

**EFFECTS OF CHEMOTHERAPY AND BLOCKING ACTIVIN  
RECEPTOR SIGNALING ON SKELETAL MUSCLE SIZE,  
OXIDATIVE CAPACITY AND FUNCTION**

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## ABSTRACT

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**Introduction.** Doxorubicin (DOX) is widely used as a chemotherapy drug for cancer. However, it is known to affect negatively skeletal muscle mass and function, which can expose to other diseases and decrease survival rate. Presently, there are no accepted drugs for muscle wasting, but myostatin and activin blockers are possible agents. The aim of this study was to investigate the effects of DOX administration alone or combined with blocking of activin receptor signaling on skeletal muscle size, oxidative capacity, mitochondrial function and running performance.

**Methods.** Two identical four-week experiments were conducted in this study. The mice (n = 19 and n = 29 in experiments 1 and 2, respectively) were randomly organized into three groups: 1) controls (Ctrl, n = 6; n = 9), 2) DOX treated group (Dox, n = 6; n = 10) and 3) DOX treated group administered with sACVR2B-Fc (Dox + sACVR2B, n = 7; n = 10). Body composition was determined with DXA imaging and incremental running test was used to examine running capacity. Oxidative capacity was investigated with static biomarkers and mitochondrial function was examined with high resolution respirometry (OROBOROS). Static biomarkers were analyzed with Western immunoblot protein analysis and enzyme assay. PGC-1 $\alpha$  gene expression was examined with RT-qPCR method.

**Results.** Skeletal muscle mass decreased significantly in Dox group ( $p < 0.01$ ), but increased following sACVR2B-Fc administration together with DOX ( $p < 0.001$ ). Running distance decreased in Dox group compared to Ctrl group ( $p < 0.01$ ), but did not alter in Dox + sACVR2B group vs. Dox. DOX did not have effect either on oxidative capacity or mitochondrial function. Some static biomarkers changed following sACVR2B-Fc administration. Of those, citrate synthase activity (Krebs cycle enzyme) and porin/VDAC1 protein content increased significantly ( $p < 0.01$ ) compared to Dox group. The opposite trend was observed in the protein content of respiratory chain subunit (OXPHOS) complexes I ( $p < 0.001$ ) and V ( $p < 0.05$ ). However, neither mitochondrial function, other static biomarkers (cytochrome c and total OXPHOS protein contents) nor PGC-1 $\alpha$  protein content and isoforms gene expression altered significantly.

**Conclusion.** This study was the first to show decreased maximal running capacity after chemotherapy. This occurred, however without skeletal muscle mitochondrial alterations. sACVR2B-Fc may be a promising strategy to treat chemotherapy induced skeletal muscle loss, without further compromises in running capacity or major mitochondrial alterations.

**Key words:** doxorubicin, function, oxidative capacity, running capacity, skeletal muscle

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## ABBREVIATIONS

Acetyl CoA	Acetyl coenzyme A
ACVR2B/2A	Transmembrane activating receptor 2B and 2A
ADP	Adenosine diphosphate
Akt	Protein kinase B
ALK4/5	Type-I receptors: activin receptor-like kinase 4 and 5
AMPK	Adenosine monophosphate-sensitive protein kinase
ATP	Adenosine triphosphate
CI-V	Mitochondrial protein complexes (I-V)
cDNA	Complementary DNA
CHF	Congestive heart failure
CS	Citrate synthase
CO <sub>2</sub>	Carbon dioxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
Dox	Doxorubicin treated study group
Dox + sACVR2B	Doxorubicin + sActRIIB-Fc treated study group
Ctrl	Control study group
DXA	Dual-energy X-ray absorptiometry
FAD/ FADH <sub>2</sub>	Flavin adenine dinucleotide /reduced flavin adenine dinucleotide
FOXO	Forkhead transcription factor
GA	Gastrocnemius muscle
GDF-8	Growth/differentiation factor-8
GTP	Guanosine triphosphate
H <sup>+</sup>	Proton
IGF-1	Insulin-like growth factor I
IgG	Immunoglobulin G
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NAD <sup>+</sup> /NADH	Oxidized nicotinamide adenine dinucleotide/ reduced nicotinamide adenine dinucleotide
OXPHOS	Respiratory chain subunits
p38 MAPK	p38-mitogen -activated protein kinase
p53	Tumor supressor p53
PBS	Phosphate buffered saline
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator-1
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RT-qPCR	Real-time quantitative polymerase chain reaction
sACVR2B-Fc	Soluble ligand binding domain of ActRIIB fused to the FC domain of IgG
SUIT	Substrate-uncoupler-inhibitor titration
TA	Tibialis anterior muscle
TGF- $\beta$	Transforming growth factor- $\beta$
TNF- $\alpha$	Tumor necrosis factor $\alpha$
UCP	Uncoupling proteins
VDAC	Voltage-dependent anion-selective channel
VO <sub>2</sub> max	Maximal oxygen consumption

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# 1 INTRODUCTION

Words cancer and malignancy are used as synonyms for neoplasm. Medical term neoplasm can be defined as a fast growing tissue, which results from uncontrolled cell proliferation. This starts, if normal cell quality and quantity control mechanisms don't work. Structural organization and function of the neoplastic tissue differ significantly from the normal tissue. (Ehrman et al. 2013, 646.) Cancer touches worldwide and it is said to be the leading cause of mortality nowadays (Ehrman et al. 2013, 379). There are four commonly used treatments for cancer; surgery, radiation, chemotherapy and biotherapy or combination of these. Chemotherapy acts against cancer cells by attenuating cell division. (Ehrman et al. 2013, 386.)

Doxorubicin is an antibiotic that belongs to a class of chemotherapy drugs called anthracyclines (Gilliam & St. Clair 2011). Doxorubicin is widely used as a treatment for breast cancer, childhood solid tumors, soft tissue sarcomas and aggressive lymphomas (Minotti et al. 2004). Its anticancer mechanisms include DNA Topoisomerase II inhibition, reactive oxygen species (ROS) generation, p53 activation, caspase cascade activation, doxorubicin binding to DNA and disruption of mitochondrial iron metabolism. (Gilliam & St. Clair 2011; Ichikawa et al. 2014.)

Doxorubicin has severe side effects that include, for example, cardiotoxicity (Swain et al. 2003). In addition, it has an influence on skeletal muscle weakness, fatigue and loss of muscle mass (Braun et al. 2014; Gilliam et al. 2009; Gilliam et al. 2013; Gouspillou et al. 2015; Hydock et al. 2011; Stone et al. 1999).

Cancer and its treatments often decrease muscle mass, thus, means to prevent muscle mass are under investigation. Myostatin and activins, that signal via activin receptor type 2B

(ACVR2B), regulate negatively muscle growth when bound to an activin receptor (Lee et al. 2001; McPherron et al. 1997). Blocking of ACVR2B signaling pathway increases muscle mass (Akpan et al. 2009; Lee et al. 2005; Pistilli et al. 2011.), but it may also negatively regulate oxidative properties in skeletal muscle (Hulmi et al. 2013a; Relizani et al. 2014).

During cancer muscle mass preservation seems to be essential for survival. (Zhou et al. 2010). In addition, aerobic capacity is strongly linked to health and longevity (Koch et al. 2011; Kokkinos et al. 2008; Myers et al. 2002; Wisløff et al. 2005). Based on this knowledge, the aim of this thesis was to study the effects of extensively used chemotherapy agent doxorubicin on skeletal muscle size, oxidative capacity at the muscle level, mitochondrial function and running performance. The second aim was to find out, if blocking of activin receptor signaling could alleviate the expected side effects of doxorubicin, when combining it with sACVR2B-Fc administration.

## **2 AEROBIC CAPACITY**

### **2.1 Effects on health**

Aerobic capacity can be examined by measurement of maximal oxygen consumption ( $\text{VO}_2\text{max}$ ). In addition, incremental running test is a common way to estimate aerobic capacity. Aerobic capacity is the maximal capacity to produce ATP via oxidative pathways at the whole body level. Aerobic capacity is determined by intrinsic (heredity) and environmental factors such as physical activity. (Koch & Britton 2001; Koch et al. 2011; Lessard et al. 2011; Little et al. 2010.) According to many studies, aerobic capacity has an effect on longevity. In other words, low aerobic capacity is associated with high mortality rate. Low aerobic capacity increases the occurrence of metabolic and cardiovascular risk factors. (Koch et al. 2011; Kokkinos et al. 2008; Myers et al. 2002; Wisløff et al. 2005.)

### **2.2 The role of mitochondria**

Mitochondria are organelles that are responsible for cellular respiration and regulation of energy metabolism in a cell. Energy is needed for all vital functions in the body. (Reece et al. 2011, 155.) Oxidative capacity can be defined as the ability of skeletal muscle mitochondria to produce energy in a form of adenosine triphosphate (ATP) by using oxygen. ATP is composed of adenosine and three phosphate groups. According to studies, oxidative capacity is determined, in part, by the efficiency and the number of mitochondria in skeletal muscles (Rivas et al 2011). The oxidative capacity at the skeletal muscle level and the aerobic capacity at the whole body level are linked.

Mitochondria are 1-10  $\mu\text{m}$  long organelles and there are hundreds to thousands of them in a cell. The cell membranes of mitochondria are formed by phospholipid bilayer; the outer

membrane is smooth and the inner membrane is folded. The inner membrane forms so called crests (Figure 1). The space inside the inner membrane is called mitochondrial matrix where many enzymes, ribosomes and mitochondrial DNA (mtDNA) are located. However, the most essential proteins for oxidative energy production are located in the inner membrane. (Reece et al. 2011, 156.)

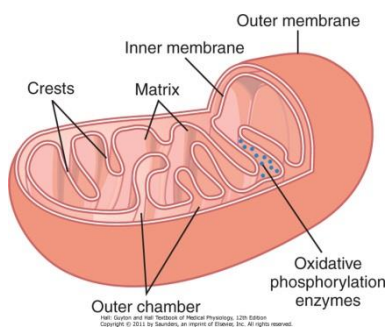


FIGURE 1. The structure of the mitochondrion (Guyton & Hall 2011, 16).

Oxidative pathway is the most effective way to produce energy in cells. Generally energy is produced from glucose, fats and to smaller extent from proteins. One glucose molecule can be converted to about 32 ATP molecules. Energy production from fats produces twice as much ATP compared to glucose. Oxidative energy production from glucose takes place in three different steps; glycolysis, citric acid cycle and oxidative phosphorylation. Further, oxidative phosphorylation can be subdivided into electron transfer system and ATP synthesis (chemiosmosis). Oxygen is used only in the last phase. The first phase takes place in the cytosol and the others inside the mitochondrion. (Reece et al. 2011, 210-223.)

During glycolysis glucose molecule is broken down into two pyruvates and are then carried into the mitochondrion. Pyruvates are oxidized to acetyl coenzyme A (acetyl CoA) molecules, which are then moved to citric acid cycle. If fats are used as an energy source, acetyl CoA is produced via beta-oxidation and moved to citric acid cycle. Glycolysis

produces two ATP molecules (or three when glycogen is the source of glucose) and, in addition to that due to redox reaction, electron carrier NAD<sup>+</sup> (nicotinamide adenine dinucleotide) accepts electrons to form two NADH molecules. Also two NADH molecules form, when pyruvates are converted into acetyl coenzyme A molecules. Beta-oxidation produces NADH and FADH<sub>2</sub>. FADH<sub>2</sub> is a reduced form of an electron carrier flavin adenine dinucleotide (FAD), which is derived from riboflavin. (Reece et al 2011, 213-218, 226.) In the next section a closer look is taken into reactions after glycolysis and beta-oxidation.

### **2.2.1 Citric acid cycle and oxidative phosphorylation**

Citric acid cycle, also called Krebs cycle, consists of eight different reactions (Figure 2). During citric acid cycle ATP is formed from guanosine triphosphate (GTP), which shares structural and functional properties with ATP. Most importantly, citric acid cycle produces more NADH and FADH<sub>2</sub> molecules, which are used for ATP formation later. In addition, carbon dioxide (CO<sub>2</sub>) is released. (Reece et al. 2011, 216-218.)

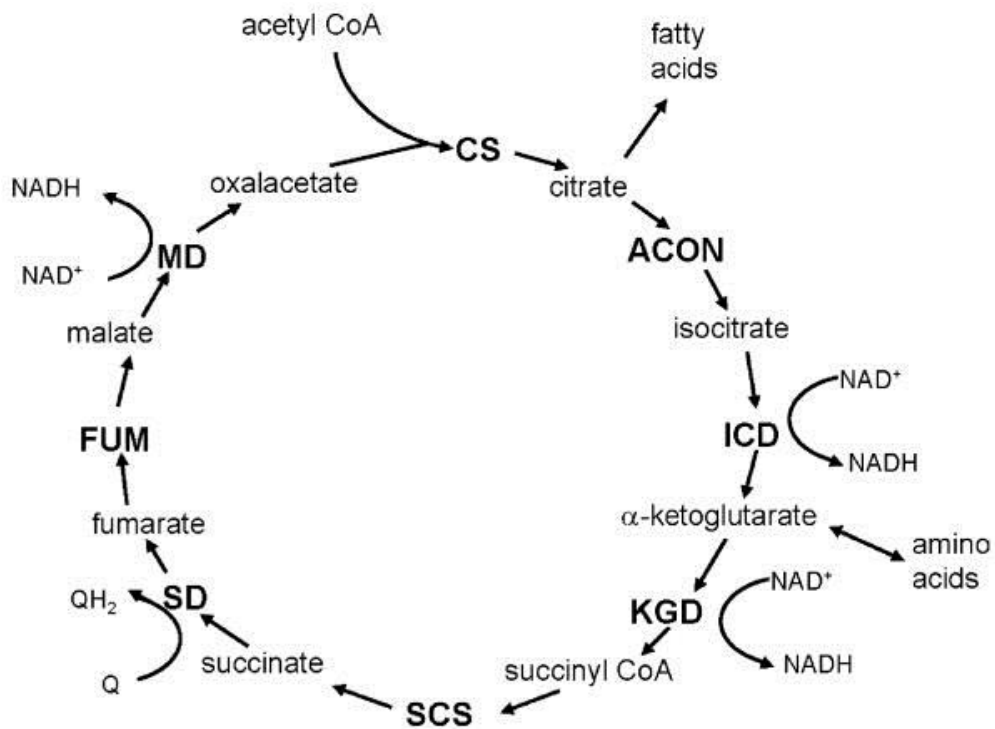


FIGURE 2. Citric acid cycle. It consists of eight reactions. CS=citrate synthase, ACON=aconitase ICD=isocitrate dehydrogenase, KGD= $\alpha$ -ketoglutarate dehydrogenase, SCS=succinyl-CoA synthase; SD=succinate dehydrogenase, FUM=fumarase, MD=malate dehydrogenase. (Yarian et al. 2006)

Later, NADH and FADH<sub>2</sub> unload their high-energy electrons into the electron transfer system. Electron transfer system consists of four protein complexes (I-IV) which are accompanied by nonprotein prosthetic groups (Figure 3). These prosthetic groups help with catalytic functions. NADH and FADH<sub>2</sub> donate their electrons first to complexes I and/or II, respectively, and then electrons travel through all complexes. At the same time, protons (H<sup>+</sup>) are pumped from the mitochondrial matrix to the intermembrane space to form a proton gradient across the inner mitochondrial membrane. (Reece et al. 2011, 218-223.)

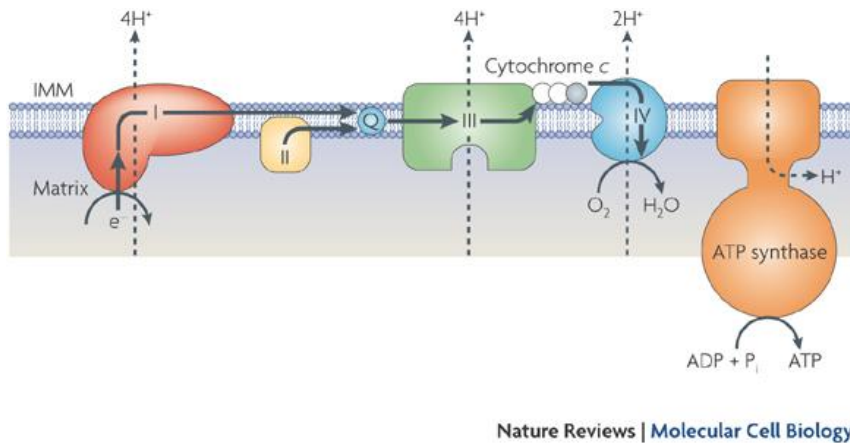


FIGURE 3. Oxidative phosphorylation. Electrons travel through electron transfer system complexes starting from complex I or II, and at the same time protein complexes pump protons (H<sup>+</sup>) from the mitochondrial matrix to the intermembrane space. Proton gradient is then used in the last step called ATP synthesis (chemiosmosis). (Ow et al. 2008.)

Proton-motive force moves protons down their gradient through enzyme called ATP synthase (complex V) back to mitochondrial matrix and this phosphorylates ADP to ATP (Figure 3.). (Reece et al. 2011, 219-223.) However, some protons are able to “leak” from ATP synthase and decrease the proton-motive force and further ATP synthesis. (Dietrich & Horvath 2010.) When electrons travel down the electron transfer system, electron carriers, which are located in the protein complexes, are reduced and oxidized one after another. Finally in the complex IV, oxygen is reduced and water is formed as a byproduct of oxidative phosphorylation. (Reece et al. 2011, 218-219.)

### **2.2.2 Biomarkers of oxidative capacity at the muscle level**

According to studies, oxidative capacity at the muscle level is determined, in part, by the efficiency and the number of mitochondria in skeletal muscles. There are many ways to study the oxidative capacity. (Larsen et al. 2012; Rivas et al. 2011.) The golden standard method to study mitochondrial content is a transmission electron microscopy imaging.

There are many biomarkers that are related to mitochondrial content and efficiency. A proper way is to study amounts and activities of enzymes, proteins and lipids, which are essential to mitochondria structure and oxidative metabolism (Larsen et al. 2012; Rivas et al. 2011). These are, for instance, citrate synthase and respiratory chain subunits (OXPHOS). In addition, mitochondrial content can be estimated by studying mitochondrial DNA copy number. (Larsen et al, 2012.) In this section a closer look is taken on citrate synthase, cytochrome c, respiratory chain subunits and porin/VDAC1.

Citrate synthase is an enzyme in the citric acid cycle and it converts acetyl CoA to citrate. This reaction takes place at the beginning of the citric acid cycle. (Ow et al. 2008.) Activity of the citrate synthase tells about the efficiency of mitochondria, and the amount of this enzyme is also a good marker of mitochondrial content in situations, when enzyme activity per mitochondria is rather unchanged. Citrate synthase activity and amount can be studied by enzyme assay. (Larsen et al. 2012; Rivas et al. 2011.)

Respiratory chain subunits (OXPHOS) are protein complexes embedded into the inner mitochondrial membrane and have a key role in oxidative phosphorylation. Subunits I-IV are electron transfer system complexes and complex V is ATP synthase. (Larsen et al. 2012; Rivas et al. 2011.) Cytochrome c is an electron carrier of the electron transfer system between complexes III and IV. Its prosthetic group is called heme group and it has an iron atom that accepts and donates electrons. (Reece et al. 2011, 219.)



Porin/VDAC1 is a protein called voltage-dependent anion-selective channel (VDAC1), which can be found from mitochondrial outer membrane and plasma membrane. Porin/VDAC1 is responsible for transporting molecules between the cytosol and mitochondria and it is also associated with apoptosis with many other factors (Lawen et al. 2005.) The protein level of porin/VDAC1 gives information of mitochondrial efficiency. Mitochondrial proteins can be studied by method called Western immunoblot protein analysis, which applies electrophoresis and immunoblotting. (Larsen et al. 2012; Rivas et al. 2011.)

To add, mitochondria have a history as independent cells and, thus, have also their own mitochondrial DNA (mtDNA). (Reece et al. 2011, 155). However, mtDNA is not considered as a good biomarker for mitochondrial content (Larsen et al. 2012). Perhaps due to high polymorphism and high variation in different mitochondrial genes.

### **2.2.3 Measurement of mitochondrial function at the muscle level**

As mentioned previously, oxidative capacity at a cellular level can be studied by measuring mitochondrial enzyme activities and protein levels. This is called a static way to study oxidative capacity (Presta & Gnaiger 2012). However, these biomarkers don't always indicate the real and complex mitochondrial functions. (Larsen et al. 2012; Jacobs et al. 2013.) There may sometimes be changes in mitochondrial function without changes in mitochondrial enzymes or proteins (Jacobs et al. 2013; Viganó et al. 2008).

There is a method to study mitochondrial function accurately and real-time at the muscle level and this method is called high resolution respirometry (Larsen et al 2012). There are some instruments designed to perform these mitochondrial respiratory protocols. The most commonly used are OROBOROS Oxygraph-2k and Seahorse XF EFA. In this literature

review the OROBOROS Oxygraph-2k is considered, as it was used as a research method in this thesis.

Respirometry is a dynamic measurement of mitochondrial function and respiration capacity. The measurement is conducted by adding electron transfer chain substrates, inhibitors and uncouplers in a sequence into a sample chamber, while observing dissolved oxygen concentration and oxygen flux in experimental solution with an oxygen sensor. This method is called SUT (substrate-uncoupler-inhibitor titration), and it is conducted with intact mitochondria. (Gnaiger 2014; Pesta & Gnaiger 2012.)

Coupling and uncoupling are important concepts for oxidative phosphorylation. In oxidative phosphorylation the conversion of ADP to ATP is coupled to generation and use of proton gradient across inner mitochondrial membrane. This proton gradient can be disturbed by physiological dyscoupling, pathological dyscoupling or experimental noncoupling. These affect energy metabolism by decreasing the rate of ATP generation. Physiological uncoupling or dyscoupling is linked to proton leak across the inner mitochondrial membrane, proton slip from improperly working proton pumps and molecular uncouplers called uncoupling proteins (UCP) that transport protons across the inner mitochondrial membrane. Decreased functions of mitochondria, due to pathological conditions, can lead to pathological dyscoupling. Noncoupled state is induced with uncouplers during experimental conditions. This noncoupled state reveals the respiration capacity of the electron transfer system by bypassing the ATP-synthase with added uncouplers. (Gnaiger 2014.)

High resolution respirometry protocol consists of different states and oxygen use is measured real-time during them. ROUTINE-state is a coupled physiological respiratory state with non-saturating ADP levels. Although this state is rather close to basal metabolic state, it cannot be considered as a way to measure basal metabolic rate due to potential influence of cell growth on respiration. LEAK-state measures respiration due to intrinsic

uncoupling or dyscoupling. LEAK-state is generated in the presence of fuel substrates from e.g. citric acid cycle, but in the absence of added ADP and with inhibition of ATP synthase (for instance with oligomycin).

OXPPOS-state measures the respiratory capacity of mitochondria in the ADP-activated state and in the presence of inorganic phosphates. This coupled OXPPOS-state reflects the function of the electron transfer system combined to ATP synthase with certain added substrates. This way, function of specific complexes, e.g. type I can be investigated. ETS-state is a noncoupled state, which reflects the maximal respiration capacity of the electron transfer system. To achieve this, ATP synthase is bypassed by uncouplers. During ETS-state the proton gradient across the inner mitochondrial membrane is partly collapsed, but oxygen use is very high. ROX-state measures residual oxygen consumption, when ETS is blocked (rotenone), and indicates oxygen consuming side reactions (enzymes and auto-oxidative reactions). Results from ROUTINE-, LEAK-, OXPPOS- and ETS-state are corrected afterwards with ROX-values. (Gnaiger 2014; Pesta & Gnaiger 2012.) After the protocol, the function and respiration of different complexes of mitochondria and maximal respiratory capacity of mitochondria can be studied.

#### **2.2.4 Regulation of mitochondrial biogenesis and oxidative metabolism: PGC-1**

Genetic code includes information needed for the control of functional and structural properties of all living organisms. Gene expression starts from DNA (deoxyribonucleic acid) transcription to messenger RNA (mRNA) and continues with mRNA translation to protein. Proteins are the main links between genotype and phenotype. The site, where transcription starts, is called promoter. Genes consist of coding parts (exons) and non-coding parts (introns). After transcription, non-coding parts are removed in a process called RNA splicing. Alternative splicing produces variable proteins by modifying exon

composition in mRNA. This means that one gene can produce several kinds of proteins. (Reece et al. 2011, 371-382.)

Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) family has a key role in regulating cellular oxidative metabolism. For instance, PGC-1 family regulates mitochondrial respiratory capacity and biogenesis. (Chinsomboom et al. 2009; Lin et al. 2002b; Miura et al. 2008; Ruas et al. 2012; Zhang et al. 2009.) Mitochondrial biogenesis increases the number of mitochondria in cells, and PGC-1 family members increase the amounts of mitochondrial protein and replication of mtDNA for fission. (Garnier et al. 2005; Wu et al. 1999.) PGC-1 family act by binding to transcription factors and coactivator complexes (for example nuclear respiratory factors), thus, PGC-1 does not bind to DNA itself. (Chinsomboom et al. 2009; Lin et al. 2002b; Miura et al. 2008; Ruas et al. 2012; Wu et al. 1999; Zhang et al. 2009.)

PGC-1 $\alpha$  was the first member of PGC-1 family, and back then it was found to regulate genes associated to thermogenesis (Puigserver et al. 1998). In addition, there are two family members called PGC-1 $\beta$  and PRC (PGC-1 -related coactivator) (Andersson et al. 2001; Lin et al. 2002a). All these three family members regulate cellular energy metabolism and there seems to some kind of co-operation between them. It has been reported that, when PGC-1 $\alpha$  levels are reduced, PGC-1 $\beta$  is increased as compensatory effect. (Szczelecki et al. 2014.)

PGC-1 $\alpha$  has isoforms, which have some different functions. Currently known PGC-1 $\alpha$  isoforms are; PGC-1 $\alpha$ -a, -b and -c, NT-PGC-1 $\alpha$ -a, -b and -c and PGC-1 $\alpha$ 1, 2, 3 and 4. Isoforms PGC-1 $\alpha$ -a and PGC-1 $\alpha$ 1 are structurally the same isoform as well as PGC-1 $\alpha$ 4 and NT-PGC-1 $\alpha$ -b. These isoforms differ from exon composition and/or alternative promoter use (Figure 4). (Chang et al. 2012; Correia et al. 2015; Martínez-Redondo et al. 2015; Miura et al. 2008; Ruas et al. 2012; Wen et al. 2014; Zhang et al. 2009.) Isoforms consisting of different compositions of exons are called splice variants. Alternative splicing and

alternative promoter use are ways to regulate gene expression and produce variable proteins. (Chinsomboon et al. 2009; Nader et al. 2014; Ruas et al. 2012; Vandembroucke et al. 2001.) NT stands for truncated isoforms, in other words these isoforms produce shorter proteins. (Zhang et al. 2009.) Some of these PGC-1 $\alpha$  isoforms are very similar to each other when considering their structure and functions.

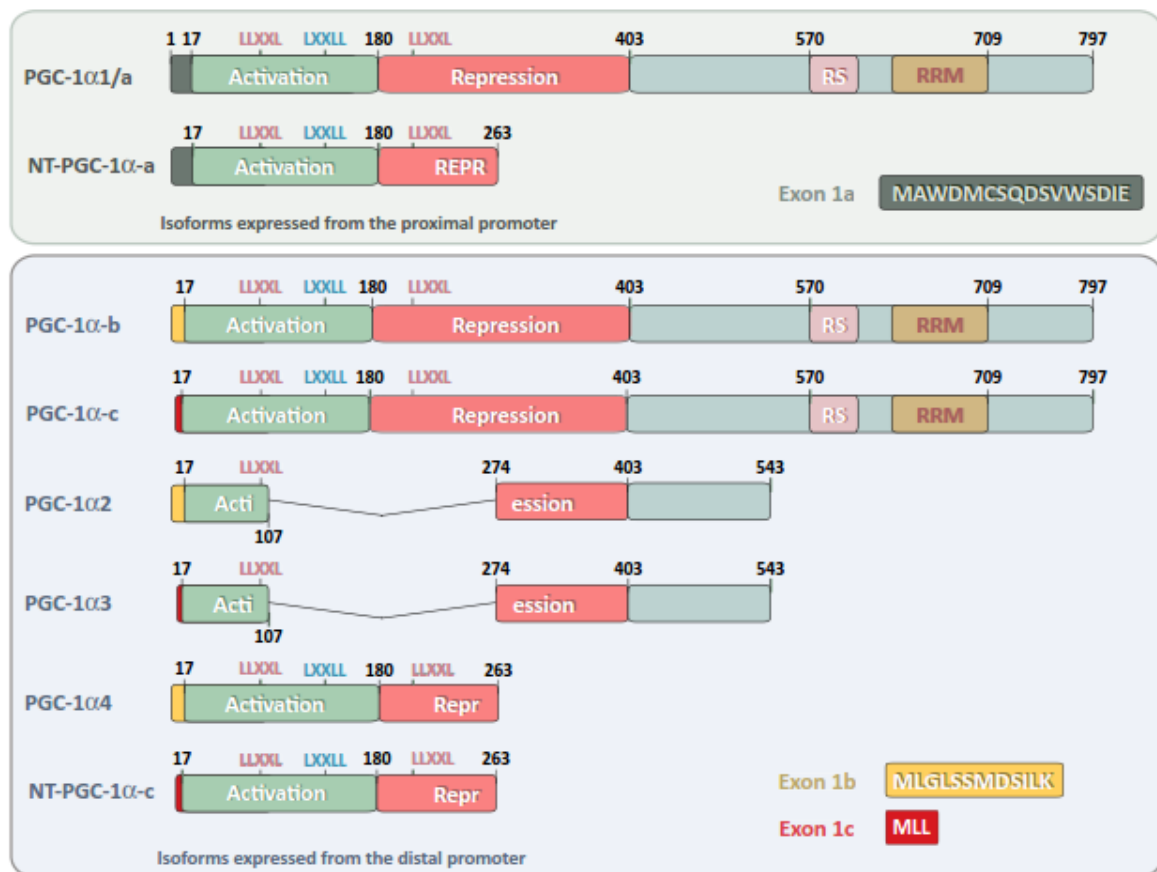


FIGURE 4. Formation of PGC-1 $\alpha$  isoforms by alternative splicing (different compositions of exons) and alternative promoter use (proximal and distal/alternative promoter). (Correia et al. 2015.) PGC-1 $\alpha$ 1 is also called PGC-1 $\alpha$ -a and in addition PGC-1 $\alpha$ 4 is also called NT-PGC-1 $\alpha$ -b.

Exercise stimulates calcium, p38 MAPK and AMPK signaling and in addition decreases promoter methylation. These response to increase PGC-1 $\alpha$  gene expression by activating different transcription factors (Akimoto et al. 2005; Barrés et al. 2012; Handschin et al. 2003; Jager et al. 2007; Wu et al. 2002). PGC-1 $\alpha$  proximal promoter is activated during endurance exercise resulting in PGC-1 $\alpha$ 1 expression (Ruas et al. 2012; Silvennoinen et al. 2015). On the other hand, resistance training activates distal promoter leading to splice variant PGC-1 $\alpha$ 4 expression (Chinsomboon et al. 2009; Nader et al. 2014, Ruas et al. 2012). However, this issue is still contradictory, because some studies report, that both promoters are activated after these two forms of exercise (Lundberg et al. 2014; Ydfors et al. 2013).

An interesting point is that neither PGC-1 $\alpha$  nor PGC-1 $\beta$  has shown any increasing effects on muscle mass or strength (Summermatter et al. 2012). Other hand, PGC1- $\alpha$ 4 may play a key role in inducing hypertrophy rather than regulate mitochondrial biogenesis (Figure 5). (Brault et al. 2010; Choi et al. 2008; Ruas et al. 2012; White et al. 2014.)

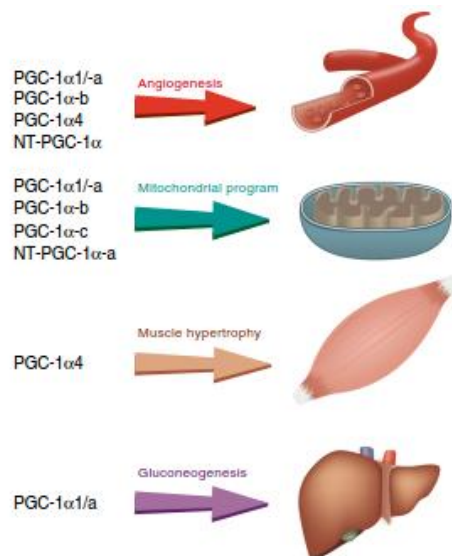


FIGURE 5. Putative functions of PGC-1 $\alpha$  isoforms. Isoforms have some overlapping functions and different exercise forms activate different isoforms. (Martínez-Redondo et al. 2015.)

## **3 CANCER AND ITS TREATMENT**

### **3.1 Cancer**

#### **3.1.1 Intro**

Cancer can originate from different organs. The uncontrolled cell growth begins from stem cells of a particular tissue that have been encountered by some carcinogenic-causing event, which leads to damaged or mutated DNA. Carcinogenic events are associated with environment, heredity, hormones and immune system. For example, tobacco, radiations and pollutants can be pointed out as environmental carcinogens. In addition, uncontrolled cell growth does not start without mutations in genes involved to regulate cell growth and apoptosis. These genes are called proto-oncogenes and tumor suppressor genes. Proto-oncogenes regulate normal cell growth and development, thus, mutation concerning these genes can be a risk for cancer. Tumor suppressor genes activate apoptosis pathways, that inhibit tumor progression, and mutations in these genes can expose to cancer. (Ehrman et al. 2013, 379-382.) In other words, without these genes cell quality and quantity control does not occur.

#### **3.1.2 Cancer treatment: chemotherapy**

There are four commonly used treatments for cancer; surgery, radiation, chemotherapy and biotherapy. The best treatment or combinations of treatments are selected individually for patients. In surgery, the whole tumor or part of its mass is removed. Radiation allocated to tumor cells can inhibit tumor growth by damaging DNA. Biotherapy increases immune response of the body against the cancer cell specific antigens. (Ehrman et al. 2013, 386.)

This literature review concentrates on chemotherapy as a cancer treatment, because it was involved in this study. During chemotherapy, cancer cell replication is attenuated by chemicals. This treatment is most valid for tumors that are growing fast, however, cancer cells may sometimes become resistant to these drugs. In this case, combination chemotherapy can be considered. (Ehrman et al. 2013, 386.)

Although treatments are effective against cancer nowadays, they have some severe side-effects including fatigue, nausea, anxiety, muscle weakness, loss of muscle mass and stress. (Ehrman et al. 2013, 390-392.) A chemotherapy drug doxorubicin and its side-effects are reviewed more closely in this thesis.

### **3.1.3 Cancer and muscle**

Approximately 50-80 % of cancer patients suffer from a syndrome called cancer cachexia. This syndrome is known to reduce quality of life and increase the mortality rate of the cancer patients. (Argilés et al. 2014.) During cancer cachexia weight decreases progressively. Skeletal muscle mass is decreased following alterations in e.g. myofibrillar protein metabolism. (Cosper et al. 2012.) Decreased muscle protein synthesis, increased protein breakdown, increased apoptosis and decreased ability to regeneration have been reported as mechanisms for muscle loss in cancer cachexia (Argilés et al. 2014). This may result in muscle weakness, fatigue and decreased skeletal muscle function (Cosper et al. 2012; Gorselink et al. 2006). According to studies, low muscle mass is also negatively related to chemotherapy toxicity and time to tumor progression (Prado et al. 2009). Most likely cancer cachexia is related to disease itself as well as to cancer treatments (Gorselink et al. 2006; Le Bricon et al. 1995).

Cancer cachexia is an energy-wasting syndrome that involves impaired mitochondrial functions. During cancer cachexia, ATP synthesis seems to decrease in mitochondria



(Constantinou et al. 2011). Mitochondrial dysfunction may result from decreased oxidative capacity (altered protein synthesis, changes in membrane fluidity and oxidatively modified mitochondrial proteins). (Antunes et al. 2014; Fermoselle et al. 2013; Padrão et al. 2013; Shum et al. 2012). In addition, activation of uncoupling proteins decreases ATP production (Busquets et al. 2005; Collins et al. 2002; Sanders et al. 2004). Uncoupling proteins increase proton leak from intermembrane space to mitochondrial matrix at the same time decreasing proton gradient across mitochondrial inner membrane (Dietrich & Horvath 2010). During cancer cachexia, PGC-1 $\alpha$  activation and production may be increased and enhance mitochondrial uncoupling and energy expenditure (Fuster et al. 2007; Miura et al. 2006).

### 3.2 Doxorubicin chemotherapy

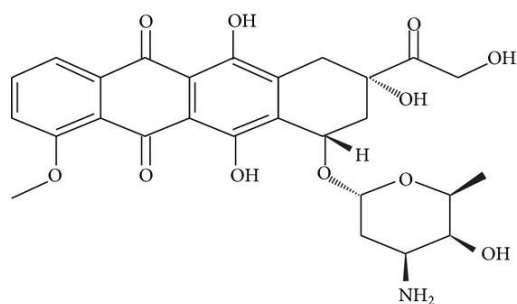


FIGURE 6. The chemical structure of doxorubicin ( $C_{27}H_{29}NO_{11}$ ) is a tetracyclic ring, which contains quinone-hydroquinone groups, a methoxy substituent, a side chain containing a carbonyl, a primary alcohol and sugar called daunosamine (Kamba et al. 2013; Minotti et al. 2004).

Doxorubicin (Figure 6) is an antibiotic that belongs to a class of chemotherapy drugs called anthracyclines. It acts against cancer by preventing tumor cell growth, cell division and metastasis. More specific mechanisms seem to involve DNA Topoisomerase II inhibition, reactive oxygen species (ROS) generation, p53 activation, caspase cascade activation, doxorubicin binding to DNA and disruption of mitochondrial iron metabolism. (Gilliam &

St. Clair 2011; Ichikawa et al. 2014.) Doxorubicin can induce apoptosis, in other words programmed cell death, via these mechanisms, (Arola et al. 2000; Ichikawa et al. 2014; Yoshida et al. 2009).

Topoisomerase II controls nuclear processes such as DNA replication (Yang et al. 2014). According to studies doxorubicin activates apoptosis-inducers, such as caspase-3, at least in cardiomyocytes (Ueno et al. 2006). Doxorubicin can activate tumor suppressor protein p53, which can lead to caspase cascade initiation (Nithipongvanitch et al. 2007). Doxorubicin tends to bind easily to nuclear as well as mitochondrial DNA and this alters DNA structure and complicates replication (Agudelo et al. 2014; Ashley & Poulton 2009). Doxorubicin also increases iron accumulation in mitochondria and cellular ROS generation (Ichikawa et al. 2014). One mechanism in ROS generation is that doxorubicin can be converted into a semiquinone free radical by redox cycling. If this molecule further donates its unpaired electron to oxygen, it turns into reactive oxygen species (ROS). (Minotti et al. 2004.) Free radicals are characterized by unpaired electrons, which make them very reactive with other molecules. Large amounts of reactive oxygen species (oxidative stress) can damage cellular structures and lead to apoptosis. However, reactive oxygen species signal some basic cell functions and are also formed in electron transfer system. (Murphy 2009.) Most of these studies above, considering doxorubicin antitumor mechanisms, have been conducted with cardiomyocytes.

Doxorubicin is used widely as a treatment for breast cancer, childhood solid tumors, soft tissue sarcomas and aggressive lymphomas (Minotti et al 2004). Doxorubicin is an effective drug for cancer, but it has also some severe side-effects that take place at surrounding healthy tissues like skeletal muscles. In the next section side effects of doxorubicin on muscle and heart are considered.

### **3.3 Side-effects of doxorubicin on skeletal muscle and heart**

Doxorubicin is very effective antitumour agent, but it has severe side-effects that include for example cardiotoxicity (Swain et al. 2003). In addition, it has an influence on skeletal muscle weakness and fatigue. (Braun et al. 2014; Gilliam et al. 2009; Gilliam et al. 2013; Hydock et al. 2011; Stone et al. 1999). It seems that doxorubicin side-effects are dose-dependent (Swain et al. 2003).

Doxorubicin is known to induce cardiotoxicity and further congestive heart failure (CHF) (Swain et al. 2003). Doxorubicin seems to decrease cardiac mass and reduced cardiac functions due to increased cardiomyocyte apoptosis (Zhu et al. 2009). Doxorubicin also causes loss of body weight, skeletal muscle mass and decreases force production (Falkenberg et al. 2002; Gilliam et al. 2009; Gouspillou et al. 2015). Doxorubicin treatment seems to decrease cross-sectional area of skeletal muscles and attenuate muscle contractile functions by lowering absolute and specific force. In addition, according to studies, test animals injected with doxorubicin are fatigued more easily compared to control animals. (Gilliam et al. 2009.) Also respiratory muscle dysfunction has been observed after doxorubicin treatment (Gilliam et al. 2011).

Doxorubicin decreases physical activity and voluntary wheel running (Gilliam et al. 2013; Zombeck et al. 2013). This may also indicate impaired aerobic capacity. There is a lack of information about effects of doxorubicin on aerobic capacity. Doxorubicin may also have a negative effect on mitochondrial function in skeletal muscle cells (Gilliam et al. 2013; Gouspillou et al. 2015). Gouspillou et al. (2015) reported decline in functions of respiratory complexes and maximal respiratory capacity after chemotherapy treatment. The maximal respiratory capacity was 36 % lower compared to control group. Same kind of results has been reported by Gilliam et al. (2013). In addition, according to Gilliam et al. (2013)

doxorubicin increased ROS formation. 2 hours following the injection ROS level was about 52 % higher than normally, but finally after 72 h it declined back in the baseline level. (Gilliam et al. 2013.)

Thus, a potential cause for skeletal muscle weakness and fatigue is the formation of reactive oxygen species after doxorubicin treatment (Gilliam et al. 2011; Gilliam et al. 2013; Min et al. 2015; Smuder et al. 2011). ROS production is also one possible antitumor mechanism of doxorubicin as previously was already reported. Mitochondria are the most common source of reactive oxygen species in skeletal muscle during doxorubicin treatment (Gilliam et al. 2012; Gilliam et al. 2013; Min et al. 2015). According to Gilliam et al. (2013), ROS can be formed by two common ways; doxorubicin reduction by mitochondrial complex I and inactivation of electron transport system. These two mechanisms have been observed in cardiomyocytes (Davies & Doroshov 1986; Xiong et al. 2006).

Doxorubicin treatment may not have an effect on mitochondrial content in skeletal muscle (Gilliam et al. 2013). This has also been observed by Gouspillou et al. (2015), reporting that either OXPHOS protein content, PGC-1 $\alpha$  gene expression or mtDNA copy number were not affected by doxorubicin. However, decrease in citrate synthase activity was observed after doxorubicin treatment. (Gouspillou et al. 2015.)

## 4 MYOSTATIN, ACTIVINS AND THEIR RECEPTORS

There are no accepted drugs for muscle wasting, but myostatin and activin blockers are recent possible agents.

### 4.1 ACVR2B and its signaling pathway

Myostatin, also known as growth/differentiation factor-8 (GDF-8), is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) family and it functions by binding to a transmembrane activating receptor 2B (ACVR2B) on a muscle cell surface. When bound to the activin receptor 2B, myostatin can regulate negatively muscle growth. (Lee et al. 2001; McPherron et al. 1997.)

Myostatin has effects on developing and adult muscles. Myostatin regulates negatively myogenesis during development by inhibiting myoblast growth and impairing differentiation. As for, when considering adults, myostatin can impair the ability to regenerate muscle and activate satellite cells. In addition, it negatively affects protein metabolism. (Langley et al. 2002; McFarlane et al. 2011; McCroskery et al. 2003.) If the signaling of myostatin is disturbed, muscle mass could overgrow and become two to three times larger than normally. This could be used as a possible clinical treatment for patients suffering muscular dystrophies and muscle wasting syndromes, for example cancer cachexia. (Lee et al. 2001; McPherron et al. 1997.) During cancer cachexia myostatin levels are known to increase (Costelli et al. 2008).

Myostatin binds to transmembrane serine-threonine receptor kinase complexes (Figure 7). These heterodimer complexes consist of two type-2 receptors (ACVR2A or ACVR2B) and two type-1 receptors (activin receptor-like kinase 4 or 5/ALK4 and ALK5). (Han et al.

2013.) Activin receptor 2B are in the majority in muscle cells compared to ACVR2A and it has a strong affinity for myostatin (Lee et al. 2001).

The signaling pathway begins from activin receptor 2B, when myostatin binds to it. This activates then receptor type 1. Followed by this, Smad2 and Smad3 are phosphorylated and Smad4 is recruited to the complex. This complex enters into nucleus and regulates gene expression leading to muscle wasting. (Han et al. 2013; Lee et al. 2001; Sartori et al. 2009.) This signaling pathway can also inhibit Akt activity, which reduces FOXO phosphorylation and increases protein breakdown (Han et al. 2013; Zhou et al. 2010).

In addition, some other ligands bind also to activin receptors (Lee et al. 2005; Souza et al. 2008). Activins are part of the activins-inhibins subfamily of TGF- $\beta$  family. Activins bind to same receptor than myostatin and activates same signaling cascade resulting to muscle wasting. (Chen et al. 2014; Han et al. 2013.)

Circulating protein, follistatin, is known to be able to bind with myostatin and activin and inhibit their actions, in other words regulation of muscle growth (Sidis et al. 2006). In addition, there are other ways to inhibit myostatin action and these will be discussed in the next session.

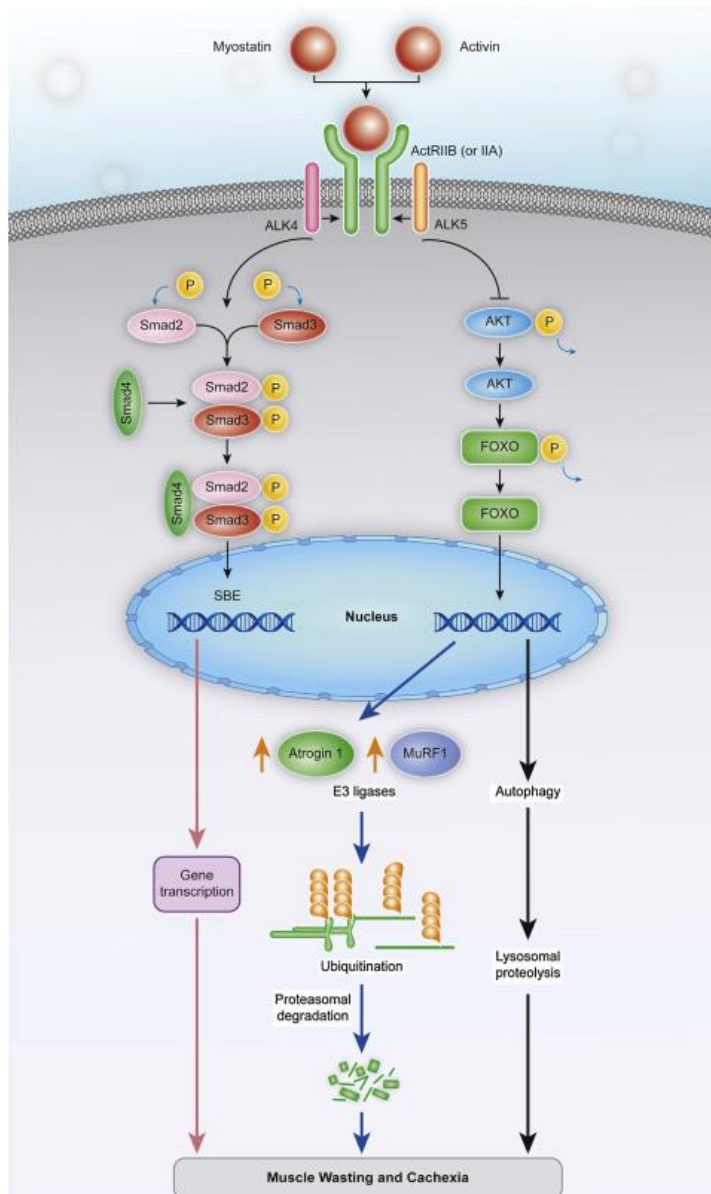


FIGURE 7. Myostatin and activin signaling pathway leading to muscle wasting and cachexia at least in part through increased proteolysis. (Han et al. 2013.)

## **4.2 Blocking of ACVR2B signaling**

The effects of ACVR2B signaling blocking have been studied mainly with myostatin knockout mice and with sACVR2B-Fc injections in Duchenne muscular dystrophy or wild-type mice. In myostatin knockout mice results can be affected by developmental change of muscle metabolic characteristics to the more glycolytic phenotype. In wild-type and Duchenne muscular dystrophy mice the phenomenon can be studied without fiber-type changes (Cadena et al. 2010.) In this literature review the main focus is on blocking of ACVR2B signaling with sACVR2B-Fc.

Myostatin can be blocked via a soluble ligand binding domain of ACVR2B fused to the Fc domain of IgG (sACVR2B-Fc). Injections with sACVR2B-Fc increase muscle mass and force production. (Akpan et al. 2009; Lee et al. 2005; Pistilli et al. 2011.) However, myostatin also seems to enhance oxidative properties in skeletal muscle and regulate muscle energy metabolism (Relizani et al. 2014). According to some studies, blocking of ACVR2B signaling may disadvantage oxidative capacity and force production in healthy or dystrophic mice (Amthorn et al. 2007; Hulmi et al. 2013a; Rahimov et al. 2011; Relizani et al. 2014).

### **4.2.1 Effects on skeletal muscle size**

According to several studies, injections with sACVR2B-Fc increase muscle mass and also absolute muscle strength in some studies. (Akpan et al. 2009; Hulmi et al. 2013a; Lee et al. 2005; Pistilli et al. 2011.) Skeletal muscle mass seems to increase both via muscle cell hyperplasia and hypertrophy (in adults) following blocking of activin receptor signaling (McPherron et al. 1997).

sACVR2B-Fc treatment can increase muscle mass up to 60 % in wild type mice (Lee et al. 2005). Amthorn et al. (2007) has reported that the weight of extensor digitorum longus



muscle was increased by 66 % in myostatin knock-out mice and the muscle cross-sectional area was increased by 53% compared to wild-type mice. According to Cadena et al. (2010) sACVR2B-Fc treatment does not alter either muscle fiber type or number, thus, body weight and muscle mass increases due to fiber hypertrophy in wild-type mice. The study of Lee et al. (2005) confirms the issue. They reported that mean fiber diameter of wild-type mice increased 20 % compared to control group after sACVR2B-Fc treatment. (Lee et al. 2005.) Duration of the sACVR2B-Fc treatment has also effects on muscle mass. Acute sACVR2B-Fc treatment (one dose) may not have substantial effect on muscle weight, however, chronic treatment (for example, four doses in two weeks) increases muscle weight. (Rahimov et al. 2011.) During cancer cachexia muscle mass preservation is essential for survival. ACVR2B antagonism may prevent muscle loss also in cancer patients. (Zhou et al. 2010.)

#### **4.2.2 Effects on oxidative capacity**

Hulmi et al. (2013) reported that daily voluntary running activity decreased after sACVR2B-Fc treatment in Duchenne muscular dystrophy mice. Daily running distance was about 50 % less compared to mice without injections during the time muscles were growing. (Hulmi et al. 2013a.) Decreased running performance may also indicate, in some part, about decreased aerobic capacity. Increased fatigability has been demonstrated also with wild-type mice treated with sACVR2B-Fc by Relizani et al. (2014). Fatigability and elevated serum lactate levels were, however, more severe in Duchenne dystrophy mice in this study (Relizani et al. 2014.) Mice suffering from muscular dystrophy are known to have already compromised oxidative properties without blocking of ACVR2B signaling. Probably this may be one reason why they respond more severely to blocking of ACVR2B signaling (Percival et al. 2013).

There are some marks of changes in muscle oxidative metabolism after blocking of ACVR2B signaling. According to Relizani et al. (2014) expression of PGC-1 $\alpha$  was downregulated both in wild-type mice and Duchenne muscular dystrophy mice after sACVR2B-Fc treatment, however, the protein level was more severely reduced in dystrophy mice compared to wild-type mice. In addition Hulmi et al. (2013) reported in their study that markers of oxidative capacity (PGC-1 $\alpha$  and cytochrome c protein content, mtDNA content and citrate synthase activity) as well as capillary density decreased after treatment in healthy wild type mice. (Hulmi et al. 2013a; Hulmi et al. 2013b.) During sACVR2B-Fc treatment also gene sets involved in oxidative phosphorylation and electron transfer system have been reported to be downregulated in Duchenne muscular dystrophy mice (Kainulainen et al. 2015). The same result was noticed by Rahimov et al. (2011). sACVR2B-Fc treatment downregulated expression of genes related to oxidative metabolism and mitochondrial function in sACVR2B-Fc treated mice. 134 genes were regulated differently in chronic treated mice compared to control mice. When considering acute treatment, the number was 38 genes. (Rahimov et al. 2011.) To conclude, the longer treatment period seems to affect more severely oxidative properties. They also noticed 1.7 fold downregulation in PGC-1 $\alpha$  gene expression, however, the difference was not significant (Rahimov et al. 2011). Conversely, according to LeBrasseur et al. (2009) myostatin inhibition may activate PGC-1 $\alpha$  gene expression.

## **5 RESEARCH QUESTIONS AND HYPOTHESIS**

### **5.1 Research questions**

1. What kind of effects does extensively used chemotherapy agent doxorubicin have on skeletal muscle size, oxidative capacity at the muscle level, mitochondrial function and running performance?
2. What kind of effects does blocking of ACVR2B signaling have on skeletal muscle size, oxidative capacity at the muscle level, mitochondrial function and running performance in doxorubicin treated mice?

### **5.2 Hypothesis**

1. Doxorubicin decreases skeletal muscle size, oxidative capacity at the muscle level, mitochondrial function and running performance.

Arguments: According to previous studies, doxorubicin treatment causes loss of skeletal muscle mass (Gilliam et al. 2009; Gouspillou et al. 2015). Doxorubicin treatment increases fatigability, which may also indicate impaired running performance in incremental running test (Gilliam et al. 2009; Gilliam et al. 2013, Zombeck et al. 2013). The result may also predict aerobic capacity at some level. To add, doxorubicin has negative effects on some oxidative capacity biomarkers (citrate synthase) and mitochondrial function (Gilliam et al. 2013; Gouspillou et al. 2015.). On the other hand, mitochondrial content seems to be unaltered, because alterations in mitochondrial protein and mtDNA contents, as well as, PGC-1 $\alpha$  gene expression level have not been observed following doxorubicin administration.

2. Doxorubicin combined with blocking of ACVR2B signaling increases skeletal muscle size but decreases oxidative capacity at the muscle level, mitochondrial function and running performance.

Arguments: According to previous studies, blocking of ACVR2B signaling increases muscle mass up to 60 % (Lee et al. 2005). Muscle mass seems to increase due to fiber hypertrophy (Amthorn et al. 2007; Lee et al. 2005). Doxorubicin treatment has a negative effect on mitochondrial functions by itself (Gilliam et al. 2009; Gilliam et al. 2013; Gouspillou et al. 2015). In addition, according to previous studies physical activity decreases and oxidative capacity biomarkers are downregulated after sACVR2B-Fc injections in healthy and dystrophic mice. These biomarkers include cytochrome c protein content, mtDNA content and citrate synthase activity, as well as, PGC-1 $\alpha$  gene expression and protein content. (Hulmi et al. 2013a; Hulmi et al. 2013b Relizani et al. 2015.) In addition, downregulation of genes involved in oxidative metabolism and mitochondrial function are associated with sACVR2B-Fc injections (Kainulainen et al. 2015; Rahimov et al. 2011). However, there is currently no information available about combination effects of doxorubicin and sACVR2B-Fc treatment and mitochondrial function measured by high resolution respirometry.

## **6 METHODS**

### **6.1 Animals**

C57BL/6 male mice (Envigo), aged 9–10 weeks, were used in this study. During the study mice were housed in standard conditions (temperature 22°C, light from 8:00 AM to 8:00 PM) in individually ventilated cages in groups of two to three mice. Mice had free access to tap water and food pellets throughout the experiments.

### **6.2 Ethics statement**

The treatment of the animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the National Animal Experiment Board.

### **6.3 Experimental design**

The aim of this study was to investigate the effects of doxorubicin administration alone or combined with sACVR2B-Fc treatment on muscle size, aerobic capacity and oxidative capacity at the muscle level. Data collection was conducted in Wihuri Research Institute and University of Helsinki.

Two experiments were conducted in this study and both of them lasted 4 weeks. Experiments 1 and 2 had identical protocols and experiment 2 was organized to confirm the results and to conduct more profound analyses on the changes in body composition and oxidative metabolism.

The mice (n = 19 and n = 29 in experiments 1 and 2, respectively) were randomly organized into three groups: 1) PBS placebo treated controls (Ctrl, n = 6; n = 9), 2) doxorubicin treated group (Dox, n = 6; n = 10) and 3) doxorubicin treated group administered with sACVR2B-Fc (Dox + sACVR2B, n = 7; n = 10).

The mice were weighed every other day and the feed consumption was monitored once during doxorubicin treatment and once at the end of the treatment in experiment 2. The body composition was determined with DXA imaging in the beginning and at the end of the experiment 2. Treadmill running test was performed on average two days before sacrifice. Three mice were euthanized prematurely during the experiments because the humane end-point criteria were fulfilled. The final group sizes are reported above.

The mice were euthanized under inhalation anesthesia (isoflurane, Vetflurane) by heart puncture followed by cervical dislocation four weeks after the first doxorubicin injection. Tissue samples were collected upon euthanization. Tibialis anterior (TA), gastrocnemius (GA) and soleus muscles were immediately excised and weighed. A thin cross-sectional sample (5–10 mg) from the middle of the left TA muscle was collected for the OROBOROS analysis. The rest of the muscle was snap-frozen in liquid nitrogen. The final muscle weights were averaged from the left and right leg muscles. In addition the length of the tibia (mm) was measured in order to normalize skeletal muscle weights.

## **6.4 Doxorubicin dosage**

Doxorubicin (DOX) hydrochloride (Sigma Aldrich®) was used in this study. Before injections doxorubicin (10mg) was diluted into 2.5 milliliters of phosphate buffered saline (PBS) and to assure the sufficient amount of DOX for each animal the dose (mg/kg) was multiplied by the weight of the individual (kg). Further, the required DOX quantity (mg)

was divided by the concentration of our standard solution (4mg/ml) in order to determine the volume of needed DOX standard solution for injections.

Group number 2 was injected four times intraperitoneally with clinical dosage of doxorubicin (6 mg/kg in PBS) every third day during the first two weeks of the experiment. The last injection was timed 19 days before the euthanization. The cumulative dosage was 24 mg/kg. Control animals were injected with identical volumes of PBS in exactly the same time-points as doxorubicin injections.

## **6.5 sACVR2B-Fc production and dosage**

The protocol for sACVR2B-Fc production was previously described by Hulmi et al (2013b). In this study the same protocol for production and purification of the recombinant fusion protein was utilized. The ectodomain (ecd) of human sACVR2B and a human IgG Fc domain were first amplified by polymerase chain reaction with the help of plasmids. Afterwards they were fused together and the fusion protein was expressed in Chinese hamster ovary (CHO) cells grown in a suspension culture.

Group number 3 was treated with doxorubicin as well as administered intraperitoneally with sACVR2B-Fc (5 mg/kg in PBS) twice a week during the first two weeks of the experiment and once a week after that. sACVR2B-Fc administration was started prior to doxorubicin administration and the last dose was injected seven days before the euthanization.

## **6.6 Sample processing**

*RNA extraction.* Total RNA was extracted from the proximal part of the left TA muscle by using TRIsure reagent (Bioline). The purification was conducted with NucleoSpin® RNA II

columns by following the manufacturer's instructions. Further, the concentration and the purity of RNA were determined spectrophotometrically by measuring the absorbance of the samples at wavelengths of 260 and 280 nm.

*cDNA production.* RNA was converted to single stranded cDNA. Reverse transcription was conducted with iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories) by following manufacturer's protocol. cDNA was further used for gene expression analysis.

*Protein extraction and content.* Part of the left TA muscle was homogenized in 15-fold volume of ice cold HEPES homogenization buffer (20mM HEPES pH 7.4/5, 1mM EDTA, 5mM EGTA pH 7.4, 10mM MgCl<sub>2</sub>, 2mM DTT, 1% NP-40, 3% protease and phosphatase inhibitor cocktail (Pierce Protein Biology Products, Thermo Scientific), 100mM β-glycerophosphate) using PowerLyzer® 24 Bench Top Bead-Based Homogenizer (MO BIO) (3500 rpm, 2 x 15s, 10s interval) with compatible ceramic bead tubes (MO BIO). After the homogenization the samples were rocked/rotated for 30 at 4 °C. For the signaling analyses the homogenates were centrifuged at 10,000g for 10 minutes at 4 °C. The supernatants were collected and stored at -80 °C for further analysis. The analysis for total protein content was conducted by using the bicinchoninic acid (BCA) protein assay (Pierce Protein Biology Products, Thermo Scientific) with an automated KoneLab device (Thermo Scientific, Vantaa, Finland).

## **6.7 Citrate synthase activity**

The activity of citrate synthase (CS) was measured by using tibialis anterior muscle homogenate and an automated KoneLab device (Thermo Scientific, Vantaa, Finland). A special kit (Sigma-Aldrich, CS0720) was used for this analysis.



## 6.8 High resolution respirometry

In experiment 2, mitochondrial function of the left TA muscle was analyzed real-time with OROBOROS Oxygraph-2k, Innsbruck, Austria (Figure 8). After dissection, the samples were temporarily stored in Biops buffer. Before the analysis, the samples were homogenized with a shredder. The analysis of each sample was initiated within 30 minutes of dissection. Carbohydrate SUI protocol was conducted with only minor changes to the protocol previously reported by Lemieux et al. (2011) (Table 1).

The sample size was 2500  $\mu\text{l}$  of TA muscle homogenate and two samples were analyzed at the time. LEAK state (MPG-L) was measured in the absence of ADP and injecting Malate and Pyruvate into the sample chambers. Later Glutamate was injected in order to increase generation of NADH. OXPHOS capacity (MPG-P) was achieved by adding then ADP and Magnesium  $\text{Mg}^{2+}$  to generate saturating levels of ADP. In the next step cytochrome c was added to make sure that the outer mitochondrial membrane was intact. (Kivelä et al. 2014; Lemieux et al. 2011.) Cytochrome c is released from mitochondria due to patophysiological conditions or mistakes during sample preparation and this may alter respiration. By adding cytochrome c in reaction quality control can be made. (Gnaiger 2014.)

The respiration capacity of electron transport system (SMPG-P) (electron input through complexes I and II) was generated by injecting Succinate and the maximal ETS capacity was measured by adding FCCP uncouplers stepwise into the sample chamber. After that complex I was inhibited by Rotenone injection (ROT) which indicates electron input only through complex II. Furthermore, complexes II and III were also inhibited with Malonic acid and Antimycin A in order to measure residual oxygen consumption (ROX). (Kivelä et al. 2014; Lemieux et al. 2011.) If  $\text{O}_2$  concentration dropped too low (below 250  $\mu\text{l}$ ) during the experiment the reoxygenation was performed with  $\text{H}_2\text{O}_2$ .

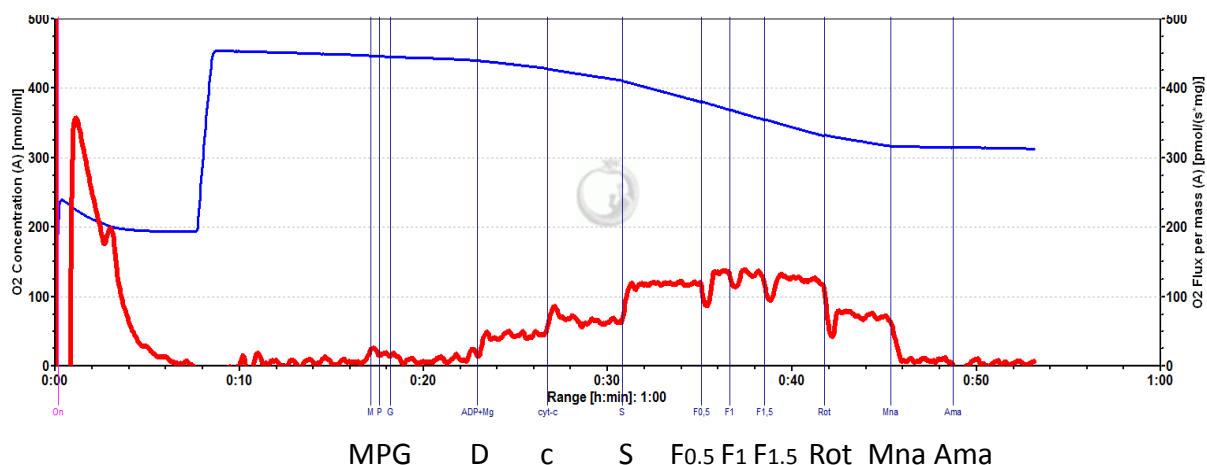


FIGURE 8. Example of the OROBOROS Oxygraph-2k high resolution respirometry analysis. The blue line represents O<sub>2</sub> concentration [nmol/ml] and the red line represents O<sub>2</sub> flux per mass [pmol/(s\*mg)]. Injection order: M, P, G, D, c, D, F0.5, F1, F1.5, Rot, Mna and Ama (Table 1).

TABLE 1. Carbohydrate SUIT-protocol. Used substrates, uncouplers and inhibitors, concentrations and injection order.

Titration (substrates, uncouplers and inhibitors)	Stock concentration	Injection volume ( $\mu$ l)	Final concentration	Abbreviation	State
Malate	0.8 M	5	2mM	M	MPG-L (LEAK)
Pyruvate	2 M	5	5mM	P	
Glutamate	2 M	10	10mM	G	
ADP + Mg <sup>2+</sup>	0.5 M	5	1.25mM	D	MPG-P (OXPHOS)
Cytochrome c	4 mM	5	10 $\mu$ M	c	
Succinate	1 M	20	10mM	S	SMPG-P
FCCP	1 mM	1	0.5 $\mu$ M	F0.5, F1, F1.5	ETS
Rotenone	0.2 mM	2	0.2 $\mu$ M	Rot	ROT
Malonic acid	2 M	5	5 $\mu$ M	Mna	ROX
Antimycin A	5 mM	1	2.5 $\mu$ M	Ama	

## 6.9 Western immunoblot protein analysis

Protein content of PGC-1 $\alpha$ , cytochrome c and OXPHOS were analyzed with Western immunoblot protein analysis, which bases on electrophoresis and immunoblotting. The homogenized TA muscle samples were first diluted with ddH<sub>2</sub>O according to total protein content of each sample. The final protein content was 30  $\mu$ g in 15  $\mu$ l of a sample. The protocol for OXPHOS varied compared to the protocol used for cytochrome c and PGC-1 $\alpha$  in some parts. The protocol for OXPHOS is discussed in the end of this section.

*SDS-page.* 15  $\mu$ l of diluted samples were mixed with 15  $\mu$ l 2 x Laemmli sample buffer (Bio-Rad # 161-0737) including 5 %  $\beta$ -mercaptoethanol. Samples were then centrifuged briefly and put on the heat block for 10 minutes at 95 °C. After that centrifugation was repeated and samples were put on ice for 5 minutes. In the next step 25  $\mu$ l of each sample (containing approximately 30  $\mu$ g of total protein) was loaded to a gel (4-20 % Criterion™ TGX™ Precast Gels, Bio-Rad # 567-1094). In addition, 6  $\mu$ l molecular weight marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad # 161-0374) was added to the first well of the gel. Next the loaded gel was put into the electrophoresis chamber (two gels at the same time) with electrophoresis running buffer (2.5 mM Tris Base, 19.2 mM glycine, 0.01 % SDS, ddH<sub>2</sub>O). Proteins were separated with electrophoresis by running gel with 270 V for approximately 40 minutes in ice bucket and at the temperature of +4 °C. SDS is negatively charged and binds to proteins in relation to their size. When bound to proteins SDS makes them negatively charged and during electrophoresis proteins migrate with different speeds from negative pole to positive pole. In this way proteins are separated according to their molecular weights.

*Blotting.* In next step separated proteins were blotted from gel to an absorbent PVDF membrane (Hybond-P, GE Healthcare Life Sciences, RPN303F). Before blotting gel was put in transfer buffer (2.5 mM Tris Base, 19.0 mM glycine, (pH adjusted to 8.3 with HCl),

10% methanol, ddH<sub>2</sub>O) for 30 minutes and PVDF membrane was activated briefly with methanol and ddH<sub>2</sub>O one after the other. Further, PVDF membrane was balanced approximately 15 minutes in transfer buffer. The blotting sandwich was built and all the parts for it were wetted with transfer buffer. The blotting sandwich was piled inside a plastic cassette in following order: a scotch-brite pad, a sheet of blotting paper, the electrophoresis gel, the PVDF membrane, a sheet of blotting paper and a scotch-brite pad. Closed cassette and an ice brick were immersed in a blotting chamber (two cassettes at the same time) filled with transfer buffer. Blotting was conducted with electric current of 300 mA for approximately 2.5 hours at the temperature of +4 °C in the ice bucket. Magnetic mixing was used to stir transfer buffer inside the blotting chamber during blotting.

*Ponceau S staining, blocking and primary antibody incubation.* Following blotting membranes were stained with Ponceau S and imaged. This was done in order to make certain that proteins were transferred properly and later to determine the relative protein content of each lane by quantification. This was conducted with Molecular Imager ChemiDoc XRS System (Bio-Rad) and Quantity One 4.6.3 –software (Bio-Rad). Next the membrane was cut into stripes containing only one protein. Membranes were blocked in blocking solution (TBS + 0.1% Tween-20 + 5% non-fat milk) at the room temperature for 2 hours with gentle rocking motion. This was done to avoid unspecific protein binding. Following blocking membranes were incubated overnight with specific primary antibodies (appendix 1.) for each protein at 4°C with gentle rocking.

*Secondary antibody incubation and detection.* Next morning membranes were washed 4 x 5 minutes in TBS-Tween and incubated in HRP-conjugated secondary antibodies (Appendix 1.) for one hour at room temperature and with gentle rocking. Secondary antibodies were washed off 5 x 5 minutes with TBS-Tween with strong rocking. After this membranes were incubated with detection kit (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Protein Biology Products, Thermo Scientific #34096) for 5 minutes and imaged with

Molecular Imager ChemiDoc XRS System (Bio-Rad) and Quantity One 4.6.3.-software (Bio-Rad).

*Stripping.* If a blotting strip was used for determination of another protein stripping was performed in order to remove bound antibodies that could prevent new antibodies to bind. Strips were incubated with Restore<sup>TM</sup> Western Blot Stripping Buffer (Thermo Scientific, #21059) for approximately 30 minutes while rocking at the room temperature. Strips were rinsed then 5 times with ddH<sub>2</sub>O and 3 x 5 minutes in TBS-Tween. After the stripping protocol was completed, strips were blocked again, which was followed by previously explained steps after blocking with new antibodies.

*Quantification and data analysis.* Proteins were quantified with Quantity One 4.6.3. - software (Bio-Rad) in order to determine the relative quantities of each studied protein. This is based on the knowledge that larger quantity of protein binds more antibodies and this can be seen as a more intensive and bigger band during detection. The quantities of GAPDH and Actin band (42 kDa) of Ponceau S staining were used to normalize the raw data and to confirm that possible variation in total protein content does not affect results. All the values were normalized to the mean of Ponceau and GAPDH. These mean values were further divided by the mean value of the whole gel in order to eliminate the gel to gel variation and normalized to DOXO-group value which was set to be 1.00.

*Protocol for OXPHOS.* The Western immunoblotting protein analysis protocol for OXPHOS differed from other proteins only in SDS-page. These samples were heated on a heat block for 7 minutes at 50 °C.

## 6.10 RT-qPCR

Expression of PGC-1 $\alpha$  isoforms were studied by using real-time quantitative polymerase chain reaction (RT-qPCR) method. RT-qPCR is considered as a golden standard for measuring gene expression. (Campbell & Farrel 2012, 377-378). In addition, RT-qPCR is a suitable method for isoform quantification (Vandenbroucke et al. 2001). This method applies polymerase chain reaction and amplification of the target sequence in complementary DNA (cDNA) samples can be followed real-time with the help of fluorescence (Real-Time PCR Applications Guide, 2-3). The reaction is performed in a thermocycler because amplification is based on the three temperature cycling phases; first high temperature denatures the DNA strands, then lower temperature anneals the primers on the template and finally temperature is raised again to start the elongation phase done by DNA polymerase. (Kubista et al. 2006.) DNA-binding dye SYBR green I and hydrolysis probe TaqMan are the most often used chemistries for the RT-qPCR. (Real-Time PCR Applications Guide, 9.) In this study SYBR green I was used.

In this study each reaction contained 2  $\mu$ l (10 ng) of a sample and 18  $\mu$ l of mix thus the total reaction volume was 20  $\mu$ l in each well. The mix contained 10  $\mu$ l iQ SYBR Supermix (Bio-Rad Laboratories), 1  $\mu$ l (0.6  $\mu$ M) 5' primer, 1  $\mu$ l (0.6  $\mu$ M) 3' primer and 6  $\mu$ l RNase free water (H<sub>2</sub>O). Primers are designed as complementary to the ends of the DNA sequence chosen for amplification and are called forward and reverse primer. (Campbell & Farrel 2012, 377-378.) (Table 2 and Appendix 2). First all the samples, primers and reagents were melted on ice. Then primers were diluted 1:10 (30  $\mu$ l primer and 270  $\mu$ l RNase free water) and the mix was made. All the procedures were conducted in laminar flow hood in order to avoid contaminations. In the next step 18  $\mu$ l mix were loaded on each well of 96-well plate for PCR. After that 2  $\mu$ l each sample were loaded. The plate was sealed with a film and centrifuged 2000 rpm for 30 seconds. The quantification was conducted with CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Temperature cycles were + 95  $^{\circ}$ C for

10 minutes, 40 cycles at +95 °C for 10 seconds, at isoform specific annealing temperature for 30 seconds, at +72 °C for 30 seconds. Amplification protocol was followed by melting curve protocol, where fluorescence was measured at temperatures between +65 °C and +95 °C with 0.5 °C increments for 5 seconds. The annealing temperature for PGC-1 $\alpha$  Exon1a-derived isoforms were +60.6 °C, for PGC-1 $\alpha$  Exon 1b-derived +60 °C, for PGC-1 $\alpha$  Exon 1c-derived +60 °C, for Total-NT-PGC-1 $\alpha$  +61°C, for 36b4 +60°C, for GAPDH +60°C and Rn18s 60 °C.

Because of the nonspecific nature of SYBR green I a melt-curve analysis was conducted to check that the primers were amplifying only the target sequence in question and no unspecific products or primer-dimers were formed. In addition to melt-curve analysis, the length of amplified sequence and possible unspecific products and primer-dimers were checked with agarose gel electrophoresis (Appendix 2). The amplification efficiencies of each gene were between 90-106 %. Every sample was analyzed in triplicate and nontemplate controls were added in each run in order to exclude potential contaminations. 36b4 was used as a housekeeping gene for the normalization of the results. This was chosen because 36b4 was the most stable of studied housekeeping genes (36b4, GAPDH and Rn18s). Data analysis was conducted by using efficiency corrected  $\Delta\Delta C_t$  method (Pfaffl 2001).

TABLE 2. Primers for PGC-1 $\alpha$  isoforms. Primers are designed as complementary to the ends of the target gene sequence and are called forward and reverse primer.

<b>Isoform</b>	<b>Forward primer 5' → 3'</b>	<b>Reverse primer 5' → 3'</b>
<b>PGC-1<math>\alpha</math> Exon1a- derived</b>	CATGTGCAGCCAAGACTCTG	ACACCACTTCAATCCACCCA
<b>PGC-1<math>\alpha</math> Exon1b- derived</b>	CCATGGATTCAATTTTGAAATGTG	GTTCGCAGGCTCATTGTTGT
<b>PGC-1<math>\alpha</math> Exon1c- derived</b>	AAGTGAGTAACCGGAGGCATTC	TTCAGGAAGATCTGGGCAAAGA
<b>Total- NT- PGC-1<math>\alpha</math></b>	TCACACCAAACCCACAGAAA	CTGGAAGATATGGCACAT
<b>36b4</b>	GGCCCTGCACTCTCGCTTTC	TGCCAGGACGCGCTTGT
<b>GAPDH</b>	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
<b>Rn18s</b>	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

## 6.11 Dual-energy X-ray absorptiometry

Changes in body composition were studied with DXA imaging. This DXA analysis was performed with Lunar PIXImus II densitometer (GE Healthcare) in the beginning and at the end of the experiment 2. Head and tail were excluded from the analysis. Before DXA analysis, the mice were anesthetized by intramuscular injection of ketamine + xylazine (3:1)



(Ketaminol® and Rompun®, respectively) to both hindlimbs (m. quadriceps femoris, 5-15 µl/limb depending on the weight of the animal).

## **6.12 Treadmill running test protocol**

Maximal running capacity of the mice was measured with a treadmill test until exhaustion. The running protocol was incremental and consisted of 5 min at 9 m/min, 5 min at 12 m/min, 5 min at 15 m/min, 2 min at 17 m/min, 2 min at 19 m/min etc. until exhaustion. All mice were familiarized with treadmill running prior to test.

## **6.13 Data processing and statistical analyses**

IBM SPSS Statistics version 22 for Windows (SPSS, Chigago, IL, USA) was used for all statistical tests. Body and muscle weight data as well as treadmill running data were analyzed with parametric one-way ANOVA and Boferroni post-hoc tests. Results of experiments 1 and 2 were pooled together for figures when possible. Non-parametric variables, such as Western Blot data, were analyzed with Kruskal-Wallis test and Mann-Whitney U was used as a post-hoc test. Post-hoc p-values were corrected afterwards with Holm-Bonferroni method. Statistical significance was set at  $p < 0.05$ . All the figures were done by Microsoft Power point and Excel software.

## 7 RESULTS

### 7.1 Body composition and muscle weight

Doxorubicin treatment seems to have a clear and predicted effect on body weight compared to Ctrl and Dox+sACVR2B groups (Figure 9). Doxorubicin treatment decreased body weight significantly ( $p < 0.001$ ) compared to control group, however, sACVR2B-Fc administration counteracted the loss of body weight. Body weight of Dox + sACVR2B group was significantly ( $p < 0.001$ ) higher compared to Dox group, body weight increased even over the level of the control group. In addition, as expected doxorubicin decreased lean body mass significantly compared to control group ( $p < 0.05$ ) and blocking of activin receptor signaling increased it significantly ( $p < 0.001$ ) over the level of the control group (Figure 10). This is consistent with the results of skeletal muscle weight indicating that skeletal muscle mass decreased significantly ( $p < 0.05$ ,  $p < 0.01$ ) after using doxorubicin and sACVR2B-Fc administration increased significantly ( $p < 0.001$ ) skeletal muscle mass over the level of the control group (Figures 11). Fat mass change (%) was significantly ( $p < 0.001$ ) negative for Dox and Dox + sACVR2B groups compared to control group (Figure 10).

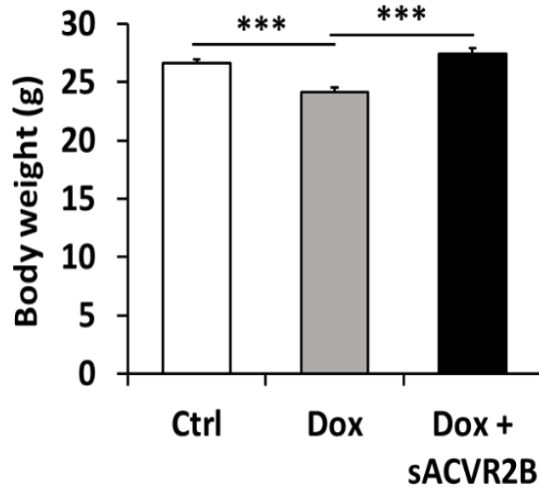


FIGURE 9. The body weight of mice at the end of the experiment. \*\*\* depicts significantly different from Dox.  $p < 0.001$ .

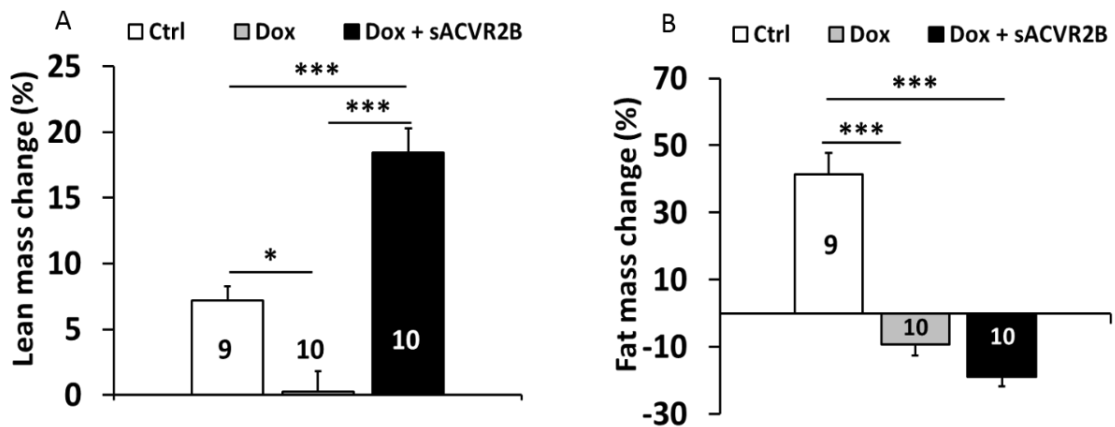


FIGURE 10. (A) Changes in lean body mass change (%) assessed with DXA at the end of the experiment. \* depicts significantly different from Ctrl  $p < 0.05$ , \*\*\* depicts significant difference Ctrl vs. Dox + sACVR2B and Dox vs. Dox + sACVR2B  $p < 0.001$ . (B) Changes in fat mass change (%) assessed with DXA at the end of the experiment. \*\*\* depicts significantly different Ctrl vs. Dox and Ctrl vs. Dox + sACVR2B  $p < 0.001$ .

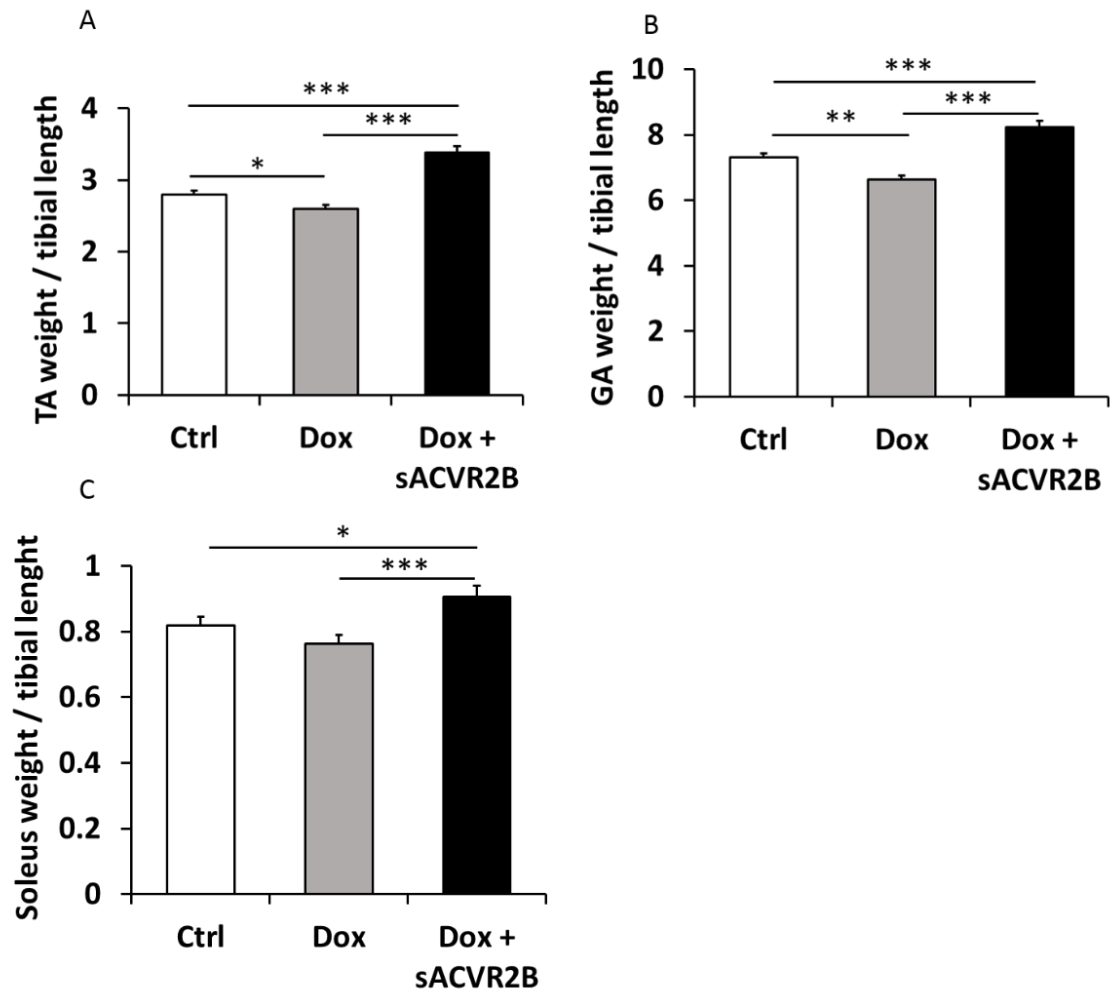


FIGURE 11. (A) The weight of tibialis anterior (TA) at the end of the experiment. \* depicts significant difference Ctrl vs. Dox  $p < 0.05$ , \*\*\* depicts significantly different from Dox + sACVR2B  $p < 0.001$ . (B) The weight of gastrocnemius (GA) at the end of the experiment. \*\* depicts significant difference Ctrl vs. Dox  $p < 0.05$ , \*\*\* depicts significantly different from Dox + sACVR2B  $p < 0.001$ . (C) The weight of soleus at the end of the experiment. \* depicts significant difference Ctrl vs. Dox + sACVR2B  $p < 0.05$ , \*\*\* depicts significant difference Ctrl vs. Dox + sACVR2B  $p < 0.001$ .

## 7.2 Running performance

The results of incremental running test show that doxorubicin treatment significantly decreased running capacity (Figure 12). However, blocking of activin receptor signalling did not further alter running performance, which was not expected.

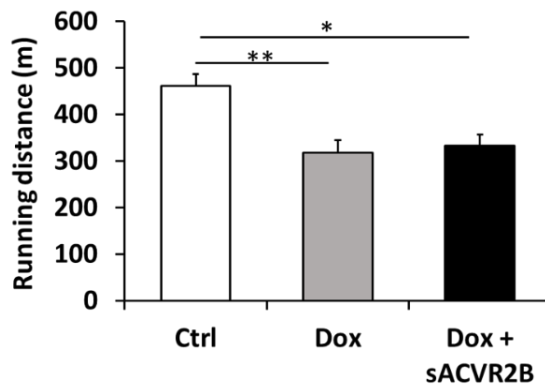


FIGURE 12. The results of incremental treadmill running test at the end of the experiment. \* depicts significant difference Ctrl vs. Dox + sACVR2B  $p < 0.05$ , \*\* depicts significant difference Ctrl vs. Dox + sACVR2B  $p < 0.01$ .

## 7.3 Oxidative capacity at the muscle level and mitochondrial function

### 7.3.1 Mitochondrial proteins and citrate synthase

This study did not show any significant differences in total respiratory subunit (OXPHOS) protein levels between study groups (Figure 13). However, some differences were observed when individual OXPHOS complexes were considered (Figure 14). There was significant difference in complex I protein level between Ctrl and Dox + sACVR2B groups ( $p < 0.05$ ),

Ctrl group having higher protein level. sACVR2B-Fc administration had also decreasing effect on CI protein level ( $p < 0.001$ ) compared to doxorubicin only treatment. In addition, complex V protein level was significantly ( $p < 0.05$ ) decreased in Dox + sACVR2B group compared to Dox and Ctrl groups.

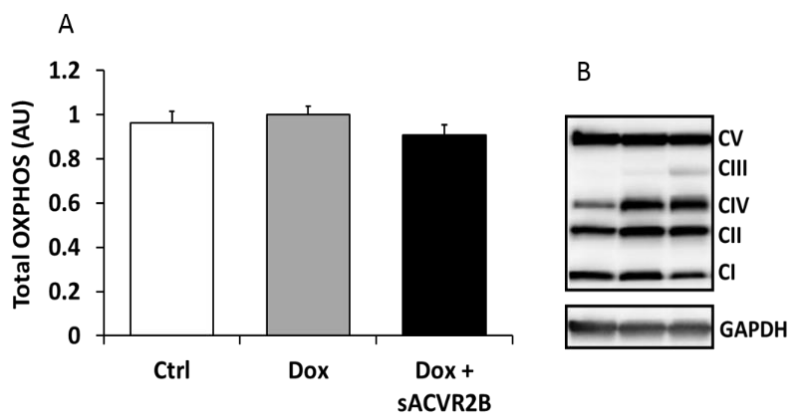


FIGURE 13. (A) Total OXPHOS protein content (AU) at the end of the experiment. (B) Western immunoblotting images of OXPHOS complexes CI-CV and GAPDH. Group order from left: Ctrl, Dox and Dox + sACVR2B.

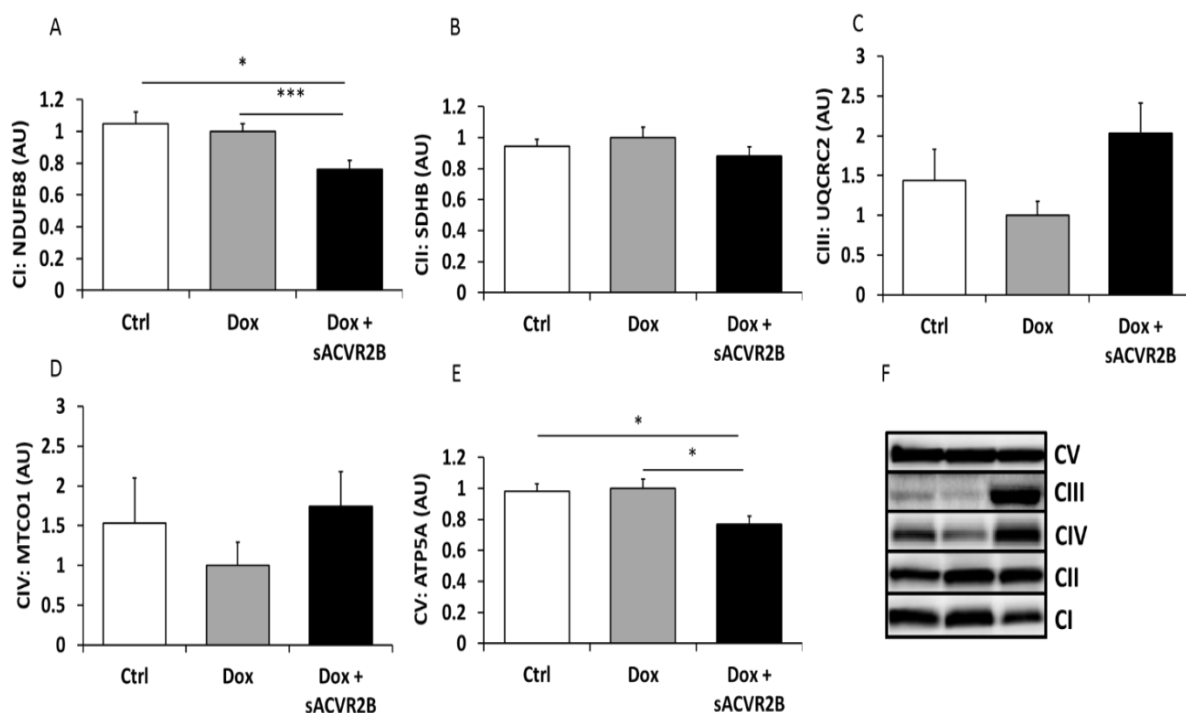


FIGURE 14. (A) Protein level of complex CI at the end of the experiment. \* depicts significant difference Ctrl vs. Dox + sACVR2B  $p < 0.05$ , \*\*\* depicts significant difference Dox vs. Dox + sACVR2B  $p < 0.001$ . (B) Protein level of complex CII at the end of the experiment. (C) Protein level of complex CIII at the end of the experiment. (D) Protein level of complex CIV at the end of the experiment. (E) Protein level of complex CV at the end of the experiment. \* depicts significant difference Ctrl vs. Dox + sACVR2B  $p < 0.05$ , \* depicts significant difference Dox vs. Dox + sACVR2B  $p < 0.05$ . (F) Western immunoblotting images of OXPHOS complexes.

There were no significant differences in cytochrome c and PGC-1 $\alpha$  protein levels between study groups (Figure 15 and 16). Porin/VDAC1 protein levels were increased significantly compared to Dox group ( $p < 0.01$ ) and Ctrl group ( $p < 0.05$ ) (Figure 17). In addition, citrate synthase activity (Figure 18) was significantly ( $p < 0.01$ ) increased in Dox + sACVR2B group compared to doxorubicin only treated group. There was also a trend ( $p = 0.054$ ) that

Dox + sACVR2B group would had increased citrate synthase activity levels compared to Ctrl.

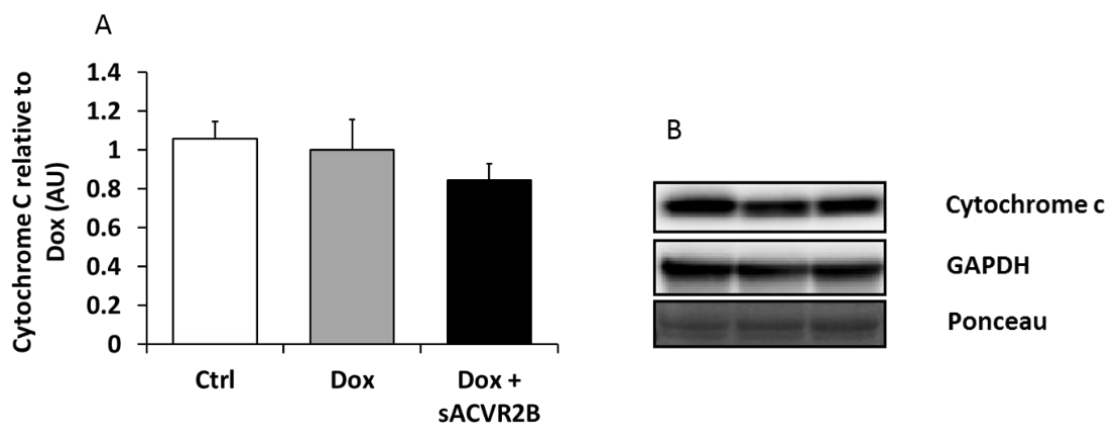


FIGURE 15. (A) Cytochrome c protein content at the end of the experiment. (B) Western immunoblotting images of cytochrome c, GAPDH and Ponceau. Group order from left to right: Ctrl, Dox and Dox + sACVR2B.

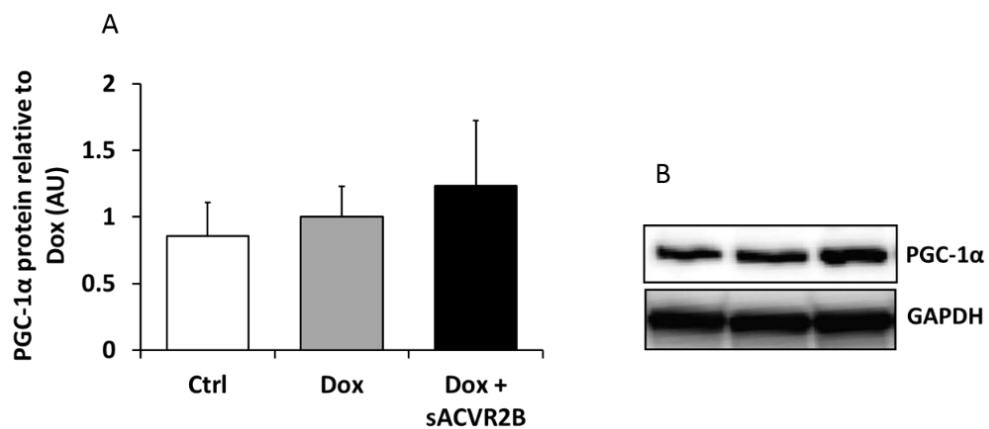


FIGURE 16. (A) PGC-1α protein content at the end of the experiment. (B) Western immunoblotting images of PGC-1α and GAPDH. Group order from left to right: Ctrl, Dox and Dox + sACVR2B.



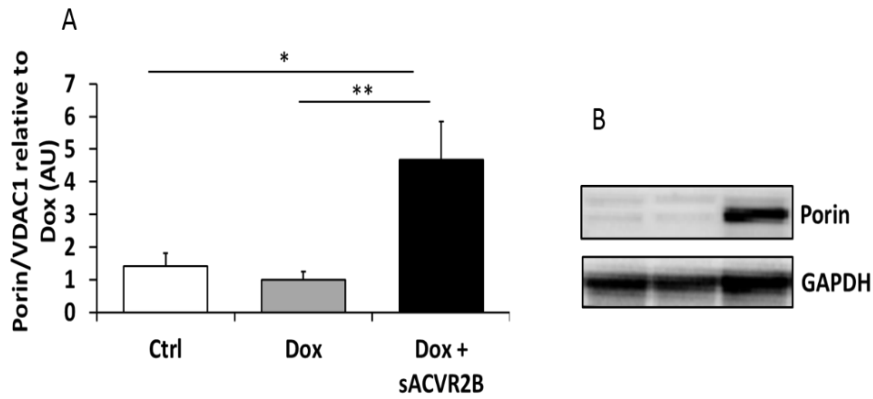


FIGURE 17. (A) Porin/VDAC1 protein content at the end of the experiment. \* depicts significant difference Ctrl vs. Dox + sACVR2B  $p < 0.05$ , \*\* depicts significant difference Dox vs. Dox + sACVR2B  $p < 0.01$ . (B) Western immunoblotting images of porin/VDAC1 and GAPDH. Group order from left to right: Ctrl, Dox and Dox + sACVR2B.

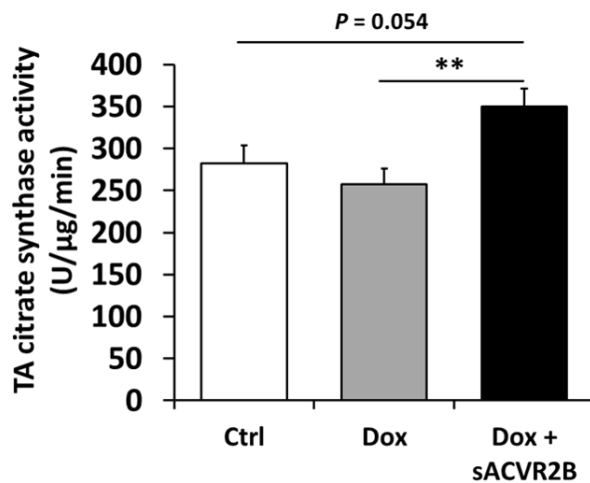


FIGURE 18. Citrate synthase activity at the end of the experiment. \*\* depicts significant difference Dox vs. Dox + sACVR2B  $p < 0.01$ , a trend between Ctrl and Dox + sACVR2B  $p = 0.054$ .

### 7.3.2 Mitochondrial function

Figure 19 presents the results from high resolution respirometry analysis (OROBOROS). These results were corrected with the measured residual oxygen consumption (ROX) values. The results of three study groups did not differ significantly from each other. The results were normalized per mg of muscle, per absolute muscle weight, mitochondria volume and ETS capacity but any significance was not observed. However, there seems to be a trend for variation of Dox + sACVR2B group from two other groups. According to results cytochrome c seems to alter respiration somewhat.

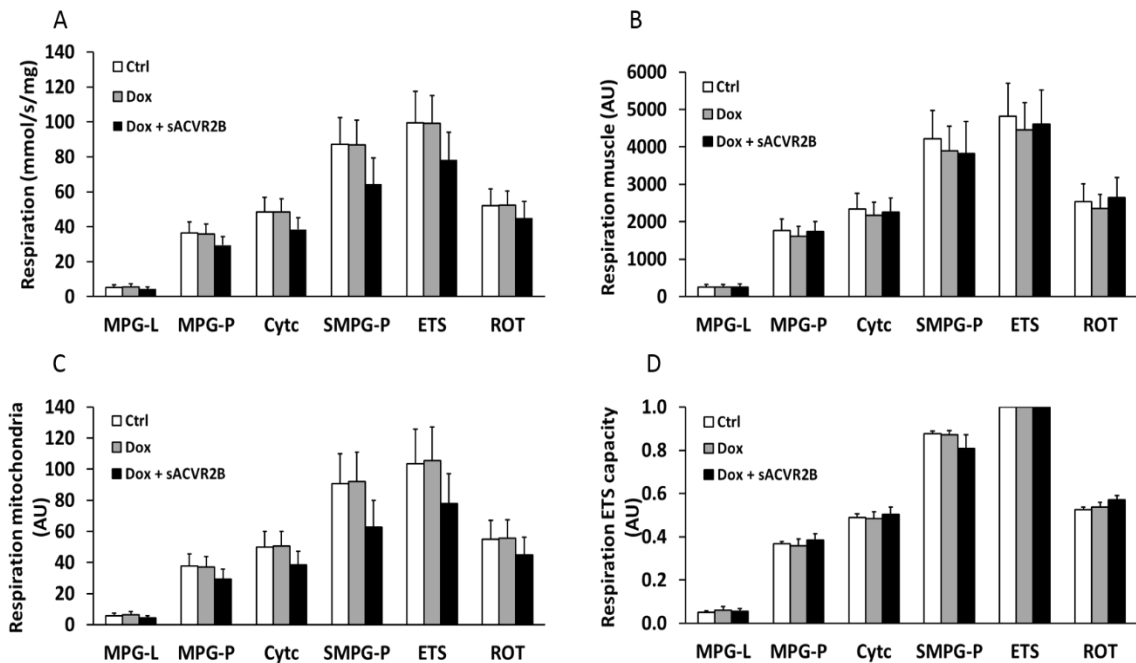


FIGURE 19. High resolution respirometry results at the end of the experiment (A) per mg of muscle, (B) per absolute muscle weight, (C) per mitochondria and (D) per ETS capacity. Results are corrected with ROX-values.

## 7.4 PGC-1 $\alpha$ gene expression

Gene expression of different PGC-1 $\alpha$  isoforms were not altered significantly after doxorubicin only and doxorubicin combined with sACVR2B-Fc treatments. The results are reported in Figure 20.

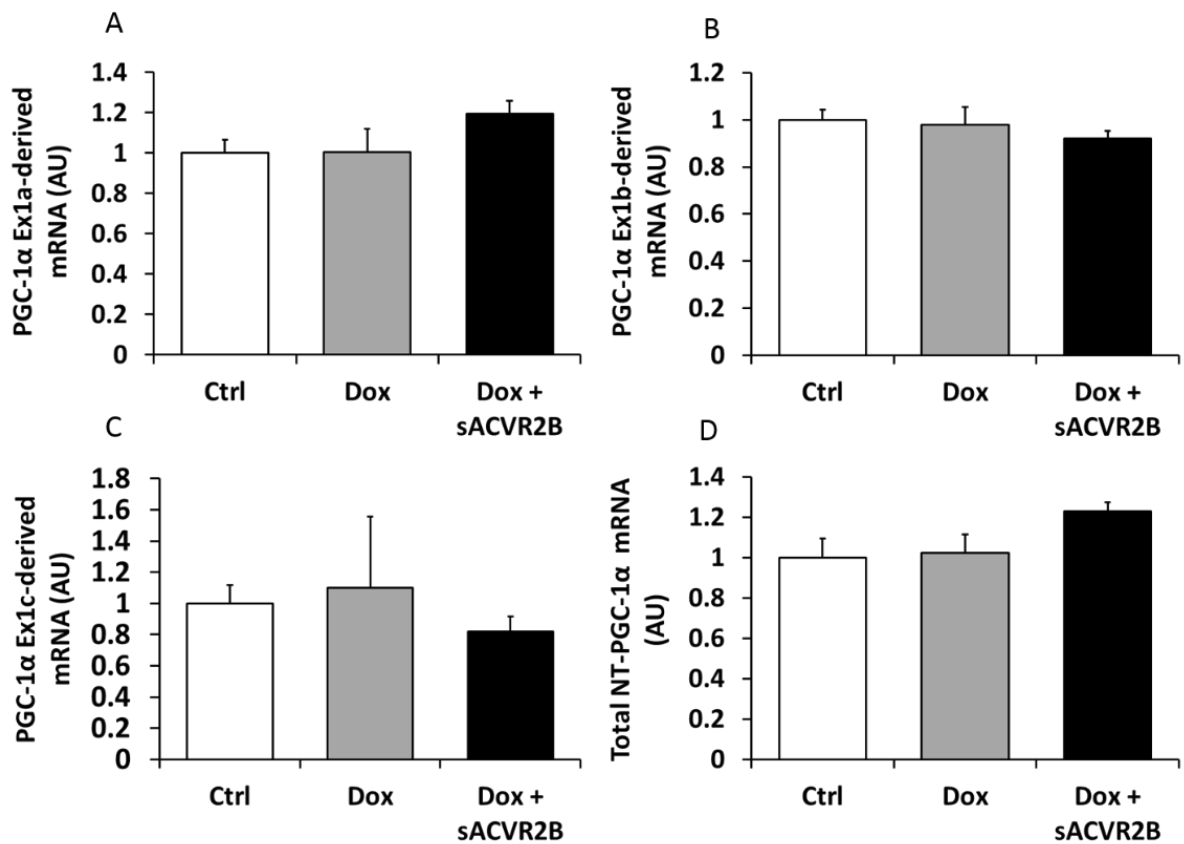


FIGURE 20. mRNA levels of PGC-1 $\alpha$  isoforms (AU) at the end of the experiment. (A) PGC-1 $\alpha$  Exon 1a-derived. (B) PGC-1 $\alpha$  Exon 1b-derived. (C) PGC-1 $\alpha$  Exon 1c-derived. (D) Total NT-PGC-1 $\alpha$ . The housekeeping gene 36b4 was used for the normalization of the results.

## 8 DISCUSSION

The aim of this thesis was to study the effects of extensively used chemotherapy agent doxorubicin on skeletal muscle size, oxidative capacity at the muscle level, mitochondrial function and running performance. The second aim was to examine, if blocking of activin receptor signaling could alleviate the expected side effects of doxorubicin, when combining it with sACVR2B-Fc administration. The study design composed of two similar four-week experiments with three different study groups; Dox, Dox + sACVR2B and Ctrl. The main findings of this study were that doxorubicin treatment significantly decreased body mass, skeletal muscle size and running performance. However, the blocking of activin receptor signaling prevented these negative changes in body composition. Even though sACVR2B-Fc administration had some negative effects on the studied biomarkers of oxidative capacity at the muscle level, the running performance and the mitochondrial function remained at the same level compared to Dox group.

*Doxorubicin administration.* According to this study doxorubicin treatment reduced severely body weight, skeletal muscle mass and fat mass, as well as impaired running performance measured by incremental running test. Cancer cachexia is characterized by decreased body weight and skeletal muscle mass. This syndrome is known to reduce quality of life and increase the mortality rate of cancer patients, due to negative relationship between low muscle mass and chemotherapy toxicity as well as time to tumor progression (Argilés et al. 2014; Prado et al. 2009). It is also one possible explanation for muscle weakness and fatigue in cancer patients (Cospér et al. 2012). Possible mechanisms for skeletal muscle loss are decreased muscle protein synthesis, increased protein breakdown, increased apoptosis and decreased ability to regeneration (Argilés et al. 2014). During cancer cachexia myostatin and activin A levels are also known to increase and negatively regulate muscle growth (Costelli et al. 2008; Lee et al. 2001; Loumaye et al. 2015;

McPherron et al. 1997). With these studied variables it is impossible to report the exact mechanism, which caused doxorubicin induced skeletal muscle loss in this study. Previous studies have proposed, that cancer cachexia is most likely related to disease itself as well as to cancer treatments (Gorselink et al. 2006; Le Bricon et al. 1995). This study demonstrated, that cancer cachexia may also occur following chemotherapy alone.

Even though running performance impaired, mitochondrial function measured by high resolution respirometry did not alter significantly after doxorubicin administration compared to control group. The same trend was observed in the biomarkers of oxidative capacity; the protein contents of respiratory chain subunits (OXPHOS) in total and individually, cytochrome c and porin/VDAC1, as well as in citrate synthase activity level. In addition, doxorubicin did not have any clear effects either on PGC-1 $\alpha$  gene expression or protein level, which are related to mitochondrial biogenesis. Thus, it seems that oxidative capacity may not explain impaired running performance.

According to current knowledge, this kind of aerobic running test has not been used in previous studies concerning chemotherapy. Previous results from alternative tests follow the same kind of trend compared to this study. Gilliam et al. (2013) reported that doxorubicin had negative effect on the whole-body oxygen consumption, voluntary activity and total energy expenditure. These variables were measured by indirect calorimetry. In addition, Zombeck et al. (2013) reported that doxorubicin treatment decreased voluntary wheel running in rats by 30 % compared to control animals.

According to this study, alterations in mitochondrial function and static biomarkers of oxidative capacity at the muscle level did not seem to explain impaired running performance. However, there were significant changes in muscle weight in the Dox group versus control group, and that could be one possible explanation for decreased physical function (Buford et al. 2012; Cicoira et al. 2001; Haykowsky et al. 2013; Prior et al. 2016).

Incremental running test is a common way to estimate aerobic capacity (Koch & Britton 2001). Impaired running performance may then also indicate, at some level, decreased aerobic capacity, which has been widely linked to increased mortality rate and occurrence of metabolic risk factors (Koch et al. 2011; Kokkinos et al. 2008; Myers et al. 2002; Wisløff et al. 2005). This is obviously an undesirable side-effect of chemotherapy, which is planned to cure patient of the disease and not to expose to other diseases.

Previously, Gilliam et al. (2013) and Gouspillou et al. (2015) have reported effects of doxorubicin on body composition, oxidative capacity and mitochondrial function. There are some differences between this study and the study conducted by Gouspillou et al. (2015). One main difference is that Gouspillou et al. (2015) combined doxorubicin with dexamethasone, which might have caused some variability in the results compared to this study. In addition, the study designs were longer and the doxorubicin dosage was also different and more periodic; so called “early group” was treated with two chemotherapy cycles and euthanized four weeks after the latter cycle and the “late group” was treated with four chemotherapy cycles and euthanized 12 weeks following the last cycle. The chemotherapy cycle composed of one injection with doxorubicin (10 mg/kg) and five injections with dexamethasone (2.5 mg/kg) and this was administered every three weeks. (Gouspillou et al. 2015.)

Gouspillou et al. (2015) reported that in the late group body weight decreased 23 % and gastrocnemius muscle mass decreased 12 %. The early group did not have any significant changes in gastrocnemius muscle mass or mitochondrial function (respiratory capacity). However, in the late group mitochondrial function reduced significantly; maximal respiration capacity was 36 % lower. However, it was reported that the coupling efficiency of mitochondrial oxidative phosphorylation was not altered in either of these groups. Gouspillou et al. (2015) also reported that OXPHOS protein content as well as PGC-1 $\alpha$  protein content and gene expression did not change due to chemotherapy in the late group.

However, the late group had significantly decreased citrate synthase activity, but this effect was not observed in the early group. They proposed that alteration in mitochondrial function is not due to decreased mitochondrial content. (Gouspillou et al. 2015.)

Gilliam et al. (2013) injected rats with one dose of doxorubicin (20 mg/kg) and studied the short term effects (2, 24 and 72 hours after injection). Also in this study doxorubicin negatively affected body composition 72 hours after injection. Mitochondrial function was studied with the same high resolution respirometry instrument that was used in this study. The results altered between different time points. Two hours after doxorubicin administration mitochondrial complexes I and II supported respiration decreased. However, according to this study mitochondrial function seemed to restore partially at the time point 24 hours after administration. At the last time point mitochondrial function decreased again. Mitochondrial function seems to vary soon after doxorubicin administration. The maximal respiratory capacity did not alter between doxorubicin treated group and control group and there were no changes in OXPHOS protein content compared to control group 72 hours following doxorubicin injection. (Gilliam et al. 2013.) In Gilliam et al. (2013) study the doxorubicin dosage was high; almost the cumulative dosage used in this study (24 mg/kg).

It can be seen that the results of this study are for the most part (body composition and protein contents) in line with the previous studies of Gilliam et al. (2013) and Gouspillou et al. (2015). There is, however, variability in the results of mitochondrial function, which may be partly explained by the differences in the study designs.

*sACVR2B-Fc administration.* sACVR2B-Fc administration seemed to counteract the loss of body weight and skeletal muscle mass in this study, which is a good result when considering cancer cachexia and the common health problems related to it. This result is also in line with previous studies with sACVR2B-Fc (Akpan et al. 2009; Hulmi et al. 2013a; Lee et al. 2005; Pistilli et al. 2011). On the other hand, according to previous studies sACVR2B-Fc

administration may compromise severely oxidative capacity and physical activity (Hulmi et al. 2013 a; Relizani et al. 2014). In this study Dox + sACVR2B group had impaired running performance compared to control mice, but there was not a significant difference between Dox + sACVR2B and Dox groups, which may be a promising result when considering the aerobic capacity and overall health of cancer patients.

There were not any significant alterations in mitochondrial function or total respiratory chain subunit (OXPHOS) protein level after sACVR2B-Fc administration. To point out, there was, however, a minor trend that Dox + sACVR2B group had decreased high resolution respirometry results compared to two other study groups. Maybe this result could have reached statistical level with bigger sample size. In addition, oxidative capacity marker cytochrome c protein level did not change significantly. The same kind of trend was observed in PGC-1 $\alpha$  gene expression and protein levels, which may indicate that mitochondrial biogenesis was not increased. On the other hand, oxidative capacity markers citrate synthase activity level and porin/VDAC1 protein content increased significantly following sACVR2B-Fc administration compared to doxorubicin only treatment. In addition, protein levels of individual respiratory chain subunit (OXPHOS) complexes I and V decreased significantly in Dox + sACVR2B group compared to Ctrl and Dox groups. Static biomarkers of oxidative capacity may, in part, explain preserved oxidative capacity and running performance in this study.

The observed alterations in the biomarkers of oxidative capacity may indicate compensating effect of citric acid cycle and porin/VDAC1 to impaired electron transport chain function. Complexes I and II accept electrons from NADH and FADH<sub>2</sub> in the beginning of the electron transport chain. Complex V is an enzyme called ATP synthase which returns electrons back to mitochondrial matrix and this phosphorylates ADP to ATP. Citrate synthase functions in the beginning of citric acid cycle. It is unknown currently, if other enzymes are activated in citric acid cycle following sACVR2B-Fc administration.



Porin/VDAC1 is an ion channel protein between cytosol and mitochondrial matrix. Porin/VDAC1 protein levels were increased significantly after sACVR2B-Fc administration compared to Dox and Ctrl groups. This may indicate that molecules, for example NADH and ADP/ATP, are transported more effectively between cytosol and mitochondria. This may compensate impaired electron transport chain function in order to maintain oxidative capacity and running performance.

The relationship between porin/VDAC1 and apoptosis is still not clear (McCommis & Baines 2012). However, there are a few studies about cancer and porin reporting that cancer is associated with increased expression of porin/VDAC1 and reduced expression inhibits cancer cell growth (Arif et al. 2014; Ko et al. 2014). This could be an important target of cancer treatment in the future, but more research is still needed to confirm this relationship. Relizani et al. (2014) reported, that reduced oxidative capacity in muscular dystrophic mice might be related to decreased porin/VDAC1 protein levels. However, in this study sACVR2B-fc administration increased porin/VDAC1 levels. It is not yet known, if this will cause different outcomes of sACVR2B-Fc administration when considering chemotherapy caused cachexia and muscular dystrophy.

In addition, in this study muscle mass was increased due to the blocking of activin receptor signaling. According to some earlier studies, increase in muscle mass and force production are linked (Akpan et al. 2009; Pistilli et al. 2011). Previous studies have also reported, that increased lean body mass is associated positively with aerobic capacity and physical function (Buford et al. 2012; Cicoira et al. 2001; Haykowsky et al. 2013; Prior et al. 2016). These may also explain preserved oxidative capacity and running performance in this study. Increased skeletal muscle mass and force production may help to continue running motion longer and, in addition, it may be associated with better capillarization which improves oxygen delivery to muscles (Buford et al. 2012; Prior et al. 2016). In other hand, there are also contradictory results; Hulmi et al. (2013a) reported that two weeks after sACVR2B-Fc

administration muscle mass increased, but running activity decreased in muscular dystrophic mice. In this study, however, the study design was longer. Maybe increased muscle mass, due to blocking of activating receptor signaling, was not so effective in oxidative metabolism compared to “normal” muscle mass.

*Practical applications.* sACVR2B-Fc administration may be a promising strategy to treat skeletal muscle loss induced by chemotherapy. Maintenance of body weight and skeletal muscle mass is important for recovery and survival during cancer. Decreased skeletal muscle mass and aerobic capacity are linked to many severe diseases. Chemotherapy also decreases mitochondrial functions and increases fatigue. Contrary to previous studies with muscle dystrophy models, in this study sACVR2B-Fc administration did not further impair mitochondrial and physical functions during chemotherapy. When considering the existing literature and the hypothesis of this study, this is an interesting and promising result that needs more research in the future.

*Limitations and strengths of this study.* The study design (timing of muscle collection etc.) needs to be taken into consideration when investigating the results of this study. For example, in experiment 1 incremental running test was conducted in some part only a day before sacrifice, which might have caused some minor acute exercise effects in some mice. In experiment 2 there were approximately two days apart, which was chosen to exclude all possible acute effects of the exercise and to confirm the results. Previous studies have reported time- and dose-dependent effects of chemotherapy on skeletal muscle (Gilliam et al. 2013; Gouspillou et al. 2015; Swain et al. 2003). For example, longer treatment period may induce more severe skeletal muscle loss (Gouspillou et al. 2015). Experiments 1 and 2 lasted 4 weeks, which should be long enough to see effects of doxorubicin on skeletal muscle according to literature. Maybe there would have been some significant changes in high resolution respirometry measurement in shorter or longer experiment, because running performance and oxidative capacity biomarkers changed already during this study. Longer

and shorter studies have previously reported alterations in mitochondrial function (Gilliam et al. 2013; Gouspillou et al. 2015). In addition, the bigger sample size might have revealed some significances, for example, in the high resolution respirometry results as was discussed earlier. However, there seems to be no link between mitochondrial function and oxidative capacity biomarkers necessarily. The results might also have changed by varying doxorubicin dosage. The dosage used in this study was clinically relevant and the results are then more easily applied. For example, in Gilliam et al. (2013) study the doxorubicin dosage was almost the cumulative dosage used in this study (24 mg/kg). In addition, according to results addition of cytochrome c seemed to alter respiration during high resolution respiration measurement somewhat, and this may tell about some damages in mitochondrial membranes due to sample preparation or effects of the study design.

In addition to the physiological doses of doxorubicin used, the strength of this study was widely studied oxidative capacity and function; static biomarkers, real-time mitochondrial function and running performance were all examined. New methods were also used; the running capacity was measured by using aerobic incremental running test, which has not been used widely in studies considering chemotherapy and its effects. Results from experiments 1 and 2 were pooled together when possible which increased the sample size and made results more reliable. In both of these experiments the results were comparable. In addition, all analyzes and measurements were designed very carefully. For example, all the samples were analyzed in triplicate during the RT-qPCR method and the anthropometric variables were measured with the very accurate device DXA, in order to confirm the results. The statistical tests were also conducted very carefully; post-hoc p-values were corrected afterwards with Holm-Bonferroni method. This study gave also new information about doxorubicin combined to blocking of activin receptor signaling. Blocking of activin receptor signaling has been widely studied among wild-type and muscle dystrophy studies, but never with chemotherapy.

*Future study proposals.* In this study only chemotherapy was involved, however, in clinical cases a tumor has an effect too. Therefore, in the future it would be interesting to clarify the basic interaction between chemotherapy and different cancer types (Kazemi-Bajestani et al. 2015). In addition, it would be important to study how cancer cachexia and cancer itself affect these studied variables. The research group has continued the study of doxorubicin effects also in cancer and has replicated doxorubicin effects in there (Nissinen et al. manuscript submitted). It is already known according to other studies, that cancer cachexia compromises functions of mitochondria (Constantinou et al. 2011).

Metabolic syndrome and its risk factors are general topics nowadays. An interesting study area in the future would be the effects of cancer and its treatments on the occurrence of metabolic risk factors. The general metabolic risk factors are elevated triglyceride and glucose levels as well as increased blood pressure and waist circumference (Alberti et al. 2009). Low aerobic capacity is related to occurrence of these factors (Koch et al. 2011; Kokkinos et al. 2008; Myers et al. 2002; Wisløff et al. 2005). In this study these risk factors were not studied after doxorubicin and sACVR2B-Fc treatments. It would be interesting to know, if these risk factors followed the trend that was seen in running performance predicting aerobic capacity in some part. There is also new information that metabolic risk factors may worsen the prognosis of cancer patients (Micucci et al. 2016).

There are also many interesting topics concerning exercise and cancer. Although cancer treatments are effective nowadays, they have severe side-effects including fatigue, nausea, anxiety, muscle weakness, muscle mass loss and stress. However, it seems that exercise could alleviate these side-effects and improve quality of life, as well as coping with daily activities. (Argilés et al. 2012; Ascensão et al. 2005; Ehrman et al. 2013, 390-392; Gerritsen & Vincent 2015; Kavazis et al. 2014, McMillian & Newhouse 2011.) Different exercise modes can counteract cancer side-effects differently. Endurance exercise promotes aerobic

capacity and resistance exercise activates the Akt/mTOR signaling cascade which is known to increase muscle mass (Argilés et al. 2012; Bondine et al. 2006; White et al. 2011).

Endurance exercise increases aerobic capacity and performance by improving oxidative capacity and buffering chemotherapy toxicity (Argilés et al. 2012; Kavazis et al. 2014). This may occur via mitochondria-mediated mechanisms (Ascensão et al. 2005; Kavazis et al. 2014). In addition, endurance exercise affects positively metabolic risk factors. (Argilés et al. 2012.) Endurance exercise is also known to increase capillarization in the skeletal muscle of healthy individuals which increases aerobic capacity (Jensen et al. 2004). Last but not least, endurance exercise improves quality of life in cancer patients (Gerritsen & Vincent 2015). There is not so much knowledge about effects of resistance training and combined training on cancer patients compared to than endurance training (McMillian & Newhouse 2011). According to some studies, resistance training is effective to improve lean body mass, muscular strength, self-esteem and chemotherapy completion rate in chemotherapy patients and to stop bone loss and general fatigue in cancer survivors (Courneya et al. 2007; Kampshoff et al. 2015, Winter-Stone et al. 2011).

To add, inflammation is linked to cancer and proinflammatory cytokine levels are increased after chemotherapy. This may be also related to chemotherapy side-effects. (Al-Majid et al. 2009; Smith et al. 2014.) Exercise may decrease inflammatory response by increasing anti-inflammatory cytokines and decreasing proinflammatory cytokines, which helps, in part, to preserve muscle mass and function. (Al-Majid et al. 2009; Argilés et al. 2012.) Resistance training is known to reduce cytokine tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Greiwe et al. 2001). Therefore, also resistance exercise may have a positive effect on inflammation response during cancer.

Fatigue, anemia as well as cardiac dysfunction and poor nutritional status can be contraindications for exercise, and exercise prescriptions needs to be planned individually

for each cancer patient. These contraindications combined with exercise can even worsen existing cachectic state. (Argilés et al. 2012.) Anemia could be treated also in combination with endurance exercise and this has been reported to enhance exercise effects such as PGC-1 $\alpha$  gene expression and mitochondrial biogenesis, as well as skeletal muscle strength (Pin et al. 2015). Figure 21. summarizes the possible effects of endurance and resistance exercise on cancer patients. Exercises as a cancer treatment would need, however, still more research (Grande et al. 2015; McMillian & Newhouse 2011).

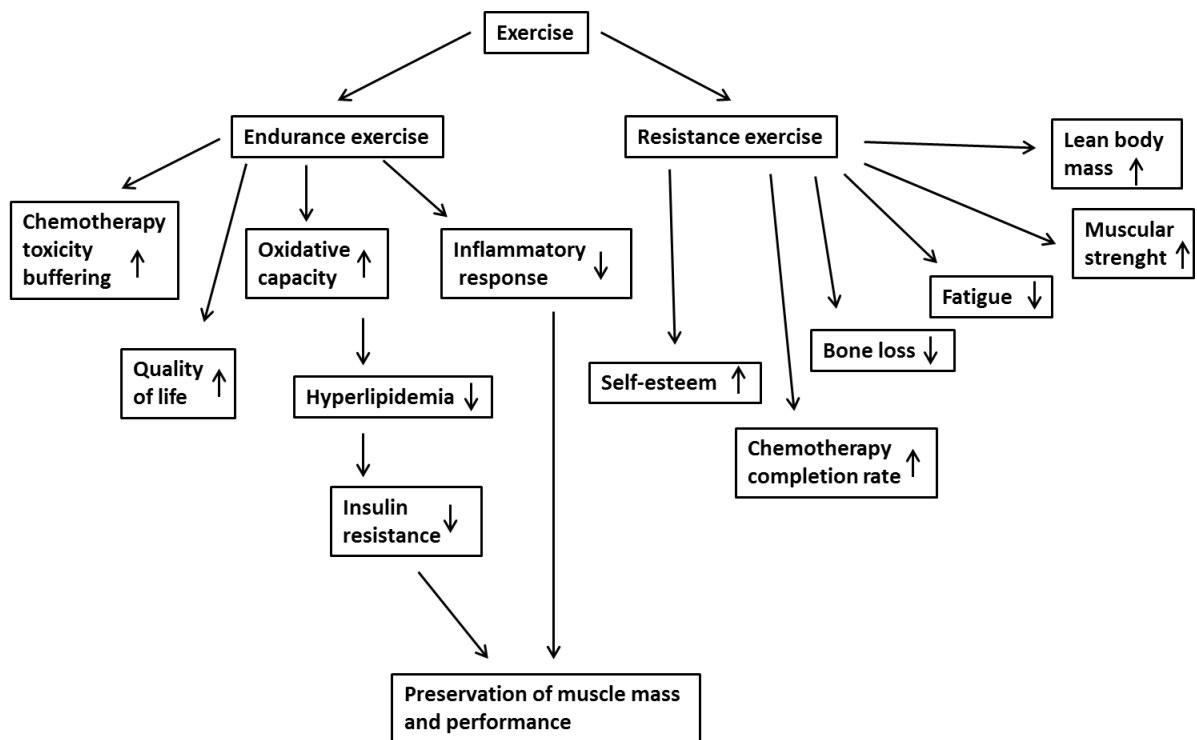


FIGURE 21. Exercise as a cancer treatment; a summary of the possible effects of endurance and resistance exercise on cancer patients. (Adopted from: Argilés et al. 2012; Ascensão et al. 2005; Courneya et al. 2007; Ehrman et al. 2013, 390-392; Gerritsen & Vincent 2015; Kampshoff et al. 2015; Kavazis et al. 2014; McMillian & Newhouse 2011; Winter-Stone et al. 2011).

During cancer cachexia muscle mass preservation is essential for survival (Zhou et al. 2010), but there are no accepted drugs for muscle wasting. Myostatin and activin blockers, however, are recent possible agents. These are still mainly studied with test animals, partly due to ethical reasons. However, there are differences in physiology of rodents and humans, and in the future there needs to be studies also with humans, in order to confirm the study results and apply this information in a field of medicine. In addition, it would need more research to specify the mechanisms leading to skeletal muscle loss during doxorubicin treatment. This information might also lead to other possible strategies to counteract skeletal muscle loss more specifically.

## 9 CONCLUSION

This study was first to show decreased maximal running capacity after chemotherapy. This occurred, however without skeletal muscle mitochondrial alterations. Blocking of activin receptor signaling may be a promising strategy to treat some of the side-effects of extensively used chemotherapy agent doxorubicin. According to previous studies doxorubicin is known to decrease skeletal muscle mass and function. In this study, doxorubicin-induced muscle loss was prevented by combinatorial treatment with sACVR2B-Fc (soluble ligand binding domain of ACVR2B fused to the Fc domain of IgG) administration. In addition, sACVR2B-Fc administration did not further compromise running performance and mitochondrial functions compared to doxorubicin only treated group, even though previous studies have reported that sACVR2B-Fc may severely compromise oxidative capacity in dystrophic mice. In that study, the researchers speculated that a possible reason may have been decreased protein content of mitochondrial protein porin/VDAC1. In this study, supporting the non-altered running capacity, porin protein was not decreased due to sACVR2B-Fc, but even increased. Running performance, oxidative capacity and mitochondrial function may be related to aerobic capacity in some part. Poor aerobic capacity and low muscle mass increase cancer related fatigue, expose to other diseases as cancer cachexia and metabolic syndrome and worsen prognosis. Maintaining skeletal muscle mass and aerobic capacity is essential for survival during cancer and blocking of activin receptor signaling is a possible strategy for that. In the future, this study needs to be replicated during cancer in order to apply results more clinically.



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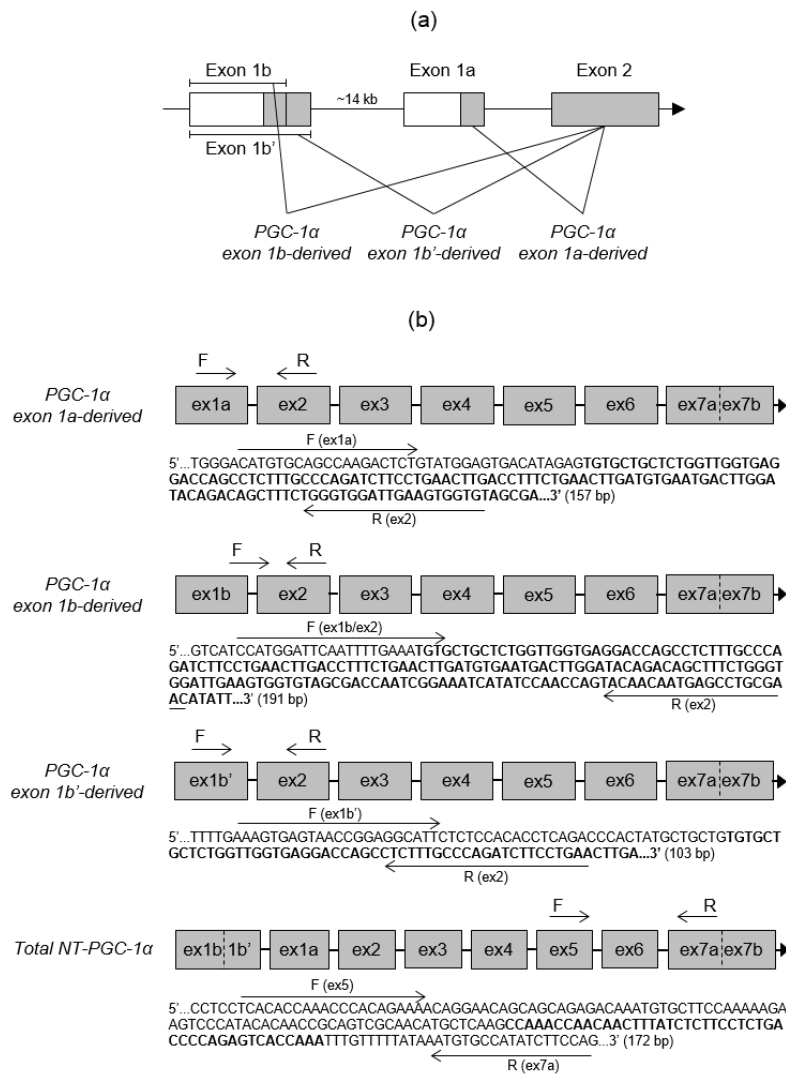
## APPENDIX 1. LIST OF PRIMARY AND SECONDARY ANTIBODIES

<b>Protein</b>	<b>Primary antibody</b>	<b>Secondary antibody</b>
GAPDH	Rabbit Ab to GAPDH (Abcam)	Horseradish peroxidase- conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)
PGC-1 $\alpha$	Rabbit Ab to PGC-1 $\alpha$ (C-terminal, Calbiochem)	Horseradish peroxidase- conjugated secondary anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)
Cytochrome c	Goat Ab to Cytochrome c (Santa Cruz Biotechnology)	Horseradish peroxidase- conjugated secondary anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)
Porin/VDAC1	Rabbit Ab to Porin/VDAC1 (Abcam)	Horseradish peroxidase- conjugated secondary anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)
OXPPOS complexes	Mouse Ab cocktail against subunits of the five OXPPOS complexes (Abcam)	Horseradish peroxidase- conjugated secondary anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, PA, USA)



## APPENDIX 2. PGC-1A ISOFORMS (A), PRIMER DESIGN (B) AND GEL ELECTROPHORESIS (C)

Figures: Mika Silvennoinen



(c)

