preload, and is therefore a stimulus for eccentric LV hypertrophy[45, 46]. The cardiac physiological hypertrophy seen with normal body growth (i.e. more muscle mass to serve) and endurance training increases contractility, SV, and cardiac performance, allowing the individual to exercise at higher workloads[47].

The heart adapts to excess hemodynamic load by compensatory hypertrophy, which, under conditions of persistent strain, over time evolves into cardiac dysfunction. Like the physiological counterpart, pathological LV hypertrophy also includes different hypertrophy patterns. Pressure-overloaded (concentric) hypertrophy results in a substantially increased wall thickness to chamber radius ratio. In contrast, volume-overloaded (eccentric) hypertrophy results in a normal wall thickness to chamber radius ratio[48]. Moreover, the course of HF is characterized by transitions between a series of interconnected phenotypes, resulting in a dilated heart with a thin wall and a large heart chamber. In addition, after myocardial infarction, loss of viable myocardium results in compensatory changes in the remaining viable muscle, which is highly heterogeneous. Pathological cardiac hypertrophy is deleterious because it increases the O_2 demand of the heart and decreases mechanical efficiency. Sustained pathological hypertrophy often leads to systolic and diastolic dysfunction, and is regarded as an independent risk factor of cardiovascular morbidity and mortality[49]. Metabolic syndrome patients may suffer from both hypertension-dependent and hypertension-independent LV pathological hypertrophy[50, 51]. In normotensive patients, both type 2-diabetes and hypercholesterolemia have been found to independently cause maladaptive cardiac growth[51].

Physiological and pathological cardiac growth, is suggested to be triggered by an interaction of mechanical forces and neurohormonal factors[52]. Most of the extracellular stimuli (ions, hormones, cell mediators, and mechanical signals) are integrated and transmitted by various intracellular signalling pathways to the cell nucleus where gene-

expression is altered[53, 54]. Pathological remodelling is mainly triggered by neurohormonal factors (angiotensin-II, endothelin-1, and catecholamines) through G-protein-coupled receptor signalling pathways[54]. Downstream, the pathway involves phosphatidylinositol bisphosphate, diacylglycerol, and inositol triphosphate (IP_3). IP_3 releases calcium (Ca^{2+}) from intracellular stores, which may activate e.g. the calcineurin pathway and trigger transcription of hypertrophic genes[55]. In addition, a complex web of signalling pathways has been implicated in cardiac hypertrophy. In contrast, physiological hypertrophy appears to be triggered by growth hormone (GH) and insulin-like growth factor (IGF) and regulated e.g. through the phosphoinositide-3 kinase-Akt pathway[56]. Changes in gene expression induced by this cascade, allow the heart to produce normal active tension at a lower cost in terms of energy expenditure.

Based on the close relationship between cardiac phenotype and VO_{2max}, differences in inborn VO_{2max} might also be related to different cardiac growth patterns[57-59]. Whether the inborn level of VO_{2max} is associated with a particular growth pattern is currently unknown.

Cardiac function, contractility and Ca^{2+} handling

In metabolic syndrome patients, the frequency and/or the severity of systolic and diastolic dysfunction seem to increase with the number of components of the metabolic syndrome[60]. Myocardial contractile dysfunction is characterized by altered Ca^{2+} handling and excitation contraction coupling that leads to impaired cardiomyocyte fractional shortening and relaxation.

Cardiomyocyte fractional shortening is initially activated by depolarisation of the sarcolemma membrane. Depolarisation of the membrane opens voltage gated Ca^{2+} channels (L-type Ca^{2+} channels), which due to the electrochemical gradient over the sarcolemma, leads to an influx of Ca^{2+} to the cytosol. This small increase in Ca^{2+} opens the Ca^{2+} sensitive ryanodine receptors located at the sarcoplasmic reticulum (SR), leading to a large Ca^{2+} release from the SR. The free intracellular Ca^{2+} binds to troponin C and initiates the adenosine triphosphate (ATP) dependent movements of actin and

myosin. For diastolic relaxation to occur, Ca^{2+} must be removed from the cytosol. The removal of intracellular Ca^{2+} occurs via transport over the sarcolemmal membrane or reuptake into the SR. In rats, 92 % of the Ca^{2+} re-enters the SR via the SR Ca^{2+} -ATPase 2a (SERCA2a). The remaining 8 % is extruded over the sarcolemma via the Na^{+}/Ca^{2+} exchanger, and the sarcolemmal Ca^{2+}- ATPase, or enters the mitochondria via the mitochondrial uniporter[61].

Disruption of intracellular Ca^{2+} handling is one of the key factors of contractile dysfunction in heart disease, and has been documented in cardiomyocytes from rats with features of the metabolic syndrome (LCR rats), and in rats and patients with HF[22, 62-65]. This may partly be explain by reduced SERCA2a levels, combined with decreased phosphorylation of phopholamban depressing the re-uptake of Ca^{2+} to the SR[66, 67]. As a compensation for lower SERCA2a content and activity the Na^{+}/Ca^{2+} exchanger is relatively given more significance as its expression is increased[68]. However, this compensation may lead to lower SR Ca^{2+} content as the Na^{+}/Ca^{2+} exchanger removes Ca^{2+} over sarcolemma and out of the cell.

Alterations in the abundance and/or activity of L-type Ca^{2+} channels and the ryanodine receptors have also been associated with abnormal Ca^{2+} regulation[69, 70]. In concert, this may together with lower SR Ca^{2+} lead to a dys-synchronised and diminished Ca^{2+} release from the SR[71]. Increased sensitivity of the ryanodine receptors to Ca^{2+}, increases the frequency of spontaneously, uncontrolled Ca^{2+} release from the SR. This feature may reduce the SR Ca^{2+} storage, decrease Ca^{2+} amplitude, reduce cardiomyocyte contraction, and lead to depressed systolic function[72]. Moreover, altered Ca^{2+} myofilamament sensitivity is also a common feature in cardiac dysfunction[73, 74].

It is previously shown that endurance training improves myofilament Ca^{2+} sensitivity, cardiac contractile performance, and Ca^{2+} handling in healthy rats, rats with features of the metabolic syndrome (LCR rats) and in rats with HF[22, 36, 59, 74]. However, further investigations are needed to fully understand the mechanism of improved Ca^{2+} handling with exercise, as well as the impaired Ca^{2+} handling in heart disease.

Cardiac metabolism

Cardiac muscle fibres have the highest mitochondrial density of all tissues, and rely almost exclusively on energy released from aerobic reactions. In healthy hearts, the preferred substrate for mitochondrial respiration is free fatty acid (FFA), accounting for 60–80% of the total energy consumption. However, after a meal and during intense exercise, the preferred energy substrates are glucose and lactate, respectively. In essence, the heart metabolizes the substrates offered by the circulation at different circumstances.

Pathological conditions of the heart often involve changes in cardiac energy metabolism like increase in glucose oxidation, and downregulation of enzymes involved in fatty acid oxidation (FAO)[75, 76]. Initially, enhanced glucose oxidation improves cardiac efficiency, since the amount of ATP produced per O_2 consumed is higher. However, as the pathological condition progresses towards an uncompensated state, the capacity of utilizing glucose decreases, and hence the efficiency of the heart[77]. Increased glucose metabolism and impaired cardiac O_2-supply leads to increased anaerobic metabolism at high work loads[75]. Anaerobic metabolism has lactic acid as a major metabolite, which consequently results in cardiomyocyte acidosis, which directly influences the cardiomyocyte contractile properties[78].

In metabolic syndrome patients, the plasma levels of glucose and FFAs are often elevated. The latter may result in increased intracellular levels of FFAs and their derivatives, which potentially inhibit insulin mediated glucose transport in cardiomyocytes. However, despite less insulin-mediated glucose entrance, glucose uptake in diabetic hearts is often normal because of the hyperglycemia[76]. Therefore, also glycolytic intermediates may accumulate in the cardiomyocytes.

Regular endurance training may improve detrimental cardiac energy metabolism in diseased hearts[79]. Regular endurance training will augment myocardial blood flow because of myocardial neovascularisation, and provide a more effective control of vascular tone and blood distribution[80-82]. However, direct evidences for beneficial effects of endurance training on cardiac energy metabolism are sparse. Hence, more

studies are needed to elucidate the changes in cardiac energy metabolism by exercise and the genetic basis for this.

Skeletal muscle

Skeletal muscle tissue constitutes about half of the body mass and play a fundamental role in whole body metabolism. In the last decade, our modern lifestyle with physical inactivity has impaired skeletal muscle contractile and metabolic functions, contributing to the epidemic emergence of the metabolic syndrome.

Although genes, sex, body size, and age are the primary determinants of muscle mass, skeletal muscles have a tremendous capacity to adapt structurally and functionally to exercise by altering gene expression that affects growth and metabolism[83]. Transcriptional alterations in skeletal muscle regulatory genes occur within hours after an exercise bout, e.g. enzymes regulating FAO and oxidative phosphorylation capacity[83-85]. In contrast, transcriptional alterations of structural genes e.g. components of mitochondria and capillaries, occurs a few weeks into the exercise program[86-88]. Increased metabolic activity is necessary for structural adaptations and increase workload in muscle with exercise training. Therefore, exercise training is accompanied by increased number and volume of mitochondria, increased mitochondrial enzyme activity, and increased production of nitric oxide (NO) that improves O_2 and nutrient availability by increasing flow and perfusion[89]. A genetically determined limitation in skeletal muscle O_2 delivery and utilisation has previously been reported in the LCR rat model[90]. The activity of skeletal muscle oxidative enzymes, e.g. citrate synthase, was significantly decreased as compared with rats with no limitations in O_2 delivery and utilisation (HCR rat model)[90]. If skeletal muscle is subjected to O_2 deficiency during work, ATP production may be insufficient and structural adaptations may not occur. Studies of exercise training at hypoxia and normoxia show evident differences in skeletal muscle gene expression depending on the availability of O_2[91, 92]. In the case of a normal, healthy muscle, decreased O_2 delivery during endurance training often involves adaptations that favour O_2 transport and utilization[91].

Skeletal muscles are targets in prevention and treatment of the metabolic syndrome, due to their important role in consuming and removing energy substrates (lipids and glucose) from the circulation. Under normal circumstances, plasma FFAs is the predominant fuel for skeletal muscle, although supplemented by circulating very low-density lipoprotein (VLDL), triglycerides, lactate and glucose[93]. Under insulin-stimulated conditions (i.e. after a meal), skeletal muscle accounts for as much as 80 % of whole-body glucose elimination, and is therefore the primary tissue responsible for peripheral disposal of glucose[94].

Evidence indicates profound beneficial effects of exercise training in metabolic syndrome patients due to cellular adaptations in skeletal muscles. However, the mechanisms are far from understood and need further evaluation. The improved whole body metabolism (Figure 1) accomplished by exercise training is likely to be a central mechanism behind improved health by exercise. During exercise, skeletal muscle lipid- and glucose metabolism increases, and contributes significantly to the peripheral disposal of glucose. Despite increased fatty acid (FA) metabolism, the level of circulating FAs remains quite stable due to a simultaneous increased hydrolysis of intramyocellular triglycerides releasing FFA to the circulation. Long-term endurance training involves alterations in metabolic substrate preference in muscle with a greater reliance on lipid, rather than carbohydrates. This has beneficial effects on the muscles due to reduced formation of lactic acid, glycogen sparing, reduces high-energy phosphate utilization, and reduces muscle fatigue. Although much is known regarding exercise-induced changes in metabolism, many of the detailed molecular mechanisms and genes involved remains to be identified.

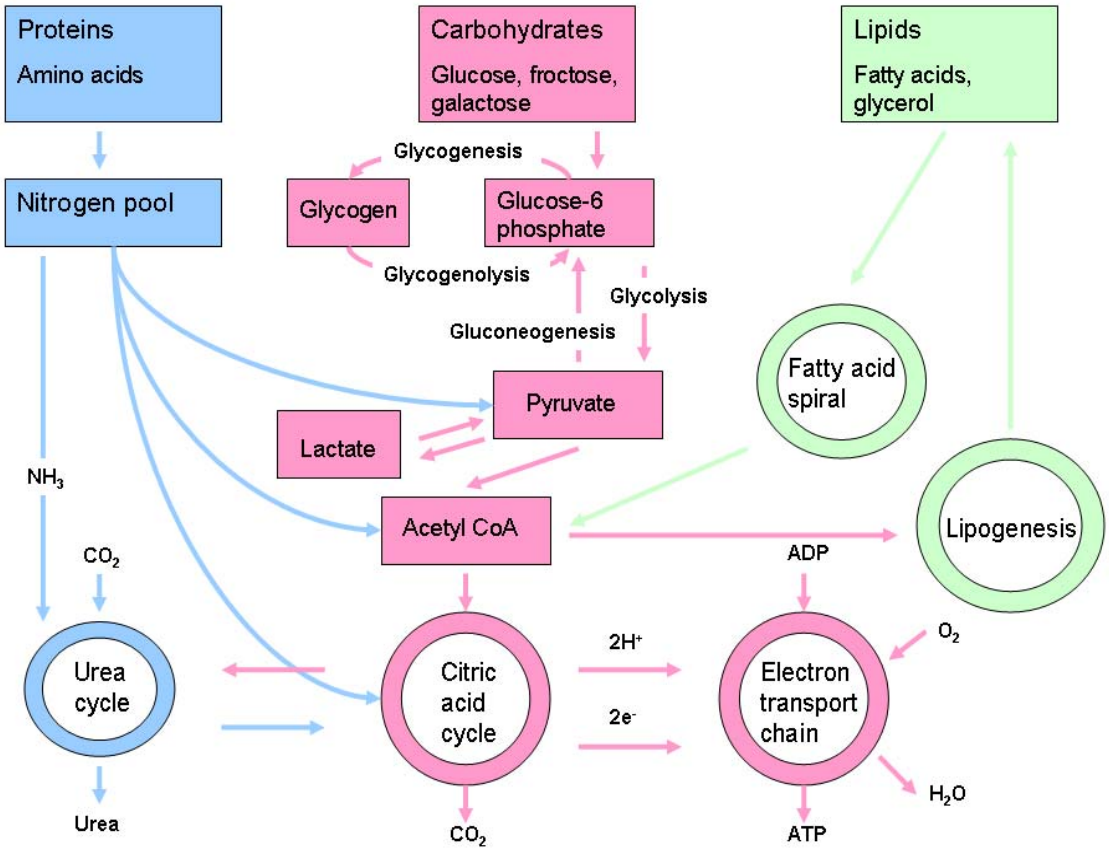

Figure 1. Summary of metabolism. CO_2: Carbon dioxide, NH_3: Ammonia, H^+: Hydrogen ion, e^-: Electron, ADP: Adenosine diphosphate, ATP: Adenosine triphosphate, O_2: Oxygen, H_2O: Water.

Circulating factors and the endothelium

Blood samples can provide useful indication of the CVD risk and contribute to a clinical diagnosis of the metabolic syndrome. Blood from metabolic syndrome patients often contains low levels of HDL, as well as elevated levels of prothrombotic- and proinflammatory factors, triglycerides, glucose and insulin. Although increased levels of prothrombotic- and proinflammatory factors are not included in the diagnostic criteria, metabolic syndrome patients often suffer from a chronic low-level inflammation process, dys-regulated coagulation system and impaired platelet function[13]. Therapies that improve insulin sensitivity, hyperinsulinemia, and metabolic abnormalities has been shown to decrease the prothrombotic state[95].

Several of the factors that constitute the metabolic syndrome, has destructive effects on the innermost surface of blood vessels, the endothelium. The endothelium regulates vascular tone, and inhibits platelet aggregation and atherosclerosis. The most important endothelium-derived factor is NO, based to the vide variety of paracrine actions, ranging from being the most potent endogenous vasodilator, to counteracting atherosclerosis[96]. NO is produced by endothelial NO synthase, and uses $_{L}$-arginine as substrate. Diminished bioavailability of NO, through decreased production or increased degradation, may lead to endothelial dysfunction. In diabetic animals, increase NO degradation by free radicals has devastating effects on cardiovascular system by inactivating NO and forming the cardio-toxic peroxynitrite[97]. Loss of proper endothelial function is an early pathogenic event of the metabolic syndrome that often appears decades before the onset of vascular disease[98]. Endothelial dysfunction is often seen in patient with hypertension, hypercholesterolemia, type 2-diabetes, and in smokers[99, 100]. The degree of endothelial dysfunction is proportional to the severity of insulin resistance; however, the cause-and-effect relationship is unclear and should be further studied[101].

Several studies suggest that improved cardiovascular health by regular physical activity is mediated, at least partly, through re-establishment of the haemostatic balance and endothelial function[102, 103]. Since substances involved in the metabolic syndrome (low-density lipoprotein (LDL), HDL, glucose, insulin, triglycerides) are constantly interacting with blood cells, blood cells might also be involved in the pathogenesis of the metabolic syndrome and the endothelial dysfunction. Studies are needed to elucidate whether blood cells alter their gene expression as a concequence of exercise training in metabolic syndrome patients, and whether altered properties of blood cells might be involved in improvements of endothelial function and the metabolic syndrome.

Fighting the metabolic syndrome

Non-pharmacological lifestyle changes, as exercise and dietary modifications, constitute the most important treatment strategies for the metabolic syndrome. The most feasible symptoms to treat by changes of lifestyle are endothelial dysfunction and insulin

resistance. Although we know how to treat the syndrome effectively with exercise and dietary modifications, the prevalence of the syndrome increases yearly, possibly because of ignorance, and lack of commitment to a healthy lifestyle. Therefore, new treatment strategies are urgently needed to cope with this expanding problem. To achieve this goal, it is essential to define a set of candidate genes involved in the development and/or progression of this syndrome. Since exercise is an efficient treatment strategy of the metabolic syndrome, genes altered by exercise also represent possible drug targets of the treatment of the metabolic syndrome.

Aims

Since VO_{2max} is a strong predictor of cardiovascular mortality, genes related to low VO_{2max} may also be related to the metabolic syndrome and CVD. As skeletal muscle, blood and the heart are involved in the pathology of the metabolic syndrome, we hypothesed that genes predisposing for CVD are expressed in these organ systems. Therefore, the main purpose was to uncover gene expression profiles associated with different levels of both inborn and acquired VO_{2max}.

The specific aims were to:

A. Determine if rats born with a great difference in running capacity and VO_{2max} (HCR/LCR model) have different LV gene expression and different soleus muscle gene expression.

B. Study whether the gene expression profile of LCR may explain the low VO_{2max} and increased cardiovascular risk.

C. Determine if rats born with different VO_{2max} respond differently to the same exercise protocol in terms of altered gene expression.

D. Determine whether transcriptional changes occur in blood cells of metabolic syndrome patients in response to 16 weeks of high intensity interval training.

E. Study whether the exercise-induced changes in gene expression (D) may explain the improved health status achieved by the metabolic syndrome patients after the exercise intervention.

Methodological considerations

Animal model

To study intrinsic VO_{2max} in a situation were environmental factors are minimised, we used an artificially selected rat model of high capacity runners (HCR) and low capacity runners (LCR) with 30% difference in inborn VO_{2max} and 432% difference in inborn running capacity (Figure 2)[22, 90, 104-117]. In the HCR/LCR-model, genes responsible for aerobic fitness are concentrated, while environmental components are minimized by maintaining a standardized environment. This makes the HCR/LCR-model of substantial value for determining the genes causative of variation in VO_{2max}. Moreover, as almost all human genes known to be associated with disease have orthologues in the rat genome, the rat is a highly applicable model for gene expression analyses in translational medical research[118].

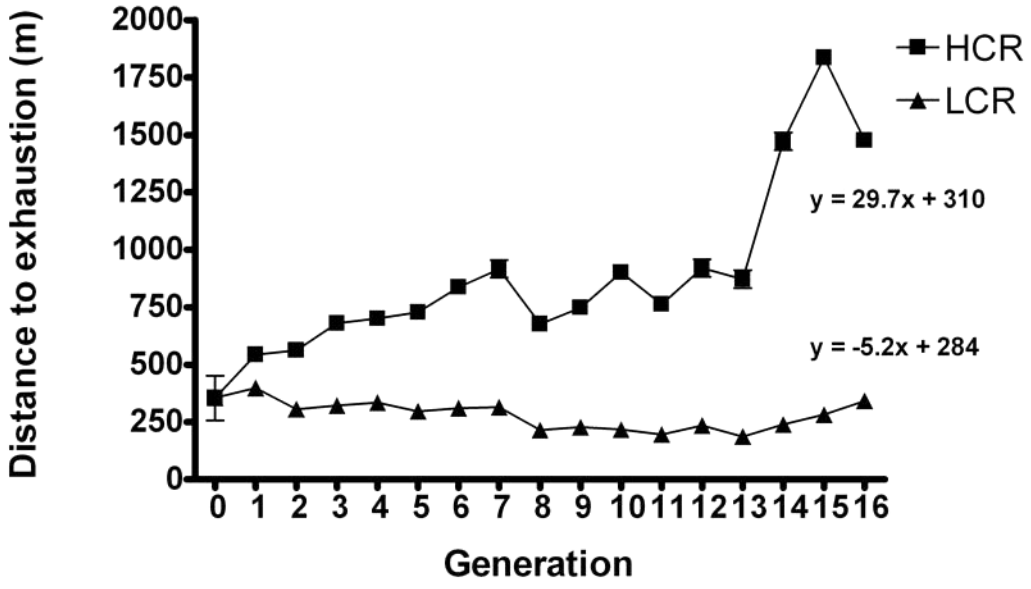

Figure 2. Response to selection for aerobic treadmill-running capacity across 16 generations. The HCR group was estimated to increase by 29.7 meters per generation, whereas the LCR group was estimated to decrease 5.2 meters per generation. HCR: High capacity runners, LCR: Low capacity runners.

Interestingly, at generation 16 (which was studied in this thesis), LCR have accumulated risk factors of CVD, such as hypertension, endothelial dysfunction, insulin resistance, impaired glucose tolerance, visceral adiposity, hyperglycemia, hypertriglyceridemia, and elevated plasma FFAs; commonly diagnosed as the metabolic syndrome[22, 107]. In addition, LCR have decreased SV, as well as impaired O_2 supply, extraction ratio and tissue diffusion capacity in skeletal muscle as compared to HCR[90, 106, 113]. LCR also have impaired systolic and diastolic cardiac function[107]. The impaired systolic function was manifested by impaired cardiomyocyte contractility, increase time to peak contraction, low levels of systolic Ca^{2+} and increased time to peak Ca^{2+} concentrations[107]. The diastolic dysfunction involved slow cardiomyocyte relaxation, less efficient Ca^{2+} removal, as well as high levels of diastolic free Ca^{2+}.

Recently, impaired tissue oxygenation due to reduced O_2 supply, extraction, and tissue diffusion capacity were reported in LCR[106, 113]. This was not surprising, since running capacity is related to the ability to deliver and utilize O_2[119]. Since the O_2 uptake of LCR is limited by supply, extraction ratio, and diffusion capacity, several systems/organs are likely to be involved in the low VO_{2max} phenotype.

Metabolic syndrome patients

In Paper III, we analysed blood samples collected from 7 males and 4 females diagnosed with the metabolic syndrome (according to the World Health Organisation criteria), before and after a 16-week high-intensity exercise program. During the exercise period, these patients had significantly improved VO_{2max}, endothelial function (in terms of flow-mediated dilatation in the brachial artery), insulin signalling in fat and skeletal muscle and blood pressure, hence reducing their risk of later developing CVD[11]. In addition, 47% of the patients were no longer classified as having the metabolic syndrome. This high success rate makes this approach an effective treatment strategy for the metabolic syndrome, and a useful model to study the relationship between improvements of the features of the metabolic syndrome and changes in gene expression. Given the growing number of persons suffering from the metabolic

syndrome, more knowledge on how genetic susceptibilities interact with exercise is urgently needed.

Interval training program

Rats performing high-intensity interval training display most of the cardiorespiratory changes observed in humans, as increased VO_{2max}, physiological cardiac hypertrophy, improved endothelial function and reduced resting heart rate[36, 59, 120]. Most of the changes occur within the first four weeks of endurance training, and VO_{2max} reaches a plateau after six to eight weeks[36, 120]. High-intensity aerobic interval training by treadmill running is a very efficient method of improving VO_{2max}[32-35]. Interval training is designed to challenge the pumping capacity of the heart to a greater extent than moderate continuous exercise. Several studies now agree that high-intensity aerobic interval training is superior to moderate training in improving VO_{2max}, in healthy individuals, healthy rats, patients with coronary artery disease, and patients with post-infarction HF[32-36]. Therefore, both laboratory animals and metabolic syndrome patients in this thesis were recruited to aerobic interval training at high-intensity, to most efficiently increase VO_{2max} and combat metabolic abnormalities.

Both rats and patients performed uphill running/walking on a treadmill, although the rats at a higher inclination than the patients. The rats ran for 1.5 hours five times a week, alternating between eight minutes at an exercise intensity corresponding to 85-90% of VO_{2max}, and two minutes active recovery at 50-60%. This model was established by Wisløff *et al.* and ensures a robust training response that involves increased VO_{2max}, physiological cardiac hypertrophy, improved contractile function, and reduced resting heart rate[35, 59, 120]. In contrast to the rats, patients performed 40 minutes of exercise three times a week, alternating between four minutes at an exercise intensity corresponding to ~90% of maximal heart frequency, and three minutes of active recovery at 70%. The exercise program for rats and patients lasted for 8 and 16 weeks, respectively. To adjust running speed in order to maintain the intended intensity for the rats throughout the experimental period, VO_{2max} was measured at the start of every training week. To

maintain the intended intensity for the patients speed or inclination was increased during the exercise period.

Tissue collection

The LV and soleus muscles were collected when the rats were approximately 7 months old, and 48 hours after their last exercise session. This was done to avoid any acute effects of exercise. Since expression of regulatory and metabolic genes tends to occur within few hours after exercise training, and often returns to baseline within 24 hours, sample collection after eight weeks of endurance training, and 48 hours after the last exercise selection means that we probably lose sight of several of the differentially expressed genes[87, 121]. However, this was intended, since we were interested in the long-term adaptations to exercise. Since we were studying effects of aerobic exercise training, we chose to analyse the soleus muscle rather than other leg muscles, because of its high concentration of aerobic slow fibres.

Blood samples from metabolic syndrome patients were collected before and after the training intervention, and 72 hours after the last exercise session to avoid acute effects like changes in number and phenotype of circulating leucocytes[122]. To minimise potential environmental factors, sample collection was performed at the same time of day after 12 hours of fasting. Blood cells possibly contributing to the gene expression profile of the metabolic syndrome patients are illustrated in Figure 3.

To avoid potential RNA degradation, samples from LV, soleus muscle and blood were treated with caution. The samples from soleus muscle and LV were snap-frozen in liquid nitrogen, whereas the blood samples were collected on specialized PAXgene tubes to ensure RNA integrity. Samples for verification of microarray data were collected at the same time as the samples for microarray analysis. This included plasma from the patients, and additional tissue from the LV and soleus from the rats. All samples were stored at -80 ^{o} C until assayed.

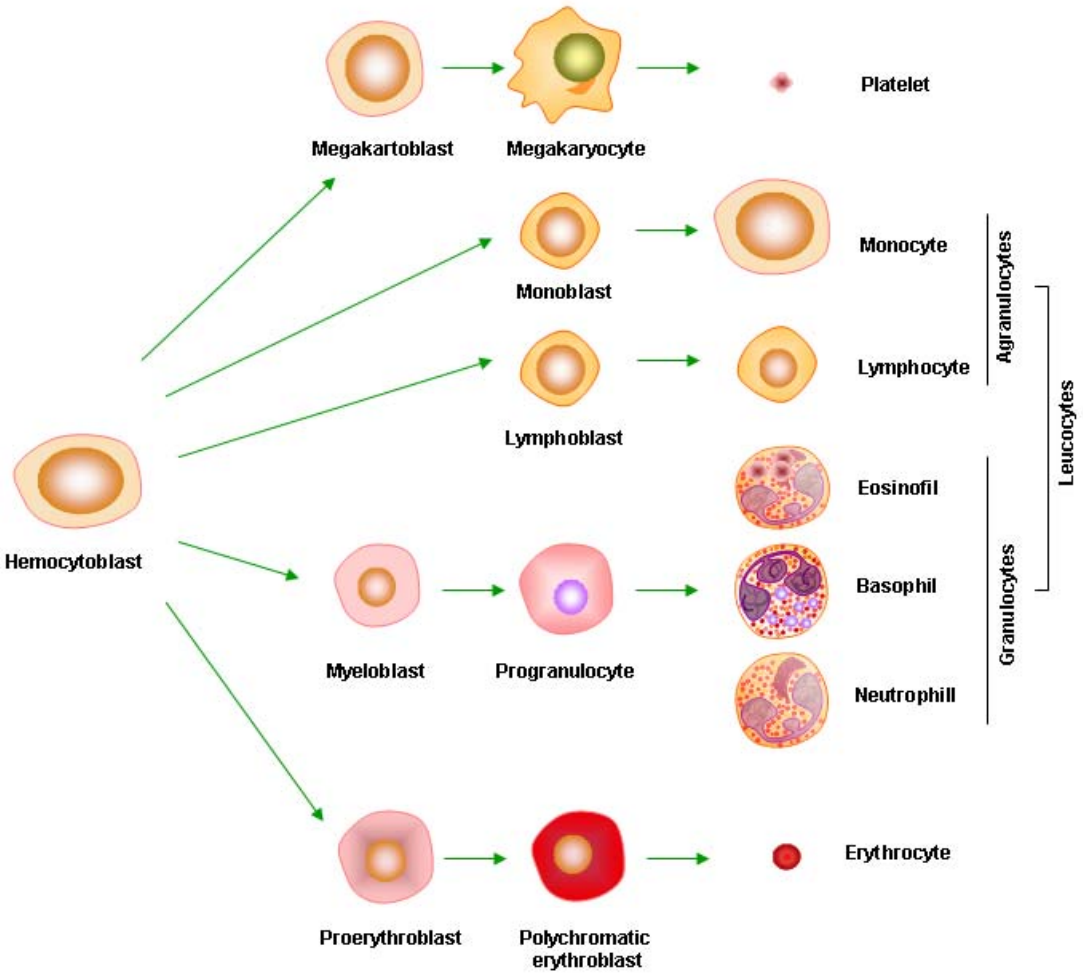

Figure 3. Blood cells developing from the hemocytoblasts, which contain DNA and may contribute to the gene expression profile of metabolic syndrome patients.

Cardiomyocyte measurements

Cardiomyocytes were isolated from the LV in a standard Langendorff retrograde perfusion system to study their size, contractility, and Ca^{2+} handling (Paper I). The protocol has previously been described in detail by Wisløff *et al*[120]. From healthy LV, this protocol recovers about 10 % of the total number of cardiomyocytes, and about 75 % are rod-shaped. Only viable, rod-shaped cells without obvious damage were selected for measurements. The advantages of using isolated cardiomyocytes as compared to the whole heart is that the cells are fully differentiated and morphologically similar to cells in the intact heart. Using isolated cells also allows for several parallel measurements

from the same animal, which is more effective and reduce the number of animals needed to be sacrificed. There are also some disadvantages with using isolated cells instead of the whole heart. The myocytes are separated from contact with other cells and are quiescent due to separation from pacemaker tissue. The myocytes could also have been modified during the cell isolation procedure, which may affect the reliability. However, to reduce this problem, myocytes from both untrained and trained rats were isolated on the same day.

RNA isolation

Messenger RNA (mRNA) is a copy of DNA and a recipe used to make different proteins. At a given time point, the mRNA level encoding different proteins reflects the cellular responses to different stimuli. In this thesis, RNA was isolated from LV (Paper I), soleus muscle (Paper II) and from whole blood (Paper III). Tissue-specific protocols for RNA isolation and RNA clean up were used for the different tissue types, according to the nature of the tissue, as viscosity and fibrousity. To ensure high mRNA quality, integrity, purity and quantity all samples were run on Bioanalyzer and Nanodrop. The Bioanalyzer system is a fully automated gel electrophoresis, analyzing the separation of bands in the sample to assess RNA quality. The Nanodrop instrument applies ultraviolet spectrophotometry at 260/280 nm to assess the concentration and quality of total RNA in the sample. Only samples with a 260/280 ratio between 1.8-2.2 and no signs of degradation were used for analysis. cDNA was later synthesized from RNA samples that fulfilled the quality criteria. cDNA was further processed to fragmented biotin-labelled cRNA and hybridized to microarrays.

Microarray gene expression analysis

The sequencing of the entire human- and rat genome has opened a new era in biomedical research[118, 123, 124]. Improvements in microarray technology have had a significant impact on large-scale studies of gene expression, and today over 30.000 transcripts can be measured on one single chip. The challenge is now to identify the biological functions of each gene, the diseases in which they are involved, and possible therapeutic targets of disease. A microarray experiment uses representative probes

corresponding to known genes, to which tissue mRNA is hybridized and quantified based on hybridization intensity. The experimental rationale is usually straightforward: hybridization data from two or more groups of samples are compared to seek evidence of differentially expressed genes. Despite the great promises of microarrays in health care, and their successes in both medical and biological research, the technology is still far away from daily use in the clinic. The reason for this is e.g. the costs of the microarrays and the costs to obtain a clean RNA sample, problems in getting tissue with satisfactory quality and quantity from hospitals after surgery, and the high variability in repeated studies. In the near future, ultrahigh-throughput sequencing technology will change the way gene expression is studied. Rather than relying on hybridization intensity from microarrays, the number of times transcripts are called in sequencing gives direct quantification of expression levels. For the time being, this method is expensive and time-consuming; therefore, microarray analysis is likely to remain the gold standard of whole-genome gene expression studies for some years to come.

There exits several systems of microarray analysis, and in this thesis we have used the Affymetrix RAE 2.0 microarray chips (Paper I and II), and the Applied Biosystems Human Genome Survey Microarray v.2.0 chips (Paper III). These are both 1-channel systems with whole-genome coverage. When comparing the ten most common microarray platforms, the Applied Biosystems platform was ranked as number one, and the Affymetrix was ranked as number two when the microarray results were compared to single mRNA measurements for 160 chosen genes[125].

Affymetrix

The Affymetrix chips consist of 31,042 probe sets that cover the entire rat genome. Each of the probe sets are represented by 11-20 probe pairs consisting of a *perfect match* and a *mismatch probe*. The expression value of each probeset is computed from background-corrected, quantile normalized and log-transformed *perfect match* values for each probe pair by use of the "*robust multi-array analysis*"[126]. To tests for significant differential expression between groups, a moderated T-tests is applied[127]. Due to the magnitude of probe sets being compared, we account for multiple testing by

the Benjamini-Hochberg step-up procedure which creates adjusted *P*-values[128]. When selecting genes with a *P*-value below 0.05, the expected proportion of genes falsely classified as differential expressed should be below 5%. No threshold for fold-change was used, based on our assumptions that a gene 25% upregulated, might be just as important as a gene found 100% upregulated. Differentially expressed genes were classified to gene networks, biological processes, molecular functions and cellular locations with the Ingenuity Pathway Analysis Application Tool and the *e*GOn web tool[129]. This approach provides a general impression of which processes separate the cases from the controls, which usually yields more information than studying single genes.

Applied Biosystems

The Applied Biosystem Human Genome Survey microarray v.2.0 contains 32,878 probes for the interrogation of 29,098 genes. The expression values are computed from filtered, quantile normalized and log-transformed signal intensities. Weak spots and outliers were filtered out, and missing values were replaced by imputation using Adaptive LSimpute[130]. Probes were collapsed to genes, using Primary Gene ID from the Applied Biosystems Human Annotation File. All genes were ranked from the most to the least significant, using the paired "*significance analysis of microarrays*" statistical test[131]. Instead of looking for differential expression of individual genes, which has been the traditional way of doing it, we focused on changes in biological processes and molecular function. We therefore used the entire ranked list in the gene set enrichment analysis (GSEA)[132] rather than selecting for instance the top 200 genes or genes with a *P*-value below a certain cut-off. This is preferred because it is difficult to decide where to set the cut-off. If the genes only change moderately it may be difficult to find significant changes by looking at each gene separately. If, on the other hand, many genes belonging to the same gene set are changed moderately this could be an interesting finding, and the defined relationship between these genes gives more statistical power to detect such small changes compared to single gene statistics. GSEA works by starting to look at the gene ranked on top of the gene list. If this gene is a member of a certain gene set, a positive score is added to an enrichment score (ES),

otherwise a negative score is added. Then the next gene on the gene list is evaluated and the ES is updated. This process is repeated for every gene in the entire gene list. The maximum value obtained during this "walk" is used as ES for the gene set. Moreover, the positive score that is added to the ES is weighted, that is, a higher score is added when a gene higher on the ranked list is found to be a member of the gene set than when a gene lower on the ranked list is marked with a hit. Therefore, a high ES means that the gene set is over-represented towards the top of the ranked list. The null hypothesis is that the genes belonging to certain gene sets are evenly spread throughout the ranked gene list. Significance of the GSEA was tested by permuting gene labels (1000 iterations). Gene sets smaller than five were excluded from the analysis.

According to the Minimum Information About Microarray Experiment (MIAME) recommendations, all the microarray data were published in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/).

Protein analysis

In the papers of this thesis, we chose to validate the microarray results on protein levels rather than on gene level. By this approach, pathogenic mechanisms of disease, which involves protein modifications, are accounted for. Since we are interested in functional changes by alterations in gene expression, protein measurements may yield more insight. In this thesis, the level of mRNA correlated, in most cases, well with the quantity of proteins synthesised. We used three different approaches to obtain quantitative or semi-quantitative protein expression levels; immunohistochemistry, Western immunoblotting, and Enzyme-Linked ImmunoSorbent Assay (ELISA).

Immunohistochemistry

Immunohistochemistry refers to the process of localizing proteins in cells of a tissue section, exploiting the principle of antibodies binding specifically to antigens in biological tissues. The proteins are localized with specific antibodies that are visualized by different detection systems. The detection systems are constantly improving, and

today, they allow for protein detection several months/years after the process was conducted. Immunohistochemistry allows for semi-quantitative detection of protein levels in tissue. Disadvantages with immunohistochemistry involve possibilities of background staining and masked epitopes by the fixation procedure.

Western immunoblotting

Western immunoblotting allows for semi-quantitative detection of a specific protein in a given sample of tissue homogenate. Denatured proteins are separated by the length of the polypeptide and electrophoresed prior to the protein detection. Proteins are then transferred to a nitrocellulose membrane, where they are detected using specific antibodies and chemiluminecence. Due to many steps in this protocol, optimizing of all the different steps is needed to obtain good reliable results. Disadvantages of this method include the fact that immunoblotting is time consuming and mainly a qualitative assay.

ELISA

The ELISA technique has a high sensitivity and can detect the amount of a given protein, antibody, or antigen in a sample. In addition to being used as a diagnostic tool in medicine and research, ELISA is also being used in plant pathology, as well as a quality control check in various industries. In simple terms, an antibody for a specific protein is fixed to the surface in a well. The specific protein binds to the antibody in the well, where it is attached. Then a second antibody is added, which binds to another site on the protein. This antibody is linked to an enzyme, which is converted to some detectable signal as the final solution is added. Today, most of the commercial ELISAs are based on fluorescence, due to the high sensitivity of the fluorogenic substrates. ELISA allow for quantitative detection of protein level in blood. Disadvantages with ELISA is the limited numbers of commercial available assays, and high purchasing costs.

Statistics

Microarray statistics constitute its own discipline in the field of statistics. It is complex and under constant development. Due to the vast amount of data created in each single microarray run, statistical tests that accounts for multiple testing is needed. Regarding the other results presented in this thesis, non-parametric procedures are the most appropriate, since each study operates with a limited number of animals and patiens per group.

Summary of the results

Paper I: Before the exercise intervention, the sedentary LCR and HCR rats differed significantly in VO_{2max} (30% higher in HCR), cardiomyocyte contractility and Ca^{2+} handling. Exercise improved all these three parameters in both HCR and LCR. In sedentary untrained rats, gene expression analysis of the LV from HCR and LCR revealed 1540 differentially expressed transcripts. Enlarged cardiomyocytes (33% wider) and upregulation of embryonic growth factors indicated ongoing pathological growth in sedentary LCR. In addition, LCR expressed high levels of genes associated with cellular stress, and low levels of contractility regulating genes and cholesterole lowering agents. The sedentary LCR also expressed higher amounts of genes involved in glucose metabolism, and less of the genes involved in lipid metabolism, as compared to the sedentary HCR. Hypoxic conditions seemed to be a common source for several of these observations, indicated by the switch in energy substrate metabolism, upregulation of hypoxia-induced growth factors, and enhanced expression of genes associated with DNA damage. No exercise-induced changes in LV gene expression were detected in either of the groups.

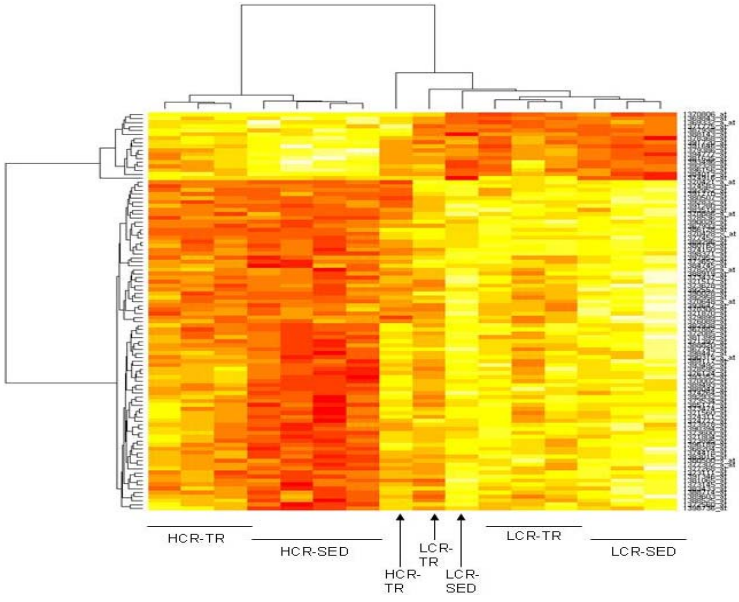

Figure 3. Heat map of the 100 most differentially expressed transcripts in Paper I. HCR-TR: Exercise-trained high capacity runners, HCR-SED: Sedentary high capacity runners, LCR-TR: Exercise-trained low capacity runners, LCR-SED: Sedentary low capacity runners.

Paper II: Before the exercise intervention, the sedentary LCR and HCR rats differed significantly in running speed at VO_{2max} (120% higher in HCR). In both HCR and LCR, 8 weeks of exercise training induced almost equal improvements in running speed, as compared to the sedentary counterparts. Fibre typing of the soleus muscle revealed a trend (P=0.07) towards less fast fibres after exercise in the HCR group. In sedentary untrained rats, gene expression analysis of the soleus muscle from HCR and LCR revealed only three differentially expressed transcripts. LCR expressed high levels of a transcript with strong homology to human leucyl-transferRNA synthetase 2 (*LARS2*). Upregulation of this gene in humans has previously been associated with a mitochondrial mutation linked to maternally inherited diabetes and mitochondrial dysfunction. The response to exercise seemed more pronounced in HCR than LCR, in terms of gene expression. A transcript similar to the cytochrome c oxidase VIIa was upregulated after exercise in both groups. In HCR, the adaptation to exercise affected genes involved in FA metabolism, FA elongation in the mitochondria, in addition to genes located in the peroxisomes. Endurance training also seemed to involve different structural adaptations and differences in improvements of fibrinolytic potential in skeletal muscle dependent on the inborn VO_{2max}.

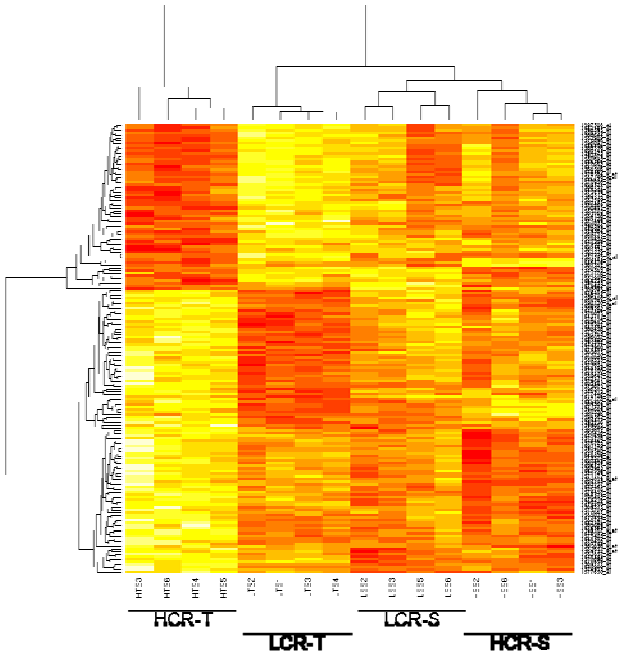

Figure 4. Heat map of the 150 most differentially expressed transcripts in Paper II. HCR-T: Exercise-trained high capacity runners, LCR-T: Exercise-trained low capacity runners, LCR-S: Sedentary low capacity runners, HCR-S: Sedentary high capacity runners.

Paper III: Eleven metabolic syndrome patients performing 16 weeks of aerobic interval training, significantly reduced their risk of CVD, in terms of improved VO_{2max} (+35%), endothelial function (+10%), mean arterial blood pressure (-5%), insulin sensitivity, fasting glucose and plasma lipid composition. Additionally, after the training period, 47% of the patients were no longer classified as having the metabolic syndrome. Gene expression analysis of blood cells from these patients revealed 18 biological processes and molecular functions altered by interval training. Eleven processes and functions were upregulated after exercise, including e.g. steroid hormone-mediated signaling. Seven processes and functions were downregulated after exercise, which included e.g. blood clotting, cell adhesion and steroid metabolism. Downregulation of arginase 1 and von Willebrand factor (VWF) was confirmed at protein level.

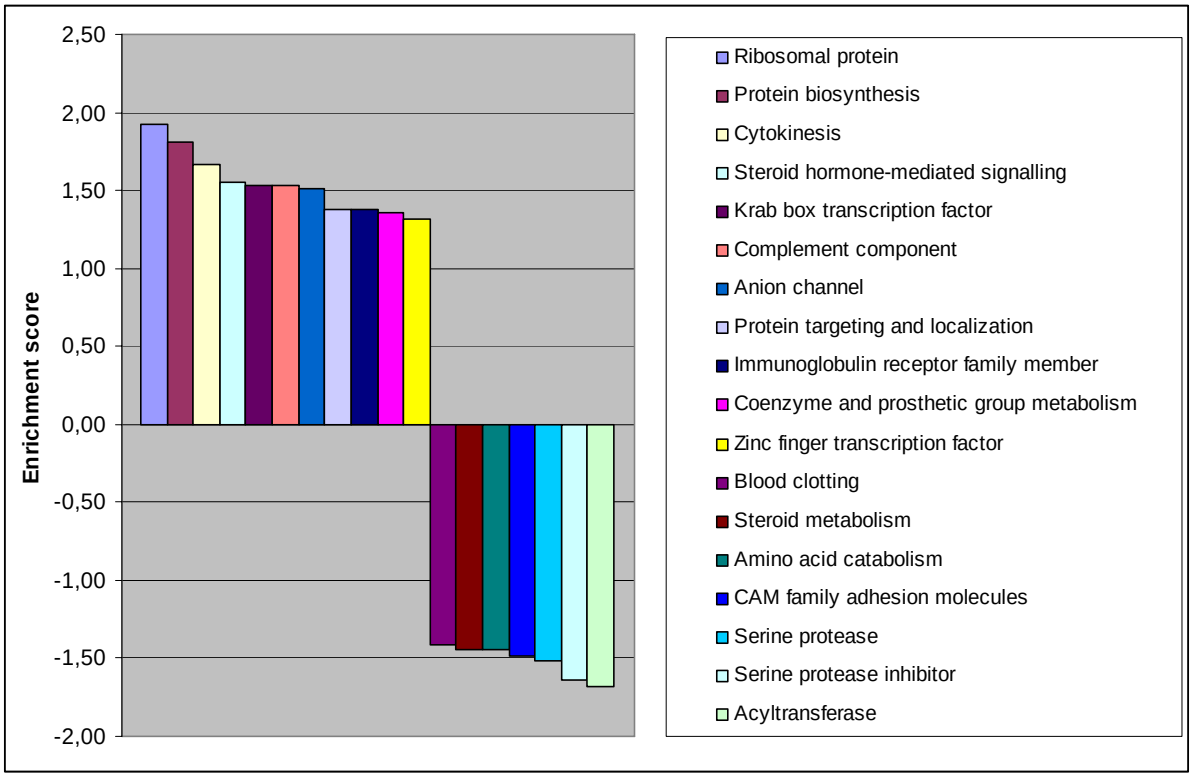

Figure 5. Biological processes and molecular functions significantly altered by exercise in metabolic syndrome patients. CAM: Cell adhesion molecule.

Discussion

Inborn low VO_{2max} was associated with a gene expression profile indicating LV pathological hypertrophy, contractile dysfunction, cardiac stress, abnormal cardiac metabolism, cardiac inflammation, and skeletal muscle mitochondrial dysfunction. The LCR gene expression profile resembled a compensatory mechanism for an inefficient heart. These features are likely to contribute to the low inborn VO_{2max} and accumulation of risk factors related to CVD.

The gene expression profile of exercise-adaptation in skeletal muscle was more pronounced in individuals with inborn high VO_{2max}, as compared to the individuals with low VO_{2max}. In the heart, no exercise-induced changes in gene expression were found at the time of sample collection. In patients with low VO_{2max} and the metabolic syndrome, the blood gene expression profile indicated an exercise-induced downregulation of genes associated with endothelial dysfunction, blood clotting, and atherosclerosis.

The gene expression profile of inherited high- and low VO_{2max}

Recently, the HERITAGE Family Study investigated the role of genes in determining inborn VO_{2max}[133]. They found familial resemblance for VO_{2max}, and suggested that both genetic and environmental factors were involved. The heritability was estimated to account for 47% of the VO_{2max}. Interestingly, a significant maternal effect was observed, potentially associated in part with mitochondrial inheritance. These results suggest that genetic and non-genetic factors, as well as maternal influences contribute to the familial aggregation of VO_{2max} in sedentary individuals.

Pathological hypertrophy

When studying the cardiac phenotype and gene expression pattern of sedentary LCR rats, we found several signs of pathological growth. LCR cardiomyocytes were 33% wider than HCR myocytes, which indicates hypertension-induced hypertrophy, in line with a higher blood pressure in LCR[134]. Upregulation of genes associated with embryogenesis further supported ongoing pathological growth in LCR LV, as

embryonic growth factors are common features in pathological cardiac hypertrophy[135, 136]. These results imply that a genetic predisposition is sufficient to induce pathological cardiac growth in relatively young subjects (7 months old).

A common feature in cardiac growth is angiogenesis. During the acute phase of cardiac growth, angiogenesis is normally enhanced, but as the heart enters the chronic phase of pathological remodelling, angiogenesis is usually impaired. This disruption of coordinated growth and angiogenesis is a contributing factor to the transition to HF[137]. Interestingly, genes promoting angiogenesis were less expressed in LCR compared to HCR, which may lead to reduced O_2 and substrate delivery to cardiomyocytes, and contractile dysfunction. This suggests that the pathological growth in LCR might be past the acute phase and is progressing towards HF. The previously reported systolic and diastolic dysfunction in LCR also suggests a possible transition towards HF in these animals[107].

Contractility regulating genes

Sustained pathological hypertrophy may lead to systolic and diastolic dysfunction, in line with the previously reported impaired contractility and Ca^{2+} handling in LCR cardiomyocytes[22, 107]. Ca^{2+} characteristics similar to those reported in LCR, are also found in diabetic rats, and in rats with HF[62, 64, 138]. Two genes that regulate heart rate and contractility were among the most differentially expressed genes in the LV of HCR and LCR. Both CD38 and the inward rectifying potassium (K^+) channel (subfamily J, member number 3) were less expressed in LCR compared to HCR. Less inward K^+ channels in LCR will potentially lower the myocyte membrane potential, hence making the myocytes from LCR more susceptible to delayed after-depolarization and ventricular tachyarrhythmia, which was recently reported in these rats[110]. Reduced density of inward rectifying K^+ channels and a lower resting membrane potential have been reported in HF[139]. CD38 is responsible for most of the synthesis of cyclic-ADP-ribose in the myocardium, which in turn controls the Ca^{2+} homeostasis in cardiac myocytes. Cyclic-ADP-ribose enhances the sensitivity of ryanodine reseptors governing the Ca^2-induced Ca^2 release from the SR, and thus the contraction[140]. This coincides

with the previously reported lower Ca^{2+} transient and contractility in LCR myocytes[22, 107]. Genes causing depressed contractility may influence SV and contribute to a reduced VO_{2max} in LCR.

Cardiac metabolism

The microarray data suggested that long-term selection for VO_{2max} resulted in different cardiac energy metabolism. LCR expressed low amounts of genes involved in lipid metabolism, and high amounts of genes involved in glucose metabolism, suggesting that LCR hearts rely more on glucose as an energy substrate, than lipids.

Differences in cardiac energy metabolism in HCR and LCR may be explained by the different compositions of energy substrates delivered to the heart, as LCR have elevated plasma levels of glucose, triglycerides, and FFAs as compared to HCR[22]. This means that LCR and HCR hearts have access to different pools of potential energy substrates, which may result in adaptation of enzymes metabolizing these different substrates. As previously mentioned, the heart metabolizes whatever substrate offered by the circulation at a certain time point. The different cardiac energy metabolism might also be a result of an underlying pathology within the myocardium. For instance, both humans and rats with hypertension-induced cardiac hypertrophy have decreased myocardial uptake, utilization, and oxidation of FAs[141, 142]. In fact, changes in cardiac energy substrate utilisation, from normal mitochondrial FAO to glucose oxidation are commonly seen in diseased hearts[76]. The changes are then often triggered by a downregulation of enzymes involved in FAO, as reported in LCR. Since the amount of ATP produced per O_2 consumed is higher in glucose oxidation than FAO, such adaptations will initially relieve the energy deficiency. However, as the condition progresses towards an uncompensated state, the capacity of utilizing glucose decreases[77].

One of the genes potentially involved in decreased FAO in LCR is carnitine palmitoyltransferase 1α (*Cpt1α*). The long-chain FA transporter *Cpt1α* was 29 % more expressed in HCR compared to LCR, and governs the most important and rate limiting

step in mitochondrial FAO[143]. As insulin is a potent inhibitor of *Cpt1α* transcription, the previously reported hyperinsulinemia in LCR may explain the downregulation of *Cpt1α*[143, 144]. A clear correlation between *Cpt1* mRNA concentrations and measured CPTI activity has previously been reported; hence, LCR are likely to have a lower CPT1α activity than HCR[144]. Since, CPT1 is crucial in regulating myocardial FAO[145], insulin-inhibition of this enzyme in LCR represents a potential reason for impaired removal of circulating FAs, less FAO and hence, cardiac energy deficiency. A potential course of events in the heart of LCR is illustrated in Figure 6.

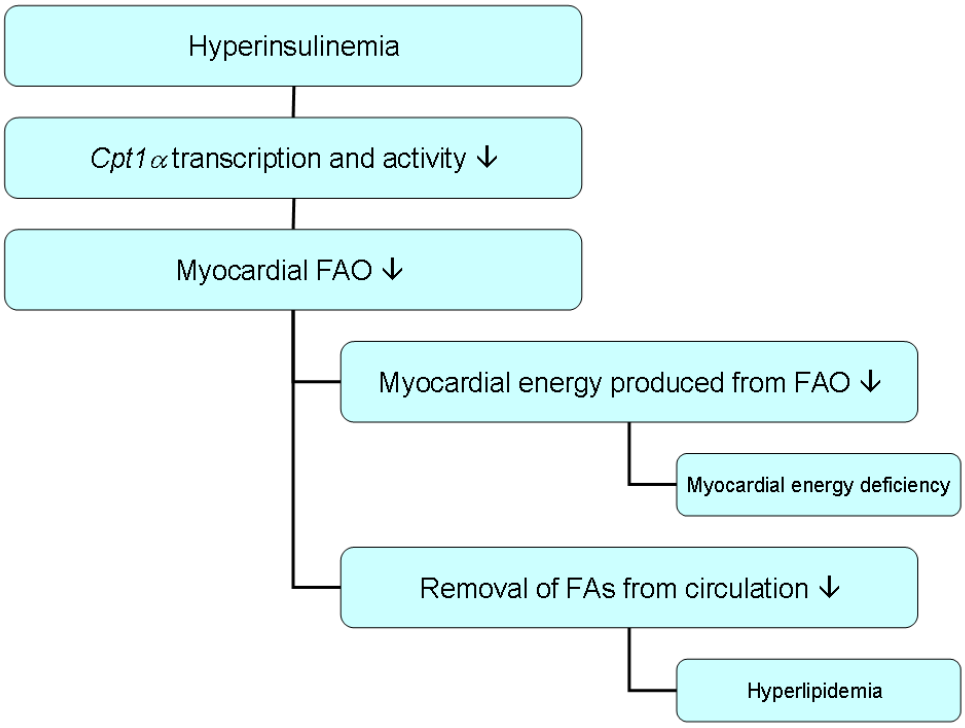

Figure 6. A potential course of events in the heart of LCR. *Cpt1α:* Carnitine palmitoyltransferase 1α, FAO: Fatty acid oxidation.

A potential compensation for impaired FAO in LCR is the upregulation of ATP synthase (mitochondrial F1 complex). ATP synthase (mitochondrial F1 complex) is an important component of Complex V and a rate-limiting step in the electron transport chain (Figure 7). This situation is often seen in HF, and might be a compensatory mechanism to meet increasing energy demands[53].

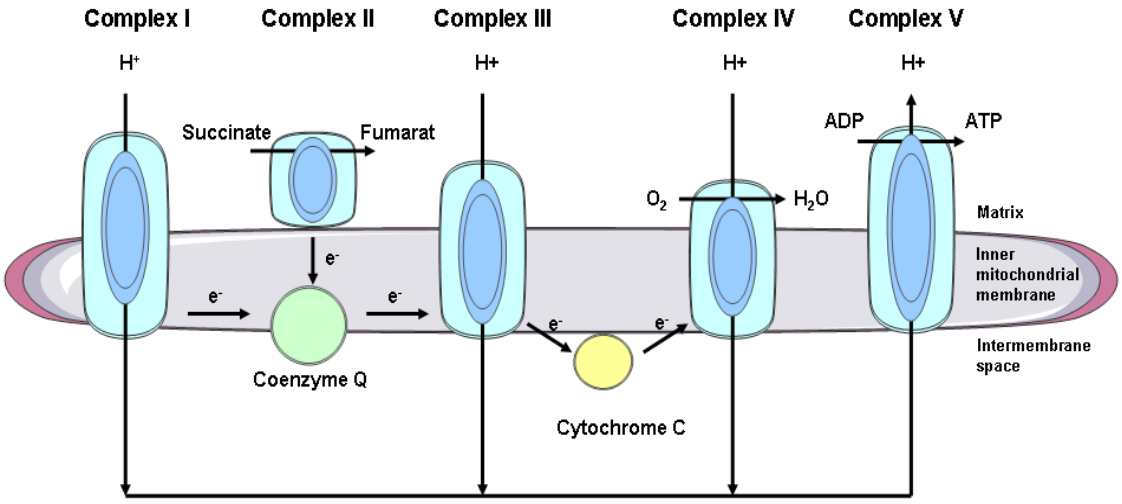

Figure 7. Schematic illustration of the electron transport chain. H^+: Hydrogen ion, e^-: Electron, O_2: Oxygen, H_2O: Water, ADP: Adenosine diphosphate, ATP: Adenosine triphosphate.

In contrast to decreased expression of genes involved in FAO, we found a evident upregulation of several genes regulating glucose metabolism in the LV of LCR rats. Increased glucose metabolism was indicated by the high expression of three important glycolytic enzymes, hexokinase 2, 6-phosphofructo-2-kinase, and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), catalyzing reaction one, two, and six in the glycolysis respectively. Reaction number two, irreversibly catalyzed by 6-phosphofructo-2-kinase, is subject to extensive regulation, because the original substrate is forced to proceed down the glycolytic pathway after this step. This leads to a precise control of glucose, galactose and fructose going down the glycolytic pathway. Before this reaction, glucose-6-phosphate can potentially travel down the pentose phosphate pathway, or be converted to glucose-1-phosphate and polymerized into the storage form glycogen. Moreover, LCR expressed high amounts of the transcription factor kruppel-like factor 15 (*Klf15*), which regulate the expression of genes involved in glucose uptake, such as glucose transporter 4 (*Glut4*)[146]. In addition to the previously possible explanations for metabolic differences between HCR and LCR, increased expression of glycolytic enzymes may also be a compensatory mechanism of insulin resistance.

Hypoxia-induced transcription

Several of the genes upregulated in the LV of LCR indicated that hypoxia-induced transcription was responsible for some of the pathological features. Upregulation of genes involved in glucose metabolism (hexokinase 2, 6-phosphofructo-2-kinase, *Gapdh*), glucose transport (*Klf15*), growth (fibroblast growth factor 1, platelet-derived growth factor 1 α), cellular stress (DNA damage inducible transcript 4), as well as downregulation of genes involved in FAO, are triggered by the hypoxia-inducible transcription factor 1α (HIF1α) (Figure 8)[147-150]. Hypoxia-induced transcription occurs when low levels of O_2 are detected in the tissue, and involves upregulation of genes responsible for increase in O_2 delivery and survival during hypoxia. HIF1α is actually one of the few transcription factors promoting upregulation of the glycolytic pathway[147]. The reduced cardiac contractility, as well as the previously reported impaired O_2 supply, extraction ratio, and tissue diffusion capacity in LCR, support our findings of hypoxia-induced transcription[106, 113].

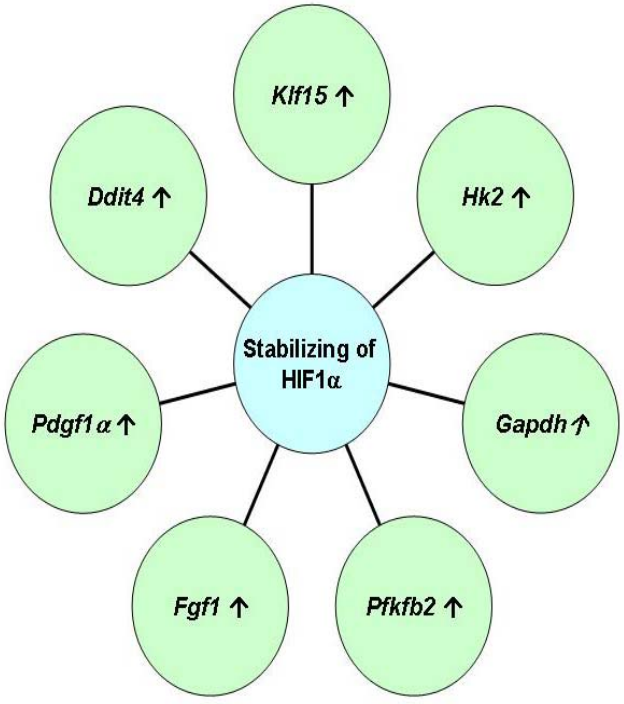

Figure 8. Genes that were among the most upregulated in sedentary LCR compared to sedentary HCR, and that are inducible by hypoxia-induced factor 1 α. HIF1α: Hypoxia-induced factor 1α, *Klf15*: Kruppel-like factor 15, *Hk2*: Hexokinase 2, Gapdh: glyceraldehyde 3-phosphate dehydrogenase, *Pfkfb2*: 6-phosphofructo-2-kinase, *Fgf1*: Fibroblast growth factor 1, *Pdgf1α*: Platelet-derived growth factor 1α, *Ddit4*: DNA damage inducible

Skeletal muscle metabolism

Based on the number of genes being differentially expressed, differences related to inborn levels of VO_{2max} seemed to be much more pronounced in the heart, than in skeletal muscle. Interestingly, Gonzalez *et al* reported that skeletal muscles were the main reason for the higher VO_{2max} in HCR compared to LCR at generation 7[90]. Based on our findings, it seems like the heart also contributes significantly to the difference in VO_{2max} when the artificial selection has reached generation 16. This is also in line with recent findings by Gonzalez *et al* based on rats from generation 15, that continuing divergence in VO_{2max} between HCR and LCR occurs largely as a consequence of changes in the capacity to deliver O_2 to the exercising muscle[106].

In the soleus muscle of LCR, proteins required for mitochondrial biogenesis and function has previously been reported to be downregulated[22]. At the gene level, however, only three transcripts were found differentially expressed in the soleus muscle of HCR and LCR. This suggests that post-transcriptional processes, including translation regulation is different in LCR and HCR. One of the transcripts that were significantly more expressed in LCR than HCR, had a strong homology with the mitochondrial *LARS2* seen in humans. Upregulation of the human homolog is regarded as a hallmark of a mitochondrial DNA A-to-G point mutation in the leucyl-transferRNA $(tRNA)^{Leu\ (UUR)}$ gene at base pair 3243[151]. The mutation generates structural and functional defects of the $tRNA^{Leu\ (UUR)}$, including impaired aminoacylation, reduced half-life, and/or decreased steady-state level[152-154]. Since $tRNA^{Leu\ (UUR)}$ is involved in the construction of proteins, such a mutation will cause translation dysfunction of the UUR leucine codons and lead to a disruption of intra-mitochondrial protein synthesis[152]. This will influence on protein synthesis of the 13 subunits of complexes I, III, IV and V in the electron transport chain, which will decrease respiration and O_2 consumption (Figure 7). This mutation is heteroplasmic (the percentage of mutated DNA vary between tissues), and causes maternally inherited diabetes and mitochondrial dysfunction[155, 156]. Humans suffering from this mutation are diagnosed with the disorder "Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, and Stroke-like episodes" (MELAS). The syndrome is associated with insufficient O_2 extraction from blood, decreased activity of complex I (Figure 7), and reduced mitochondrial ATP production.

These malfunctions leads to hyperglycaemia, muscle weakness, high resting and exercise-induced lactate concentrations, increased fatigability, and exercise intolerance[155, 157, 158]. Several of these symptoms are previously reported in LCR[22, 106-108, 111, 113]. In line with our findings, a growing body of evidence suggests that, compared to HCR, LCR have compromised mitochondrial function[22, 109, 112]. In LCR, this might be a contributing factor to the low inborn VO_{2max} and the metabolic syndrome. Interestingly, only a short period of exercise training remarkably increases the ratio of wild type–to–mutant DNA and the proportion of muscle fibres with normal respiratory chain activity[159]. For this reason, endurance training might be particularly important for LCR.

Because of the strong link between low VO_{2max} and the metabolic syndrome, the $tRNA^{Leu\ (UUR)}$ mutation should be further studied as a possible CVD risk factor. Subjects diagnosed with MELAS are associated with increased CVD risk; however, as the ratio of wild-type-to-mutant DNA varies, asymptomatic subjects might also carry the mutation. New technology has made it possible to detect this mutation by analysing blood samples, which makes it feasible to study the connection between the $tRNA^{Leu\ (UUR)}$ 3243 A→G mutation and CVD risk in a large population[160].

Blood lipid status

We have previously reported high levels of plasma triglycerides and FFAs in LCR compared to HCR[22]. From our microarray analysis, cholesterol-lowering agents as the VLDL receptor and colony-stimulating factor 1 (*Csf1*) were both less expressed in LCR compared to HCR[76, 161-163]. Administration of CSF1 has been tested as a potential therapy for hypercholesterolemia, and favourable results have been reported[164]. Low expression of *Csf1* and the VLDL receptor might contribute to the reported accumulation of serum triglycerides and FFAs in LCR.

Another potential reason for high serum levels of glucose and FA in LCR was their low expression of uncoupling protein 4 (*Ucp4*). Previous studies have reported that uncoupling proteins are involved in thermoregulation, metabolism, and obesity[165-168].

Uncoupling proteins create proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis. As a result, energy is dissipated in the form of heat, which diminishes ATP production, forcing the cells to oxidize more nutrients to obtain energy. In LCR, a low expression of *Ucp4* may contribute to less glucose and FFAs disposal from the circulation. Acceleration of mitochondrial respiration, via induced uncoupling protein activity in appropriate tissues, has previously been suggested as a pharmacological target to counteract obesity[169].

The gene expression profile of acquired VO_{2max}

In the soleus muscle, significant transcriptional changes occurred in response to exercise training in both HCR and LCR. However, the changes were much more pronounced in HCR than LCR, indicating a substantial difference in the ability of transcriptional adaptation to exercise. Less genes upregulated by exercise in LCR is either a result of inborn genetic factors or running speed during the exercise bouts (less mechanical stress), since LCR were unable to maintain the same absolute speed as HCR, although working out at the same relative intensity[170].

In the LV, no annotated genes were differentially express after exercise training in either of the groups, despite improved contractility, Ca^{2+} handling, and VO_{2max}. Originally, we expected to find upregulation of structural genes involved in physiological LV hypertrophy, as cardiac growth has been shown to persist beyond eight weeks of training by this type of exercise[36].

In both LV and the soleus muscle, the low number of genes induced by exercise may also be due to the chosen time of sample collection. Since the plateau of VO_{2max} is reached a week or two before sample collection, genes augmenting VO_{2max} might no longer be induced[36].

Since exercise has more pronounced effect on gene expression in animals born fit, it seems likely that some of the genes that determine inborn VO_{2max} also determine the potential of training induced adaptations. This means that the LCR may be equipped

with a set of genes similar to the 10-15 % of humans that have little or no effect of exercise training, in terms of VO_{2max}[171, 172]. Interestingly, we are about to establish new rat lines that are either high or low responders to training. These rats may be helpful to identify whether the "high responder genes" are similar to those observed in HCR.

Structural adaptations

Endurance training seemed to involve different structural adaptations in skeletal muscle dependent on the inborn VO_{2max}. After endurance training, LCR expressed significantly more of the negative regulator of growth "A Disintegrin and Metalloproteinase with ThromboSpondin motifs 1 (*Adamts1*). Upregulation of *Adamts1* is associated with muscle weakness, muscle wasting, and various inflammatory processes[173]. Hence, upregulation of *Adamts1* in the soleus muscle of LCR suggests an ongoing inflammatory process and impaired growth.

Another regulator of growth, *Igf1* was significantly more expressed in the soleus muscle of exercise trained LCR than exercise trained HCR. IGF1 plays a major role in exercise-induced skeletal muscle hypertrophy and strength improvements. IGF1 is highly inducible with exercise, and the level often keeps increasing the two following days after one single exercise bout[174] At first, a higher exercise-induced increase in *Igf1* mRNA in the LCR group compared to the HCR group was not easily explained. However, when performing Western blot, we found twice as much IGF1 in the sedentary HCR compared to the sedentary LCR. That is, the LCR had a considerably lower basis of IGF1 before the exercise intervention. Reduced levels of IGF1 have previously been reported in HF[175, 176]. Skeletal muscle IGF1 levels correlate with muscle cross-sectional area, and low levels of IGF1 may contribute to the development of muscular dysfunction and atrophy[175]. The low levels of IGF1 in sedentary LCR may originate from a GH deficiency, and may contribute to impaired running speed and VO_{2max}. The potential for exercise-induced increase in IGF1, by means of work-overload and passive stretch, does however seem to be maintained in LCR. The reason why exercise had no impact on the IGF1 levels in the HCR group remains unknown.

Surprisingly, the mRNA level of myosin heavy-chain 4 was 34-times upregulated after endurance training in HCR. Upregulation of this fast-twitch myosin might cause a shift in fibre type towards more fast fibres. However, when performing fibre-typing of formalin-fixed soleus muscles, there were no signs of an increased number of fast fibres in exercise-trained HCR, but rather a trend towards less fast fibres (P=0.07). In line with our results from the fibre typing, stimuli like endurance training most often result in a shift from fast to slow fibres. The reason for exercise-induced upregulation of myosin heavy-chain 4 in HCR remains unknown.

Skeletal muscle metabolism

Several genes associated with metabolism were upregulated in the skeletal muscle of HCR after exercise training, indicating a normal adaptation to increased workload. The tendency of less fast fibres after the exercise intervention in these animals also points towards a more pronounced effect of the exercise program and a greater potential of improving O_2 consumtion in the skeletal muscle, as compared to LCR. As the VO_{2max} of LCR is limited both by the heart (reduced SV) and by O_2 extraction in skeletal muscle, this may restrict the muscular adaptations to exercise[106, 113]. Even so, in both HCR and LCR, exercise upregulated a transcript similar to the cytochrome *c* oxidase (*Cox*) VIIa (a subunit of Complex IV in the electron transport chain). Increased transcription and translation of different COXs is a common feature of exercise training, and a marker of mitochondrial content and biogenesis[177, 178].

Accumulating evidence indicate that exercise-trained muscles oxidize more FAs, both during and after exercise[179-181]. Consequently, glycogen stores are spared, hypoglycaemia-induced fatigue is delayed, and exercise capacity is increased[180, 181]. A variety of processes, like lipolysis, lipid delivery, lipid transport across membranes, lipid transport within the cell, and FAO, could contribute to the increase in fat disposal[180, 182]. In HCR, we found indications of increased FA metabolism after exercise, represented by upregulation of genes like carnitine o-octanoyltransferase (*Crot*) and enoyl CoA hydratase (*Auh*). *Crot* is important for the transfer of chain-shortened FAs from the peroxisomes to the mitochondria, making more FAs available

for mitochondrial FAO[183]. There is controversy whether CROT contributes to enhanced FAO in skeletal muscle with exercise training. A previous study concluded that CROT was not involved in increased FAO with exercise training[184]. However, they argued that the muscle studied (vastus lateralis), might have been unable to reflect the training adaptations induced by distance running to a similar degree as compared to another muscle group such as the gastrocnemius. Furthermore, the increased expression of the FAO enzyme *Auh* indicates increased energy production in the mitochondria. Since mechanisms responsible for enhanced FAO in exercise-trained muscle are not completely elucidated, both *Crot* and *Auh* should be further studied to elucidate their role in skeletal muscle FAO after exercise.

Interestingly, genes regulating FA elongation in mitochondria, and genes belonging to the peroxisomes were significantly upregulated by exercise in the soleus muscle of HCR. Peroxisomes have largely been overlooked with respect to maintaining a healthy cellular lipid environment in the cells. Peroxisomes are ubiquitously expressed and have a wide range of cellular functions, including a primary role in FAO[185]. Since peroxisomes can oxidize all types of FAs, whereas the mitochondria oxidizes only short chain FAs; increased peroxisomal activity might be important for enhanced FAO in exercise-trained muscle.

The only gene downregulated by exercise in HCR was adenylate cyclase 6 (*Ac6*). AC6 is a membrane-associated enzyme that catalyzes the formation of cyclic adenosine monophosphate (cAMP). cAMP promotes intracellular glucose production and inhibits the expression of GLUT4, and thus obstructs glucose transport into the muscle[186]. Downregulation of *Ac6* in exercise-trained HCR and a probable decrease in cAMP may therefore enforce expression of GLUT4, which is a common feature of endurance training and important for maintaining normoglycemia[187]. Mechanisms that regulate the expression of GLUT4 are important targets in the treatment of hyperglycaemic disorders as diabetes; for this reason, *Ac6* should be further studied as a possible trigger of improved health by exercise.

Endothelial function

Endothelial dysfunction is an early pathogenic event of the metabolic syndrome that often appears decades before the onset of vascular disease[98]. It is characterized by reduced bioavailability of NO that arises from decreased production or increased degradation of NO, or both. Regular endurance training, however, has the potential of effectively restoring endothelial dysfunction and NO bioavailability in metabolic syndrome patients[11, 188]. For the exercise trained metabolic syndrome patients (Paper III), improved endothelial function was accompanied by decreased expression of arginase 1, increased expression of genes associated with steroid hormone signalling, as well as decreased transcription of cell adhesion molecules (CAMs). All these changes are potential contributors to the improvements seen in endothelial function. Blood levels of arginase 1 were significantly decreased by the exercise intervention, both at gene and protein level. Arginase is present in endothelial cells, erythrocytes, lymphocytes and neutrophils, and catalyzes the conversion of $_{L}$- arginine to $_{L}$-ornithine and urea. A high level of arginase decreases the availability of $_{L}$-arginine for NO synthesis, and has previously been associated with endothelial dysfunction in aging[189, 190]. Decreased levels of arginase 1 after exercise may lead to increased levels of $_{L}$-arginine, and thereby increased NO bioavailability, as in line with the previously reported trends (P=0.07) towards increased levels of NO in blood after endurance training in these patients[11, 191]. Downregulation of arginase 1 may therefore contribute to improved endothelial function and CVD risk profile. As NO contributes considerably to exercise-induced increase in limb blood flow, increased levels of NO may also be involved in increased VO_{2max} in these patients[192].

Another common feature in endothelial dysfunction is inflammation. Activated endothelial cells increase their expression of CAMs, which further enhances the inflammation response[193]. Carcinoembryonic antigen-related CAM 5 and carcinoembryonic antigen-related CAM 8 (CD66B) were both downregulated in the blood of metabolic syndrome patients after exercise. High levels of CD66B has previously been associated with leukocyte activation, atherosclerosis and type 2-diabetes[194, 195]. Exercise-induced decrease in *CD66B* transcription suggests improved vascular conditions and a reduction in factors contributing to endothelial inflammation.

Further studies should be carried out to determine the clinical potential of CAMs as potential sensitive and early markers of endothelial dysfunction.

Another potential candidate for improved endothelial function by exercise, is the estrogen receptor β (*ERβ*). It is well known, that middle-aged women are much less likely than men to develop CVD, and that the difference is mainly estrogen mediated. Although the atheroprotective effects of estrogen are well recognized, the underlying mechanisms responsible are still not well understood. Interestingly, we found increase expression of the *ERβ* after exercise in metabolic syndrome patients. *ERβ* is expressed in the vasculature of both men and woman, and mediates nearly all of the known biological effects of estrogen[196]. Recently, increase estrogen receptor-signalling has been suggested to counteract CVD through beneficial effects on blood pressure, endothelial function, plasma lipids composition, antioxidant system, coagulation system, and carbohydrate and lipid metabolism[197-201]. Endothelial effects of estrogen receptor-signalling involves increased NO production in the vasculature, through increased expression and activation of endothelial NO synthase. This is in line with the earlier discussed trends (P=0.07) towards increased level of NO in blood after endurance training[11]. Increased expression of *ERβ* therefore potentially contributes to the improved CVD risk profile seen after endurance training.

Blood clotting

Each of the risk factors that constitute the metabolic syndrome appear to uniquely promote atherosclerosis; yet, the mechanism is not fully understood[202]. Increased levels of pro-thrombotic factors is not included in the diagnostic criteria, however, metabolic syndrome patients often suffer from both an impaired coagulation system and platelet function[13]. Regular physical exercise has proved effective in restoring the haemostatic imbalance in individuals with CVD risk factors[102, 103]. This is in line with our findings, as genes controlling blood clotting, like coagulation factor XIII, thrombin, *VWF*, integrin 3β and gamma-glutamyl carboxylase were less expressed in metabolic syndrome patients after long-term endurance training.

VWF is an important biomarker of endothelial damage and dysfunction, and has strong correlation to diabetes and CVD[203-206]. In metabolic syndrome patients, endurance training reduced both mRNA and protein level of VWF in plasma. As estrogen is a potential inhibitor of coagulation factor transcription, the increased expression of *ERβ* might be involved in reduction of VWF and other coagulation factors after exercise[207]. Decreased transcription of pro-thrombotic factors is likely to contribute to the improved vascular function and CVD risk profile observed in metabolic syndrome patient after 16 weeks of exercise.

Increased fibrinolytic potential is a well-known beneficial effect of long-term endurance training, as large amounts of clot-destroying fibrinolytic proteins are produced by the exercise-trained muscles[14, 102]. When comparing soleus muscle gene expression after exercise in HCR and LCR, HCR expressed less fibrinogen-like 2, a recently discovered pro-thrombinase[208]. The superior fitness in exercise-trained HCR may contribute to a superior anti-thrombotic status, as compared to LCR.

Study limitations

We were unable to fully determine whether the differential gene expression patterns reported in Paper I and Paper II represent the cause or the consequence of the inborn differences in VO_{2max}. Further follow-up studies with modification of particular genes are needed for this purpose.

In Paper III, the small number of patients makes it difficult to draw definite conclusions; hence, further studies are needed to verify our findings in a larger population. The low number of patients has also made it hard to detect group differences, due to paired-samples statistics.

Further perspectives

While gene expression profiling do not tell the whole story of what might be happening in your sample, metabolic profiling (metabolomics) can give an instant snapshot of the physiology of the cell. Metabolomics is a systematic study of the unique chemical

fingerprints that a specific cellular processes leave behind in the form of small-molecule metabolites[209]. The metabolome represents the collection of all metabolites in a biological organism, which are the end products of the gene expression. To give a more complete picture of the biology of a sample, gene expression profiling and metabolomics should be integrated in future studies.

Since a growing number of genes are associated with risk factors of CVD, as low VO_{2max} and the metabolic syndrome, we are getting closer to finding new possible drug targets of these pathological conditions. Also by studying exercise-induced changes in gene expression in these patients, we can get an indication of which genes that mediates the beneficial effects of exercise. Therefore, screening the entire genome for changes in gene expression may lead to the discovery of new pharmacological drug targets of these complex diseases. Recently, a new promising treatment strategy involving RNA interference (RNAi) has emerged. RNAi is a naturally occurring mechanism that suppresses the expression of a specific gene and provides potential for treatment of the metabolic syndrome, e.g. reducing plasma cholesterol levels[210, 211]. RNAi is induced by small (21–23 nucleotides) homologous RNA molecules, like double-stranded small interfering RNA (siRNA) and single-stranded micro RNA. To further develop this treatment strategy, it is essential to define a set of candidate genes that are involved in the development and/or progression of the metabolic syndrome, as well as determining a safe delivery of the siRNA to the diseased tissue. We hope that some of the genes associated with risk factors of CVD, and improvements with exercise reported in this thesis, may be potential candidates for the management of the metabolic syndrome.

Main Conclusions

A. Rats born with different VO_{2max} show a great difference in LV gene expression. The LV gene expression patterns associated with an inborn low VO_{2max} involved activation of survival mechanisms to meet the body's demands. First, the low VO_{2max} is associated with upregulation of embryonic growth factors and increased cardiomyocyte width, which suggests pressure-induced pathological hypertrophy. Second, the low VO_{2max} is associated with a metabolic switch from oxidation of FAs to glucose, thus improving the energy efficiency of the heart, e.g. in early stages of HF. Hypoxia-induced changes in transcription seem to be a common source for the cardiac adaptations associated with inborn low VO_{2max}. Gene expression analyses of the soleus muscle indicated that inborn low VO_{2max} is linked to a mitochondrial DNA mutation causing impaired translation of mitochondrial genes and metabolic dysfunction. In humans, such a mutation involves impaired O_2 extraction from blood, hyperglycaemia, and exercise intolerance, which is in accordance with the previous reported characteristics of LCR. Because of the strong link between low VO_{2max} and CVD, this DNA mutation should be further studied as a possible risk factor of CVD.

B. The low VO_{2max} and increased cardiovascular risk in LCR is probably a result of both impaired skeletal muscle function and impaired cardiac function. In the LV, genes that contribute to cardiac dysfunction, e.g. the embryonic growth factors, contractility regulating genes, and inflammatory factors may influence on the SV and hence VO_{2max}. In the soleus muscle, the possible DNA mutation causing impaired translation of mitochondrial genes may result in less O_2 consumption and energy production. The same genes that contribute to cardiac dysfunction will make the heart more susceptible to CVD. In addition, the potentially depressed mitochondrial function in skeletal muscles may cause accumulation of energy substrates, creating an unfavourable accumulation of nutrients in blood and inside the cells. Such conditions may contribute to vascular disease and increased CVD risk.

C. Rats born with different VO_{2max} respond differently to the same exercise protocol in terms of soleus muscle gene expression. The rats born with high VO_{2max} upregulated several genes, and seemed to adapt well to exercise training. The rats born with a low VO_{2max} seemed to be less adaptive to exercise training in terms of gene expression. In LV, no exercise-induced changes in gene expression were detected in either of the groups. The time of tissue collection, might be a contributing factor.

D. Endurance training altered blood cell gene expression in subjects diagnosed with the metabolic syndrome. 16-weeks of high intensity interval training was sufficient to increase transcription of genes involved in steroid hormone-mediated signalling, reduce the levels of arginase 1 and vWf, as well as reduce transcription of genes involved in cell adhesion, blood clotting and steroid metabolism.

E. Decreased transcription of arginase 1 and several pro-thrombotic factors might be involved in exercise-induced improvements of endothelial function and cardiovascular risk profile of the metabolic syndrome patients.

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Paper I

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Paper II

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Paper III

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Transcriptional changes in blood after aerobic interval training in patients with the metabolic syndrome

Running title: Exercise and the metabolic syndrome

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Abstract

Background: Regular physical activity has beneficial effects on the metabolic syndrome. Eleven metabolic syndrome patients performing 16-weeks of aerobic interval training, significantly reduced their risk of cardiovascular disease, in terms of improved VO_{2max}, endothelial function, blood pressure, insulin signaling and plasma lipid composition. The knowledge on underlying mechanism of exercise-induced improvements is sparse, and a broad spectrum of methods is needed to gain more insight.

Design: The aim was, for the first time, to determine whether transcriptional changes occurred in blood cells of metabolic syndrome patients after participating in an exercise program.

Methods: Blood were collected on PAXgene and EDTA tubes before and after 16 weeks of exercise. RNA was extracted and run on microarrays.

Results: Eleven biological processes and molecular functions were upregulated after exercise, whereas seven were downregulated. Blood clotting, cell adhesion and steroid metabolism were among the downregulated processes, whereas steroid hormone-mediated signalling was upregulated. Downregulated protein levels of arginase 1 and von Willebrand factor confirmed microarray results.

Conclusions: 16 weeks of exercise induced transcription of genes involved in steroid hormone-mediated signalling, decreased plasma levels of arginase 1, and reduced transcription of genes involved in cell adhesion, and blood clotting in metabolic syndrome patients. These changes are likely to be involved in exercise-induced improvements of endothelial function and the total cardiovascular risk profile. These findings have provided new insights on exercise-induced improvement of cardiovascular health, and may have important implications for exercise training in rehabilitation programs and for future studies.

Abstract: 243 words

Keywords: endothelial function, nitric oxide, cell adhesion molecules, microarray, RNA

Introduction

The metabolic syndrome is defined as a cluster of factors that predispose for future cardiovascular disease (CVD) and includes hypertension, dyslipidemia, impaired glycemic control, and abdominal obesity [1]. Accumulating evidence indicate that regular physical activity has profound beneficial effects on the metabolic syndrome [2-5]. Recently, we demonstrated that aerobic interval training partly or fully reversed most of the factors that constitutes the metabolic syndrome, such as hypertension, insulin sensitivity, impaired glucose tolerance and dyslipidemia [6], in addition to improving endothelial function. In fact, 47% of the patients were no longer classified as having the metabolic syndrome after 16-weeks of exercise. Thus aerobic interval training seems to be an effective treatment strategy in patients with the metabolic syndrome. Despite this, the mechanisms behind exercise-induce improvements are unclear and specter of analytical methods are needed to better understand the beneficial effects of exercise training.

Recently, promising results from gene expression studies, using high quality ribonucleic acid (RNA) isolated from whole blood have revealed unexplored fields of biomarker discovery and gene expression profiling of disease [7-10]. Due to easy accessible and minimally invasive sample collection, gene expression profiling of whole blood might turn out to be a promising tool in molecular diagnostics and clinical medicine. To our knowledge, whole-genome transcriptional changes have not previously been studied in metabolic syndrome patients undergoing a high intensity exercise program. We hypothesized that biological processes significantly altered by the exercise program, would include a set of genes at least partly responsible for the improvement seen in this patient group.

Methods

Patient group

Eleven patients (7 males and 4 females) diagnosed with the metabolic syndrome, according to the WHO-criteria [1] were recruited to aerobic interval training. All subjects provided written, informed consent, and the regional ethics committee of medical research approved the protocol. Exclusions criteria were unstable angina, recent coronary arrest (≤4 weeks), uncompensated heart failure, severe pulmonary disease, uncontrolled hypertension, kidney failure, orthopedic and/or neurologic limitations, cardiomyopathy, pregnancy, drug or alcohol addictions, and participations in parallel studies. Detailed description of the patient population has recently been published [6] and their main characteristics are presented in Table 1. The investigation is conducted according to the principles expressed in the Declaration of Helsinki.

Exercise program

The participants performed aerobic interval training by walking or running "uphill" on a treadmill 3 times per week for 16 weeks. They warmed-up for 10 minutes at 70% of maximal heart frequency (Hf_{max}) before performing 4 intervals of 4 minutes at 90-95% of Hf_{max}, with 3 minutes of active recovery at 70% of Hf_{max} between each interval. In the end, they had a 5-minute cool-down period, giving a total of 40 minutes.

Sample collection

To avoid the acute effect of exercise, as changes in number and phenotype of circulating leucocytes [11], venous blood samples were collected 72 hours after the last exercise session. Blood were collected on PAXgene (Qiagen, Germantown, MD) and EDTA tubes (Vacuette, Kremsmuster, Austria) after 12-hours fast, at the same time of day for all patients, before and after the training period. The EDTA tubes were immediately centrifuged at 3000 rpm for 10 minutes, whereas the PAXgene tubes were handled according to manufacturer's instructions. All samples were stored at -80 ^{o}C until assayed.

RNA isolation

Total RNA was isolated from whole blood using the PAXgene Blood RNA Kit (Qiagen, Germantown, MD), and globin RNA was removed with GLOBINclear (Ambion, Austin, TX) according to the manufacturer's instructions. RNA integrity, purity and quantity were assessed by Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Nanodrop (NanoDrop Technologies, Baltimore, MD). Only samples with a 260/280 ratio between 1.8-2.2 and no signs of degradation were used for analysis.

Microarray analysis

RNA from five patients (3 males and 2 females) satisfied our strict requirement for high RNA quality. Samples from these patients were processed and hybridized to Applied Biosystem Human Genome Survey microarrays v.2.0. Raw-data was filtered and quantile normalized in J-Express Pro v.2.7 [12]. Signal intensities were log transformed and missing values were replaced by imputation using Adaptive LSimpute [13]. Genes with more than 10% missing values were rejected. Finally, probes were collapsed to genes, using Primary Gene ID from the Applied Biosystems Human Annotation File.

Gene set enrichment analysis (GSEA)

Genes were ranked from the most to the least significant, using the paired SAM (significance analysis of microarrays) statistical test, and used as an input to the GSEA[12] [14]. GSEA works by starting at the gene ranked on top of the gene list. If this gene is a member of a certain gene set, a positive score is added to an enrichment score (ES), otherwise a negative score is added. Then the next gene on the gene list is evaluated and the ES is updated. This process is repeated for every gene in the entire gene list. Therefore, a high ES means that the gene set is overrepresented towards the top of the ranked list. Significance of the GSEA was tested by permuting gene labels (1000 iterations). Gene sets smaller than five were excluded from the analysis.

Gene sets were created using the Panther biological processes and Panther molecular functions (http://www.pantherdb.org). This information was extracted from the Applied Biosystem Human Annotation File, dated September 30th 2006.

Database submission

The microarray data was prepared according to "minimum information about microarray experiment" (MIAME) recommendations, and deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE10540.

Enzyme-linked immunosorbent assay (ELISA)

The plasma levels of arginase 1 and von Willebrand factor (vWf) were measured before and after the exercise period by Human Arginase 1 ELISA (Abnova, Taipei City, Taiwan) and Imubind vWf ELISA (American Diagnostica Inc, Stamford, CT) respectively. All samples were analyzed in triplicate according to the manufacturer's instructions.

Statistics

Due to a limited number of biological processes and molecular functions in our GSEA analysis, 151 and 147 respectively, processes and functions with a nominal *P* value below 0.05 were considered significant. To assess differences in plasma protein levels we applied the Wilcoxon Signed-Ranks Test in SPSS 14.0. *P* values below 0.05 were characterized as significant. All variations are shown as standard deviation.

Results

Physiological characteristics of the patients in this study have previously been reported in detail [6]. An outline of these characteristics is summarized in Table 1. Shortly, the exercise-period induced a 35 % increase in aerobic capacity (VO_{2max}), 10 % improvement in endothelial function (in terms of flow-mediated dilatation in the brachial artery), 5 % reduction in mean arterial blood pressure, improved insulin sensitivity, increased levels of HDL, and reduced fasting glucose. Additionally, after the 16-week training period 47% of the patients were no longer classified as having the metabolic syndrome.

Gene expression

Out of 298 biological processes and molecular functions, eleven were upregulated after the exercise period (Table 2), and included processes involved in transcription, translation, and steroid signalling. Seven processes were downregulated after the exercise period (Table 2), and included blood clotting, steroid metabolism, cell adhesion molecules (CAMs) and amino acid catabolism. Decreased mRNA levels of arginase 1 and vWf after exercise contributed to the downregulation of the biological processes termed amino acid catabolism and blood clotting, respectively.

Four biological processes were considered of special interest, regarding the previously reported exercise-induced, cardiovascular risk reduction and improved health status achieved by the metabolic syndrome patients. These processes included blood clotting, steroid hormone-mediated signalling, and amino acid, in addition to CAMs. Genes contributing their high ES are presented in Table 3.

Protein expression

The plasma protein levels of arginase 1 and vWf were measured to validate the microarray results. The protein levels of arginase 1 and vWf, were both found significantly lower after the exercise period ($p<0.05$) (Figure 1a and 1b), in line with the microarray data.

Discussion

The main findings in this present study was that 16-weeks of exercise seemed to increase steroid hormone-mediated signalling, decrease the plasma levels of arginase 1, and reduce transcription of genes involved in cell adhesion and blood clotting in patients diagnosed with the metabolic syndrome. We believe that these changes are at least partly responsible for the improved endothelial function and cardiovascular risk profile of the metabolic syndrome patients after the exercise period.

Endothelial function

Endothelial dysfunction is an early pathogenic event of the metabolic syndrome that often appears decades before the onset of vascular disease [15]. It is characterized by reduced bioavailability of nitric oxide (NO) that may arise from decreased production or increased degradation of NO, or both. Regular endurance training, however, has the potential of effectively restoring endothelial dysfunction and NO bioavailability in these patients [16] (Table 1). Arginase 1, which catalyzes the conversion of $_{L}$-arginine to $_{L}$-ornithine and urea, was less expressed after exercise in the metabolic syndrome patients. A high level of arginase decreases the $_{L}$-arginine availability for NO synthesis, through endothelial NO synthase, and has been associated with endothelial dysfunction in aging [17, 18]. Decreased levels of arginase 1 after exercise may lead to increased levels of $_{L}$-arginine, and thereby increased NO bioavailability [19], in line with the previously reported trends ($p=0.07$) towards increased level of NO in blood after exercise training (Table 1).

Endothelial dysfunction often involves endothelial inflammation and activation. When endothelial cells are activated, they increase their expression of CAMs, which further promotes the inflammation response [20]. Carcinoembryonic antigen-related CAM (CEACAM) 5 and 8 were both downregulated after exercise. A high level of CEACAM8 has previously been associated with atherosclerosis, and type 2 diabetes [21]. Exercise-induced decrease in CEACAM8 transcription suggests improved vascular conditions and a reduction in factors promoting endothelial inflammation.

Another potential source of improved endothelial function is increased steroid hormone-mediated signalling after exercise. One of the genes responsible for the upregulation of steroid signalling was estrogen receptor β (ERβ), which is expressed in the vasculature of both men and woman [22]. Augmentation of estrogen mediated processes has beneficial effects on blood pressure [23, 24], endothelial function [25], plasma lipids composition [26], antioxidant system, coagulation system, carbohydrate- and lipid metabolism [27], as well as the levels of high-density lipoproteins (HDL) [26], which is in line with the findings in this study (Table 1). In the vasculature, increased ER-signaling also enhances NO production, which is in line with the previously reported trends ($p=0.07$) towards increased levels of NO in blood after exercise training (Table 1). Interestingly, as NO contributes considerably to exercise-induced increase in limb blood flow [28], increased NO bioavailability through increased ER-signalling and decreased arginase 1 might contribute to the improved VO_{2max} observed after the exercise period.

Blood pressure

Several studies agree that regular endurance training has beneficial effects on blood pressure [6, 29, 30], even so, the mechanism is not fully determined. Recently, mice lacking ERβ have been reported to be hypertensive [23]. Although this is an extremity, increased transcription of ERβ after the exercise intervention might be involved in lowering of blood pressure. Since increased ER-signaling and decreased arginase 1 may contribute to the increased NO bioavailability after the exercise period, both factors has the potential of reducing blood pressure through NO evoked dilatation of arteries and blood vessels.

Blood clotting and atherosclerosis

Each of the risk factors that constitute the metabolic syndrome appears to uniquely promote atherosclerosis [31]; yet, the mechanism is not fully understood. Increased levels of pro-thrombotic factors is not included in the diagnostic criteria, however, metabolic syndrome patients often suffer from both impaired coagulation system and platelet function [3]. Regular physical exercise has proven effective in restoring the haemostatic imbalance in individuals with CVD risk factors [32]. This is in line with

our findings, as genes controlling blood clotting, like coagulation factor XIII (fibrin stabilizing factor), thrombin, vWf, integrin 3β and gamma-glutamyl carboxylase were less expressed after the exercise training. vWf is a highly relevant biomarker of endothelial damage and dysfunction [33], and has a strong correlation to diabetes and cardiovascular disease [34, 35]. An inverse relationship between FMD and vWf has previously been reported in congestive heart failure patients [36], which, according to our results, also seems to be the case for metabolic syndrome patients. Estrogen therapy, increasing ER-signalling, has previously been associated with lower plasma concentrations of vWf and other coagulation factors [37]. Therefore, increased ER-signalling may contribute to increased transcription of vWf and other coagulation factors after the exercise intervention.

In conclusion, metabolic syndrome patients performing 16-weeks of high intensity interval training increased expression of genes involved in steroid hormone-mediated signalling, reduced the levels of arginase 1, as well as reduced transcription of genes involved in cell adhesion and blood clotting. These changes are likely to contribute to the exercise-induced improvements of endothelial function and total cardiovascular risk profile. Since the number of patients was low, further follow-up studies are needed to confirm these results in a larger patient population.

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Figure

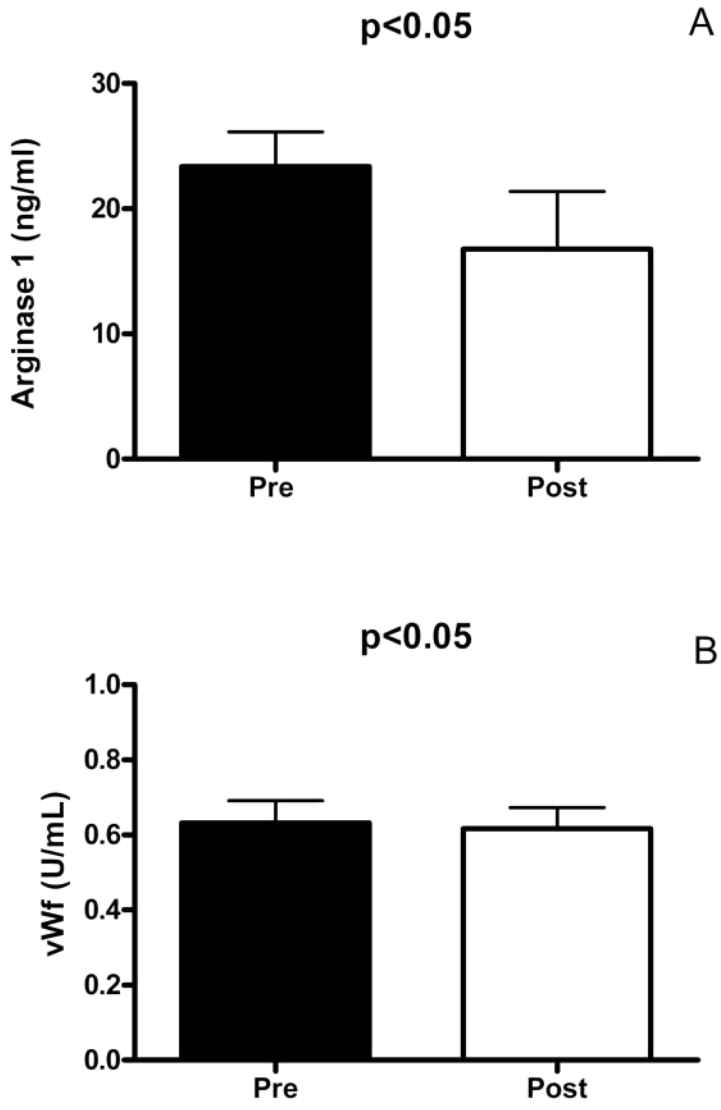

Figure 1: Plasma levels of Arginase 1 (ng/mL) and von Willebrand factor (vWf) (U/mL) in metabolic syndrome patients pre and post the exercise period.

Tables

Table 1: Physiological characteristics of the patients[6].

	Interval training	
	Pre	**Post**
No. of patients with metabolic syndrome	11/11	6/11*
BODY COMPOSITION AND MAXIMAL OXYGEN UPTAKE		
Body weight, kg	91.8 ± 5.3	89.5 ± 4.9*
Body mass index, $kg \cdot m^{-2}$	29.8 ± 1.7	29.1 ± 1.5*
Waist, cm	105.5 ± 4.1	100.5 ± 3.6*
Maximal oxygen uptake, $ml \cdot kg^{-1} \cdot min^{-1}$	33.6 ± 2.5	45.3 ± 3.3**
ENDOTHELIAL FUNCTION AND BLOOD PRESSURE		
Flow mediated dilatation (%)	3.9 ± 2.8	14.2 ± 1.5**
Mean arterial blood pressure, mmHg	111 ± 3	105 ± 3*
BLOOD VARIABLES		
Fasting Glucose, $mmol \cdot L^{-1}$	6.9 ± 0.6	6.6 ± 0.6*
Insulin sensitivity, (HOMA, %)	62.2 ± 8.0	77.2 ± 4.9*
β-cell function (HOMA, %)	76.8 ± 12.6	97.0 ± 9.2*
High density lipoprotein, $mmol \cdot L^{-1}$	0.69 ± 0.07	0.84 ± 0.10*
Nitric oxide, $\mu mol \cdot L^{-1}$	17.0 ± 6.35	22.1 ± 8.1#
Oxidized low density lipoproteins, $mmol \cdot L^{-1}$	102 ± 8	85 ± 7**
Adiponectin, µg/mL	7.8 ± 2.3	9.4 ± 3.0*

HOMA: Homeostasis Model Assessment. An estimation of steady state beta cell function and insulin sensitivity, as percentages of a normal reference population.

Data are presented as mean ± SEM.

Significant different from pre to post: * $p<0.05$; ** $p<0.01$; # $p=0.07$.

Table 2: Biological processes and molecular functions significantly altered by exercise

	Absolute enrichment score	*P* value
UP-REGULATED AFTER EXERCISE		
Protein biosynthesis	1.81	0.00
Ribosomal protein	1.92	0.00
Krab box transcription factor	1.53	0.00
Cytokinesis	1.67	0.01
Zinc finger transcription factor	1.32	0.02
Complement component	1.53	0.03
Protein targeting and localization	1.38	0.04
Coenzyme and prosthetic group metabolism	1.36	0.04
Immunoglobulin receptor family member	1.38	0.04
Steroid hormone-mediated signalling	1.55	0.05
Anion channel	1.51	0.05
DOWN-REGULATED AFTER EXERCISE		
Acyltransferase	1.68	0.00
Serine protease	1.52	0.00
Serine protease inhibitor	1.64	0.02
CAM family adhesion molecules	1.49	0.03
Steroid metabolism	1.44	0.03
Amino acid catabolism	1.44	0.04
Blood clotting	1.41	0.05

Table 3: Gene sets of special interest and the genes that have contributes most to their high enrichment score.

Gene ID	
	STEROID HORMONE-MEDIATED SIGNALING
2100	Estrogen receptor β
	CAM FAMILY ADHESION MOLECULES
1088	Carcinoembryonic antigen-related cell adhesion molecule 8
1048	Carcinoembryonic antigen-related cell adhesion molecule 5
257194	Neuronal growth regulator 1
4045	Limbic system-associated membrane protein
	AMINO ACID CATABOLISM
144193	Amidohydrolase domain containing 1
383	Arginase 1
144193	Arylformamidase
	BLOOD CLOTTING
7450	Von Willebrand factor
2677	Gamma-glutamyl carboxylase
2162	Coagulation factor XIII, A1 polypeptide
3690	Integrin 3β
2147	Thrombin

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