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FUNCTIONAL AND METABOLIC ALTERATIONS IN SKELETAL MUSCLE IN RESPONSE TO PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STRESSORS

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Functional and metabolic alterations in skeletal muscle in response to physiological and pathophysiological stressors.

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family and friends.

POPULAR SCIENCE SUMMARY OF THE THESIS

In our everyday lives we use our body's muscles both consciously and unconsciously. From getting out of bed in the morning, to jogging through the park or cooking dinner. There is not one daily activity where skeletal muscles are not involved. The moment when we realized how often we use them is probably when they start to fail us. When we experience a weakness, consequently, all tasks seem to be much more difficult to perform. The quality of life will not be the same once every task becomes a struggle.

This is something that breast cancer patients are experiencing. Breast cancer is the most commonly diagnosed cancer form in women, with 19.3 million new cancer cases diagnosed every year worldwide, meaning approximately 12.9 % of women will be diagnosed with breast cancer during their lifetime. Rightfully, a lot of attention is focused on treating cancer, however, at the same time many patients experience comorbidities and side-effects in association with breast cancer and its treatment. For instance, weakness and reduction in muscle strength are a frequently reported complication among patients with breast cancer. The weakness even persists after completion of cancer treatment, and thus afflicting their life at home after their battle with breast cancer. Patients with breast cancer are not prone to lose a lot of weight or muscle mass during disease progression and treatment, therefore the weakness must have a different cause, which currently is not well understood. Apart from the experienced muscle weakness, breast cancer and skeletal muscle are also linked on another level. It has been long known that muscle training and physical exercise benefit breast cancer patients. Already moderate exercise can improve the chances of the breast cancer treatment. But what is the exact interplay between breast cancer and skeletal muscle? In the clinic, this question is not so easy to answer, as it is difficult to distinguish between the effects of the breast cancer itself and the cancer treatment. The interaction would be important to know, to find a way to reverse muscle weakness, or how to help those breast cancer patients who feel too unwell to exercise themselves.

In the first part of the thesis (**paper I**), we investigated how breast cancer affects skeletal muscle and the beneficial effects of exercise. In particular, this study used a mouse model of breast cancer (PyMT mice). These mice mimic the situation of human patients with breast cancer, including weaker muscle force and reduced endurance, without any sign of the muscles being smaller. Interestingly, we could show that if the mice had access to a running wheel for four weeks, the training increased the endurance and muscle strength of the breast cancer model to an equal level of a healthy mouse control group. Additionally, we observed that the tumor became less aggressive, although the tumor size remained the same.

Next, we were interested in finding explanations on a molecular level. The capability of the human or mouse body to perform muscular movements and exercise depends greatly on their inner cellular powerhouse, the mitochondria. The food we eat is broken down to sugar or fats and converted by the mitochondria to provide energy that can be utilized by tissues or organs, such as skeletal muscle. For this energy supply, a series of biochemical reactions in the

mitochondria is required. Mitochondria and their functions can be damaged by certain molecules. Among those cellular factors are so called cytokines. Cytokines are small signals which the body usually uses to communicate between tissues or organs. Breast cancer is known to hijack these signals for its own advantage. For example, it has been shown that breast cancer can signal to the body that the tumor needs more blood flow, hence more oxygen and nutrients, for its own survival. Subsequently, an inflammatory environment will be created around the tumor. These inflammatory signals are then sent along in the blood stream, where they can also reach and affect the muscle. In this thesis, we found higher levels of these inflammatory signals in the blood stream, as well as in the skeletal muscle itself, in the investigated mouse model of breast cancer. Additionally, we found that certain "building blocks" of the mitochondria were constantly less available in the mouse model of breast cancer, which indicated that the stress or inflammatory signals sent by the cancer affected the energy-producing mitochondria in the skeletal muscle and thus the muscles' ability to produce force. Impressively, the exercise training (four weeks access to a running wheel) was partially able to reverse the inflammatory effects of the cancer by reducing the cellular stress and increasing the mitochondria energy production in the muscle. However, the exercise was not able to revers all the cellular stress and mitochondrial deficits back to normal non-cancer mice levels. This suggests that the cancer still interferes with the exercise effect of the muscle. In **paper I** we could show for the first time that stress signals by the breast cancer might be responsible for the muscle weakness in breast cancer mice or patient, and that exercise was able to counteract the stress signals and the breast cancer- induced muscle weakness.

As described in **paper I**, in addition to the breast cancer, comorbidities (associated conditions) such as above-described muscle weakness, can afflict patients with breast cancer. Additional to the effect of the breast cancer itself, the anti-cancer therapy can cause side-effects as well. However, not all side-effects are well understood, and some might develop years after the patient received anti-cancer treatment. In the second part of my thesis (**paper II**) we therefore focused on a novel treatment (CX), which is currently in clinical trials as a new cancer treatment. In the mouse model of breast cancer CX was able to decrease the tumor size significantly within four weeks of treatment. To further investigate any unknown side-effects of CX treatment we observed the behavior of the mice, including how much they move or eat, how much oxygen (O_2) they breathed in and how much carbon dioxide (CO_2) they exhaled. Surprisingly we found that mice treated with the drug (CX) ate more, but also used more O_2 and exhaled more CO_2, independent of tumor presence. Based on those values we could calculate that the CX-treated mice rather use sugars for their energy production in the mitochondria instead of fat. Interestingly, the evaluation of blood glucose in CX-treated mice showed an excessive increase of blood glucose levels, meaning that CX-treated mice had high levels of sugar circulating in their blood. This is an indication usually observed in patients with diabetes. Next, we wanted to understand why these blood sugar levels were so high. Since the mice had not received any high sugar-content food it must come from the way how the sugar in the regular food was utilized in the body. Skeletal muscle is a tissue which uses a lot of sugar since it needs the energy to produce force. There are special molecules which transport the

sugar from the blood stream into the muscles. In the case of CX-treated mice, the sugar transport into the muscles was decreased. That means that CX treatment impacts the sugar transport into the skeletal muscle and by that influences the availability of sugar for the muscles to produce energy. Furthermore, we observed that the CX treatment exerts the same pharmacological inhibition on skeletal muscle than on breast cancer, which might be the mechanism how the CX drug affects the skeletal muscle after a treatment injection is given. In **paper II** we could show that a novel anti-breast cancer treatment CX, despite having great anti-cancer effects, leads to metabolic changes likely to cause diabetes. This is especially important, as high blood glucose is known to fuel the tumor cells further and have a negative impact on the cancer progression. For the future, a better understanding of the side-effects of the treatment might lead to a better follow-up of the patients or to different combinational therapies to prevent patients from developing diabetes in combination with anti-cancer treatment.

Skeletal muscles can be affected by different stressors. As discussed in **paper I and II** the stress can stem from "external stressors" such as breast cancer or breast cancer treatment. But skeletal muscle is also exposed to "internal stressors", like oxidative stress. Oxidants are regularly produced during the cellular function, for example during muscle contractions, and usually cleared away by the body's own antioxidant system. This is a normal and constant process within cells and can be seen as a seesaw, where the sides of the oxidants and the antioxidants have to be kept in balance. However, in the case of excessive amounts of oxidants the balance tips over and it generates an unbalanced state, so called oxidative stress.

In the last part of this thesis (**paper III**) we examined how an enzyme, responsible for releasing stored glucose in skeletal muscle, is affected by a specific oxidant called peroxynitrite ($ONOO^-$). When glucose is available in abundance, it can be transported into the skeletal muscle and saved in its storage-form, glycogen. When in sudden need of energy, like a sprint, glycogen can quickly be converted back to glucose. This is done by another special molecule of the cell, the so-called glycogen phosphorylase to create energy quickly without the series of reactions in the mitochondria. We aimed to investigate how this molecule (phosphorylase) is affected by oxidative stress in the muscle. Hence, we directly exposed skeletal muscle from mice to different oxidants. When the muscles were broken down, and the oxidants could easily access the muscle, we found that the function of the molecule phosphorylase was inhibited by the oxidants, since no glycogen could be broken down to glucose and the muscle could not draw energy from its own glucose-storage. This inhibition was particular to the oxidant peroxynitrite, and not to other oxidants. When intact muscles were used for the same experiment, we could not measure an inhibition of the of molecule phosphorylase was not measurable. But intact muscles treated with the oxidant peroxynitrite were weaker and produced less force. Next, we wanted to know if added antioxidants (DTT, NAC) would tip the balance and let us observe cellular produced oxidants. Interestingly, although the antioxidants did not inhibit the phosphorylase, the muscle force was reduced by the antioxidants, and they appeared weaker. In **paper III** it was demonstrated how the delicate position of the oxidants and antioxidants scale can have considerable impact on the force production and energy supply of skeletal muscle.

This thesis aimed to shed more light on how different stressors, both internal and external, can affect the performance and function of skeletal muscle. The three paper (**paper I-III**) have created more knowledge on the cellular processes through which stress is generated and how it can influence the force and energy supply of skeletal muscle. With this thesis I hope to contribute to a better understanding of these processes and thereby, in the future, impact the lives of patients suffering from muscle-related afflictions.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG DER DISSERTATION

In unserem Alltag nutzen wir jederzeit bewusst und unbewusst unsere Körpermuskulatur. Beim morgendlichen Aufstehen, beim Joggen im Park bis hin zum Kochen des Abendessens. Es gibt keine einzige tägliche Tätigkeit, bei der die Körper- oder Skelettmuskulatur nicht beteiligt sinf. Wahrscheinlich bemerken wir erst wie oft wir sie benutzen, wenn die Muskeln beginnen zu versagen. Wenn die Muskeln versagen, wenn wir uns schwächer fühlen, sind alle alltäglichen Aufgaben viel schwieriger zu meistern. Folglich leidet die Lebensqualität des Betroffenen darunter stark.

Dieses beschriebene Gefühl kennen Brustkrebspatientinnen gut. Brustkrebs ist die am häufigsten diagnostizierte Krebsform bei Frauen, mit jährlich 19,3 Millionen neu diagnostizierten Krebsfällen weltweit, was bedeutet, dass etwa 12,9 % aller Frauen im Laufe ihres Lebens mit Brustkrebs diagnostiziert werden. Der Behandlung oder Therapie des Brustkrebses wird zu Recht viel Aufmerksamkeit geschenkt, jedoch leiden viele Brustkrebspatientinnen unter Begleiterscheinungen und Komorbiditäten im Zusammenhang mit der Brustkrebs Erkrankung. Beispielsweise ist die Schwächung oder Verringerung der Muskelkraft eine häufig genannte Begleiterscheinung bei Patientinnen mit Brustkrebs. Diese bleibt oft auch lange nach Abschluss der Krebsbehandlung bestehen und beeinträchtigt somit das Leben zu Hause weiter, sogar nach dem eigentlichen Kampf gegen den Brustkrebs. Allerdings neigen Patientinnen mit Brustkrebs nicht dazu, während des Krankheitsverlaufs und der Behandlung viel Gewicht oder Muskelmasse zu verlieren, daher muss die Muskelschwäche der Patientinnen andere Ursachen haben, die derzeit noch nicht ausreichend erforscht wurden. Abgesehen von der genannten Muskelschwäche der Patientinnen sind Brustkrebs und Muskeln auch auf einer anderen Ebene miteinander verknüpft. Es ist seit langem bekannt, dass Bewegung und körperliche Aktivität eine Reihe positiver Effekte für Brustkrebspatientinnen haben. Bereits moderate Bewegung kann die Chancen auf eine erfolgreiche Brustkrebsbehandlung verbessern. Doch wie sieht das genaue Zusammenspiel zwischen Brustkrebs und Skelettmuskulatur aus?

In der Klinik ist diese Frage nicht so einfach zu beantworten, da es schwierig ist, zwischen den Auswirkungen des Brustkrebses selbst und den der Krebstherapie zu unterscheiden. Dies ist allerdings essentiell zu wissen, um einen Weg oder eine Methode zu finden die Muskelschwäche umzukehren, oder um jenen Brustkrebspatientinnen zu helfen, die sich durch die Erkrankung nicht mehr in der Lage sind Sport zu treiben.

Im ersten Teil meiner Dissertation (**Paper I**) untersuchten wir, wie sich Brustkrebs auf die Skelettmuskulatur auswirkt und welche positiven Auswirkungen körperliche Aktivität auf den Krankheitsverlauf und die Muskelschwäche hat.

Diese Studie wurde mit Hilfe eines Mausmodells für Brustkrebs (PyMT Mäuse) durchgeführt. Dies erlaubte uns, sich nur auf die Auswirkungen des Brustkrebses ohne

Medikamenteneinfluss zu konzentrieren. Mit diesen Mäusen konnten die Tumore und den Krankheitsverlauf menschlicher Patientinnen mit Brustkrebs nachgeahmt werden, einschließlich schwächerer Muskelkraft und verringerter Ausdauer, jedoch ohne eine einhergehende Verkleinerung oder Abbau der Muskelmasse. Interessanterweise fanden wir heraus, dass, wenn die Mäuse mit Brustkrebs vier Wochen lang Zugang zu einem Laufrad hatten, das Laufradtraining die Ausdauer und Muskelkraft der erkrankten Mäuse auf das gleiche Niveau wie das der gesunden Maus-Kontrollgruppe steigerte. Außerdem stellten wir fest, dass der Brustkrebs weniger aggressiv wurde nach dem Training, obwohl die Größe des Brusttumors gleichblieb.

Als nächsten wollten wir diesen Vorgang auf molekularer Ebene verstehen. Die Fähigkeit des menschlichen Körpers oder des Mauskörpers, Muskelbewegungen und körperliche Aktivitäten auszuführen, hängt stark von dessen inneren zellulären Kraftwerk, den sogenannten Mitochondrien, ab. Unsere aufgenommene Nahrung, wird vor allem in Zucker und Fette im Köper zerlegt und von den Mitochondrien weiter umgewandelt, um Energie bereitzustellen, die dann von körperlichen Geweben oder Organen wie Skelettmuskeln genutzt werden kann. Für diese Energiebereitstellung sind eine Reihe biochemischer Reaktionen in den Mitochondrien erforderlich. Generell, können Mitochondrien und deren Funktionen auch durch bestimmte Moleküle oder Faktoren geschädigt werden. Zu diesen zellulären Faktoren gehören unter anderem sogenannte Zytokine. Zytokine sind kleine Signalstoffe, die der Körper normalerweise verwendet, um zwischen verschiedenen Geweben und Organen zu kommunizieren. Brustkrebs ist dafür bekannt, diese Signale zu seinem eigenen Vorteil zu nutzen.

So wurde beispielsweise bereits in Patientinnen herausgefunden, dass wachsender Brustkrebs Signale an den Körper sendet, die ein vermehrtes Wachstum von Blutgefäßen in der Krebsumgebung ermöglichen, welches wiederrum das Wachstum des Brustkrebses fördert, da dieser mit mehr Sauerstoff und Nährstoffen aus der Blutbahn versorgt werden kann. Durch diese Signale wird um den Brustkrebs herum eine entzündliche Umgebung geschaffen. Diese Entzündungssignale gelangen in die Blutbahn, worüber sie auch den Muskel erreichen und beeinflussen können. Auch in den Mäusen mit Brustkrebs fanden wir erhöhte Konzentrationen dieser Entzündungssignale sowohl im Blutstrom als auch im Skelettmuskel selbst. Außerdem stellten wir fest, dass bestimmte „Bausteine“ der Mitochondrien in den Mäusen mit Brustkrebs weniger vorhanden waren. Dies deutete darauf hin, dass die vom Brustkrebs gesendeten Entzündungs- oder Stresssignale die energieproduzierenden Mitochondrien im Skelettmuskel, und damit die die Fähigkeit der Muskeln Kraft zu erzeugen, beeinflussen.

Bemerkenswerterweise konnte das Laufradtraining die entzündlichen Auswirkungen des Brustkrebses teilweise umkehren, indem es die zellulären Entzündungs- oder Stresssignale reduzierte und die Energieproduktion der Mitochondrien im Skelettmuskel erhöhte. Das Laufradtraining der Mäuse mit Brustkrebs konnte allerdings nicht die gesamten zellulären Stresssignale und alle mitochondrialen Defizite wieder auf das normale Niveau von Nicht-

Brustkrebsmäusen umkehren. Das weist darauf hin, dass der Brustkrebs immer noch die Wirkung des Laufradtrainings auf den Muskel beeinträchtigt.

In der 1. Studie (**Paper I**) konnten wir erstmals zeigen, dass Stresssignale, ausgelöst durch Brustkrebs, für die Muskelschwäche bei Mäusen oder Patentinnen mit Brustkrebs verantwortlich sein könnten, und dass moderate Bewegung den Stresssignalen und der Brustkrebs-induzierten Muskelschwäche entgegenwirken kann.

Wie in der 1.Studie beschrieben, können Patientinnen mit Brustkrebs an weiteren Begleiterscheinungen und Komorbiditäten, wie der oben beschrieben Muskelschwäche leiden. Zusätzlich zu den Auswirkungen des Brustkrebses selbst, kann die Krebsbehandlung auch Nebenwirkungen für die Patientinnen haben. Allerdings sind nicht alle Ursachen der Nebenwirkungen von Krebsbehandlungen erforscht und einige Nebenwirkungen treten erst Jahre nach der abgeschlossenen Krebsbehandlung auf.

Im zweiten Teil meiner Dissertation (**Paper II**) untersuchten wir daher ein Medikament (CX), welches sich derzeit als neuartige Krebsbehandlung in klinischen Studien befindet. Im gleichen Mausmodell für Brustkrebs (PyMT Mäuse) verringerte eine vierwöchige Behandlung mit CX die Brustkrebsgröße deutlich. Um bislang unbekannte Nebenwirkungen von diesem Medikament zu untersuchen, wurde das Verhalten beobachtet und bestimmte Vitalwerte dokumentiert. Überraschenderweise stellten wir fest, dass alle Mäuse, die mit dem Medikament CX behandelt wurden, mehr aßen, aber auch mehr O_2 einatmeten und dadurch mehr CO_2 ausatmeten, diese Ergebnisse waren unabhängig vom Vorhandensein des Brustkrebses (sowohl bei Brustkrebs-Mäusen als auch bei Nicht-Brustkrebs-Mäusen). Anhand der gemessenen Werte konnten wir ermitteln, dass die behandelten Mäuse eher Zucker statt Fett für ihre körperliche Energiegewinnung in den Mitochondrien verwenden. Interessanterweise zeigte die Auswertung des Zuckerspiegels im Blut bei behandelten Mäusen einen übermäßigen Anstieg des Blutzuckerspiegels, was bedeutet, dass das Medikament CX zu einem hohen Blutzuckerspiegel in Mäusen führt, welcher normalerweise bei PatientInnen mit Diabetes auftritt.

Als nächstes wollten wir verstehen, warum die Blutzuckerwerte in behandelten Mäusen so hoch waren. Da die Mäuse nicht mit erhöhtem Zuckergehalt gefüttert wurden, ist ein veränderter Stoffwechsel möglicherweise die Ursache. Der Skelettmuskel ist ein Gewebe oder Organ, das viel Zucker verbraucht, da es die Energie benötigt, um Kraft zu erzeugen. Um den Zucker aus der Blutbahn in die Muskeln transportieren, nutzen die Muskelzellen spezielle Transport-Moleküle. Bei den behandelten Mäusen war die Zuckeraufnahme über diese Zuckertransporter in die Muskeln verringert. Das bedeutet, dass die Behandlung mit CX den Zuckertransport in die Skelettmuskulatur und damit die Verfügbarkeit von Zucker für die Energiegewinnung der Muskeln beeinflusst. Darüber hinaus fanden wir heraus, dass die Behandlung mit CX die gleiche pharmakologische Wirkung auf den Skelettmuskel wie auf den Brustkrebs ausübt. Dies könnte der Mechanismus sein, wie das CX-Medikament, zusätzlich zu den Brustkrebszellen, auch direkt die Skelettmuskelzellen beeinflusst.

In der 2. Studie (**Paper II**) konnten wir zeigen, dass das neuartiges Krebs-Medikament CX trotz seiner guten Wirkung gegen Brustkrebs, zu Veränderungen im Energiehaushalt des Körpers führt, die wahrscheinlich Diabetes verursachen. Diese Ergebnisse sind besonders wichtig, da ein hoher Blutzuckerspiegel im Körper bekanntermaßen das Wachstum von Krebszellen fördern kann und damit auch ein weiteres Ausbreiten des Brustkrebses bewirkt. Ein besseres Verständnis der Nebenwirkungen von Krebsbehandlungen kann zu einer besseren Nachsorge der PatientInnen oder auch zu verschiedenen Kombinationstherapien bei der Krebsbehandlung führen, um zu verhindern, dass PatientInnen an Diabetes, in Kombination mit einer Krebsbehandlung, erkranken.

Wie in der 1. und 2. Studie untersucht, kann die Skelettmuskulatur durch verschiedene Stresssignale beeinträchtigt werden. Diese Stresssignale können durch „externen Stress“ wie Brustkrebs oder der Brustkrebsbehandlung entstehen. Die Skelettmuskulatur ist allerdings auch „inneren Stress“ wie oxidativem Stress durch freie Radikale (Oxidantien) ausgesetzt. Körpereigene Oxidantien sind essentielle Botenstoffe und werden regelmäßig durch die normale Funktion der Körperzellen, zum Beispiel bei der Energiegewinnung in Mitochondrien oder bei der Kontraktion von Muskeln produziert. Einer Überproduktion von körpereigenen Oxidantien wird normalerweise durch das körpereigene Antioxidantien-System (Radikalfänger) entgegengewirkt. Die Produktion von Oxidantien und die Abwehr durch Antioxidantien wird häufig als eine Waage in Balance beschrieben. Bei zu hohen Mengen an Oxidantien kippt jedoch das Gleichgewicht in den Körperzellen und es entsteht ein unausgeglichener Zustand, der sogenannte oxidative Stress.

Im letzten Teil meiner Dissertation (**Paper III**) haben wir untersucht, wie der Verbrauch der Zucker-Reserven im Skelettmuskel von Mäusen, durch ein spezifisches Oxidant namens Peroxynitrit ($ONOO^-$) beeinflusst wird. Nach einer Mahlzeit ist Zucker häufig im Überfluss in der Blutbahn vorhanden, daher wird er (wie oben beschrieben) in die Skelettmuskulatur transportiert und kann dort in der Speicherform namens Glykogen als Energiereserve angelegt werden.

Bei einem plötzlichen Energiebedarf, wie bei einem schnellen Sprint, kann Glykogen schnell wieder in Zucker bzw. Glukose umgewandelt werden. Dies geschieht durch ein weiteres spezielles Molekül der Zelle, die sogenannte Glykogen-Phosphorylase. Der Vorgang wird eingesetzt, um schnell Energie zu erzeugen ohne dass der Zucker die langwierige Reaktionsfolge in den Mitochondrien durchlaufen muss. Wir wollten untersuchen, wie dieses Molekül (Phosphorylase) durch oxidativen Stress im Muskel beeinflusst wird und haben daher die Skelettmuskeln von Mäusen direkt verschiedenen Oxidantien ausgesetzt. Bereits zerkleinerte Muskeln, erleichterten den Oxidantien den Zugang zu den Zellen, daher konnten wie eine Hemmung des Phosphorylase Moleküls durch die Oxidantien, insbesondere durch Peroxynitrit, in den Muskelzellen beobachten. Als Ergebnis der Hemmung, konnten die Glykogen Energiereserven nicht zu Glukose umgewandelt werden und die Muskelzellen konnten keine zelluläre Energie aus den Reserven beziehen.

Als allerdings ganze, intakte Muskeln für das gleiche Experiment verwendet wurden, konnten wir keine Hemmung des Phosphorylase Moleküls feststellen. Aber intakte Muskeln, die mit dem Oxidationsmittel Peroxynitrit behandelt wurden, waren durchaus schwächer und erzeugten weniger Kraft.

Als nächstes wollten wir untersuchen, ob zugesetzte Antioxidantien (DTT, NAC) die Waage von der „anderen Seite“ ins Ungleich Gewicht bringen können. Obwohl die Antioxidantien das Molekül Phosphorylase nicht hemmten, wurde überraschenderweise die Muskelkraft auch durch die Antioxidantien verringert und die Muskeln waren schwächer.

In der 3. Studie (**Paper III**) wurde gezeigt, wie beide Seiten des sensiblen Oxidantien- und Antioxidantien-Gleichgewichtes einen erheblichen Einfluss auf die Krafterzeugung und Energieversorgung der Skelettmuskulatur in Mäusen haben können.

Meine Forschungsstudien während des PhDs, hier in der Dissertation zusammengefasst, hatten zum Ziel zu erforschen wie verschiedene Stressoren – sowohl interne als auch externe – die Leistung und Funktion der Skelettmuskulatur beeinflussen können. Durch die Ergebnisse der drei Studien (**Paper I-III**) konnten mehr Kenntnisse über die zellulären Stresssignalwege, und wie diese die Muskelkraft und Energieversorgung beeinflussen können, gewonnen werden. Mit meiner Forschungsarbeit hoffe ich zu einem besseren Verständnis dieser zellulären Prozesse im internationalen Wissenschaftskreis beizutragen und dadurch in Zukunft das Leben von Patienten mit Muskelerkrankungen zu verbessern.

ABSTRACT

Skeletal muscle performance is essential for our body's movement as well as for the whole-body metabolism. In health and disease, skeletal muscle is exposed to various endogenous and exogenous stressors, influencing its physiological functions.

In **paper I**, we showed that exercise performance and muscle force is affected by the stressor breast cancer, in a mouse model of breast cancer (PyMT). Mimicking the experienced muscle weakness of human breast cancer patients, PyMT mice performed poorly in a treadmill exhaustion run and their muscles produced less force than wildtype (WT) mice, although no difference in morphology, fiber type distribution or diameter was found. The muscle weakness was associated with an increase of pro-inflammatory cytokines, such as TNF-α in the skeletal muscle, activating the p38 mitogen-activated protein kinase (MAPK) stress-response pathway and decreasing the expression of mitochondrial electron transport chain (ETC) genes as well as antioxidant genes. After the mice had access to four weeks of voluntary running *ad libitum*, skeletal muscle force as well as the time and distance of the treadmill exhaustion run improved drastically for PyMT mice. The exercise also reduced the intramuscular stress, improved both the expression of mitochondrial ETC genes and the activity of key mitochondrial enzymes, such as citrate synthase (CS) and especially β-hydroxyacyl-CoA dehydrogenase (β-HAD) and restored the antioxidant defense system including superoxide dismutase (SOD 1,2). Additionally, we could show that the breast cancer blunted the exercise-induced expression of PPARγ coactivator-1α (Pgc-1α) in PyMT mice. Our results showed that breast cancer-induced weakness is linked to increased intramuscular stress signaling and that voluntary, moderate exercise was able to counteract the weakness in PyMT mice.

Patients with breast cancer are treated with various systemic anti-cancer treatments, and while aiming to treat cancer, these treatments often cause side-effects. In **paper II**, we aimed to study the effect of a novel anti-tumorigenic compound (CX-5461) on the whole-body as well skeletal muscle-specific metabolism. Four weeks of CX-5461 treatment effectively reduced the breast cancer tumor in PyMT mice, but also resulted in increased food intake, energy expenditure and a higher respiratory exchange ratio (VCO_2/VO_2), indicative of a substrate shift towards carbohydrate utilization in both WT and PyMT mice. Moreover, basal blood glucose levels were increased, and we observed a slower glucose clearance from the blood stream in both WT and PyMT mice after CX-5461 treatment. Skeletal muscle is an important tissue involved in maintaining the body's glucose homeostatic. In WT mice, CX-5461 treatment reduced the basal glucose uptake, whereas in PyMT mice the insulin-stimulated glucose uptake was affected in extensor digitorum longus (EDL) muscles. We found that CX-5461 not only exerts its mechanism of action, the inhibition of the RNA-Polymerase I (Pol I) pre-initiation complex, in breast cancer cells, but also directly in skeletal muscle and through that alters the glucose and fat metabolism in skeletal muscle. The results indicate that the novel drug CX-5461 affects the whole-body metabolism including elevated blood glucose levels and reduced glucose uptake into skeletal muscle, independently of the tumor development.

Skeletal muscle is a highly metabolic tissue which functions can also be regulated by oxidative stress, cause by an imbalance in the endogenous oxidative and antioxidative system. In **paper III**, we investigated the role of the intermuscular redox state on glycogen phosphorylase activity and glycogenolysis, which supply the muscle with energy from glycogen storage during exercise. Glycogen phosphorylase was strongly inhibited by incubation with the reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$), contrary to the reactive oxygen species (ROS) H_2O_2 in muscle extracts. In intact muscles, $ONOO^-$ incubation resulted in inhibition of glycogenolysis in resting and contracting as well as a reduction of muscle force in slow-twitch oxidative soleus (SOL) and fast-twitch glycolytic EDL muscles, despite not exerting a direct effect on phosphorylase activity. Moreover, post-translational nitrate modification was observed in EDL muscle after $ONOO^-$ incubation. Incubation with two antioxidants N-acetylcysteine (NAC) and dithiothreitol (DTT) did not affect phosphorylase activity or glycogenolysis, but reduced the force of EDL and SOL muscle. These results suggest that exogenous $ONOO^-$ inhibits phosphorylase activity in muscle extracts and glycogenolysis in intact contracted muscles, whereas antioxidants such as DTT and NAC only play a minor role in inducing endogenous ROS and regulate the phosphorylase activity.

All results from these three studies in this thesis investigate how the performance and function of skeletal muscle can be affected by different stressors. Taken together, a better understanding of the responsible underlying molecular mechanisms, might lead to targeted therapy approaches for afflicted patients in the future.

LIST OF SCIENTIFIC PAPERS

I. **Exercise reduces intramuscular stress and counteracts muscle weakness in mice with breast cancer**
J Cachexia Sarcopenia Muscle, 2022.
PMID: 35170227 DOI:10.1002/jcsm.12944

THERESA MADER, Thomas Chaillou, Estela Santos Alves, Baptiste Jude, Arthur J Cheng, Ellinor Kenne, Sara Mijwel, Ewa Kurzejamska, Clara Theresa Vincent, Helene Rundqvist, Johanna T Lanner

II. **CX-5461 treatment results in metabolic alteration in mice with breast cancer and healthy control mice.**
Manuscript

THERESA MADER, Ellinor Kenne, Thomas Chaillou, Julie Massart, Alexander Chibalin, Melissa Borg, Maarten M. Steinz, Zhengye Liu, Ewa Kurzejamska, Serge Ducommun, Theresa C. Vincent, Jorge L Ruas, Anna Krook, Juleen R Zierath, Johanna T Lanner

III. **Role of nitration in control of phosphorylase and glycogenolysis in mouse skeletal muscle**
Am J Physiol Endocrinol Metab, 2021. 320(4): p. E691-e701.
PMID: 33554777 DOI: 10.1152/ajpendo.00506.2020

Sarah J Blackwood, Baptiste Jude, THERESA MADER, Johanna T Lanner, Abram Katz

SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

I. **Mitochondrial NDUFA4L2 is a novel regulator of skeletal muscle mass and force**
Faseb j, 2021. 35(12): p. e22010.
PMID: 34724256 DOI: 10.1096/fj.202100066R

Zhengye Liu, Thomas Chaillou, Estela Santos Alves, THERESA MADER, Baptiste Jude, Duarte M S Ferreira, Heidi Hynynen, Arthur J Cheng, William O Jonsson, Gianluigi Pironti, Daniel C Andersson, Ellinor Kenne, Jorge L Ruas, Pasi Tavi, Johanna T Lanner

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LIST OF ABBREVIATIONS

2-DOG	2-deoxyglucose
AMPK	Adenosine monophosphate-activated protein kinase
ANK1	Ankyrin repeat domain 1
CAT	Catalase
CD36	Fatty acid translocase
CLAMS	Comprehensive lab animal monitoring system
CO_2	Carbon dioxide
CS	Citrate synthase
CTPS1	Cytidine triphosphate synthase 1
DTT	Dithiothreitol
DXA	Dual-energy X-ray absorptiometry
EDL	Extensor digitorum longus
ETC	(mitochondrial) Electron transport chain
FCSA	Fiber cross-sectional area
FVB	Friend leukemia virus B strain
GAS	Gastrocnemius
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
GTT	Glucose tolerance test
H_2O_2	Hydrogen peroxide
HSL	Hormone-sensitive lipase
i.p.	Intraperitoneal injection
IκB-α	Inhibitor of kB-α
LIPE	Hormone-sensitive lipase
MAPK	Mitogen-activated protein kinase
MHC	Myosin heavy chain
MMTV	Mouse mammary tumor virus
NAC	N-acetylcysteine
NaCN	Sodium cyanide
NFκB1	Nuclear factor-κB1

$NO^{\bullet}$	Nitric oxide
O_2	Oxygen
$ONOO^-$	Peroxynitrite
PCr	Phosphocreatine
PCR	Polymerase chain reaction
PDK4	Pyruvate dehydrogenase kinase 4
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1alpha
P_i	Inorganic phosphate
PIC	Pol I pre-initiation complex
PKB	Protein kinase B
POL I	RNA polymerase I
PyMT	Polyoma middle tumor-antigen
RER	Respiratory exchange ratio
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rplp0	Ribosomal protein lateral stalk subunit P0
rRNA	Ribosomal RNA
RT-qPCR	Real-time quantitative PCR
SCL27A1	Fatty acid transport protein 1
snoRNA	Non-coding small nucleolar RNAs
SOD	Superoxide dismutase
SOL	Soleus
TA	Tibialis anterior
TNF-α	Tumor necrosis factor α
TOP2β	Topoisomerase II beta
TRAF2	TNF receptor-associated factor 2
WB	Western blot
WGA	Wheat germ agglutinin
WT	Wildtype
β-HAD	β-hydroxyacyl-CoA dehydrogenase

1 INTRODUCTION AND BACKGROUND

1.1 SKELETAL MUSCLE

Skeletal muscles are muscles that are attached to the bones of the skeleton via its tendons. Skeletal muscles are crucial for our everyday movement and physical activities. They comprise ~30-40% of the body weight in humans and do not only produce force but are also a central player in whole-body metabolism [1, 2]. Skeletal muscles are required for the body's ability to generate force and movement and are therefore important in all physiological motions, including breathing and walking.

1.1.1 Skeletal muscle fiber types

A muscle is made of numerous single and multinucleated cells, so called fibers, which are kept together in bundles, surrounded by interstitial connective tissue. Skeletal muscles fibers appear in different classification or types, depending on their contraction speed and myosin composition. Roughly they are divided in slow-twitch (type I) and fast-twitch (type II) muscle fibers. Based on their myosin heavy chain (MHC) isoform expression, type II fibers can be further divided into IIB (MHC-IIb, *Myh4*), IIx (MHC-IIx, *Myh1*) and IIA (MHC-IIa, *Myh2*) fibers [3]. Type I fibers express MHC-I (*Myh7*) and are most effective for long-endurance activities. They depend on aerobic respiration (slow-twitch oxidative) for ATP production and appear red due to the abundance of myoglobin. Skeletal muscle fibers exhibit different molecular features for the utilization of different substrates for ATP production. Type I fibers contain greater number of mitochondria and have a higher triglyceride content than type II fibers [4]. Type II fibers are most effective for short and rapid movements and have ~20-30% more glycogen storage than type I fibers [5, 6]. Type IIa also mainly depend on aerobic respiration (fast-twitch oxidative), whereas type IIb (fast-twitch glycolytic) are predominantly anaerobic glycolytic and use glycogen and glucose for energy production [7-9]. In the hind limbs of mice both fast- and slow twitch muscles are present. For instance, soleus muscle (SOL) contains predominantly slow type I fibers, extensor digitorum longus muscle (EDL) and flexor digitorum brevis muscle (FDB) contain mainly type II fibers and gastrocnemius (GAS) and tibialis anterior muscle (TA) muscles consists of mixed type I and II fibers [10, 11].

1.1.2 Structure

Skeletal muscle fibers appear striated, as they consist of highly organized thick and thin filaments [12]. The overlapping thick and thin filaments form the functional, contractile unit of a sarcomere (see **Figure 1 A**). The main unit of the thick element is the large molecular weight protein myosin. Myosin consists of a "tail" and two myosin "heads" including the actin-binding site. The thin filament consist mainly of filamentous actin (F-actin, see **Figure 1 A,B**) [13].

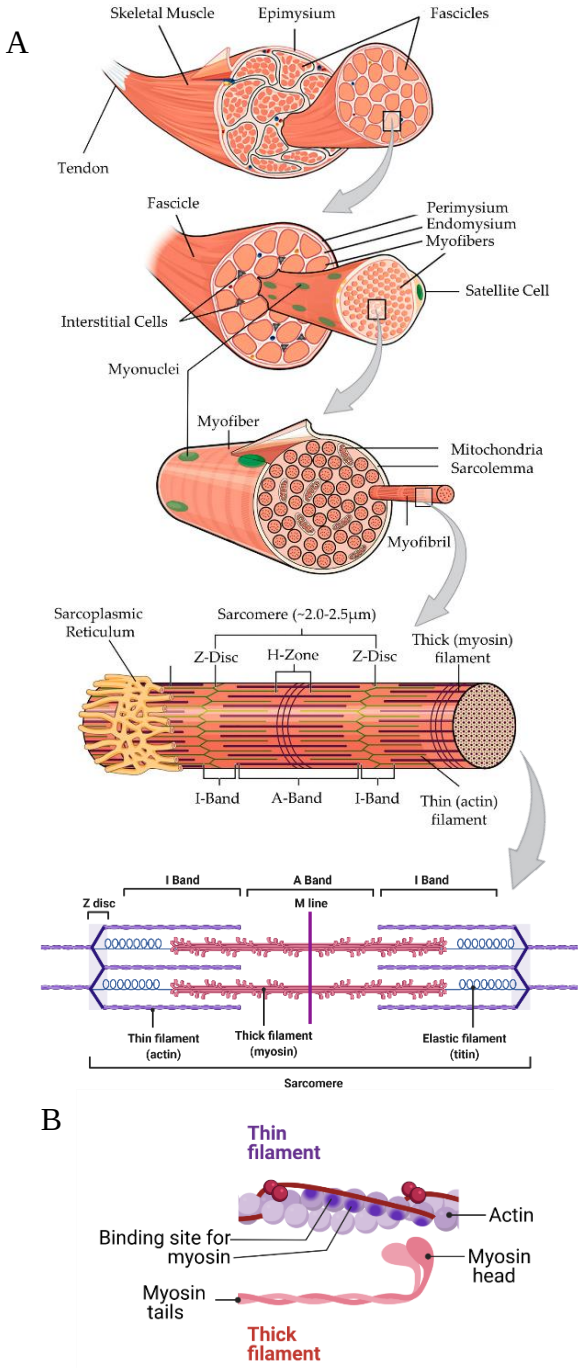

Figure 1: Schematic overview of the mammalian skeletal muscle structure and of the sarcomere arrangement. Illustration of skeletal muscle structure (A) from [14], originally from OpenStax available online: https://openstax.org/books/anatomy-and-physiology/pages/10-2-skeletal-muscle (accessed on 6 January 2020). (B) Created with BioRender.com.

1.1.3 Excitation-contraction coupling and force production

Skeletal muscle is an excitable tissue and hence activated by electric stimulation through nerves. Excitation-contraction coupling (ECC) is the process through which a stimulation leads to the contraction of the skeletal muscle fiber [9] (schematic illustrated in **Figure 2**). At a neuromuscular junction, the motor neuron releases acetylcholine into the inter-synaptic space, activating the acetylcholine receptor on the motor end plate on the muscle sarcolemma. This will initiate local membrane depolarisation, inducing the activation of the voltage-gated sodium channels responsible for the spread of the action potential along the sarcolemma membrane

(myofiber membrane) and then further through invaginations of the sarcolemma into the interior of the muscle fiber, the so-called transverse (T) tubular system. The action potential activates the voltage-sensitive dihydropyridine receptor (DHPR) in the t-tubular system which undergoes a conformational change [9]. The conformational change of DHPR leads to a mechanical activation of the ryanodine receptors (RyR1) which is the calcium ions (Ca^{2+}) release channel localized the sarcoplasmic reticulum (SR) membrane [15]. Activation of RyR1 leads to Ca^{2+} release from the SR into the intracellular space (myoplasm) [16]. The released Ca^{2+} enables the interaction of the contractile proteins, actin and myosin (cross-bridge cycling). Hereby, myosin with ADP bound, binds to actin and upon ADP release the actin filaments are pulled past the myosin filaments ("power stroke"). Binding o new ATP dissociates myosin from actin, and conversion to ADP and inorganic phosphate (P_i) causes myosin to bind a new site on actin and by that enable force production. Finally, muscle relaxation occurs by decrease in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [13, 16].

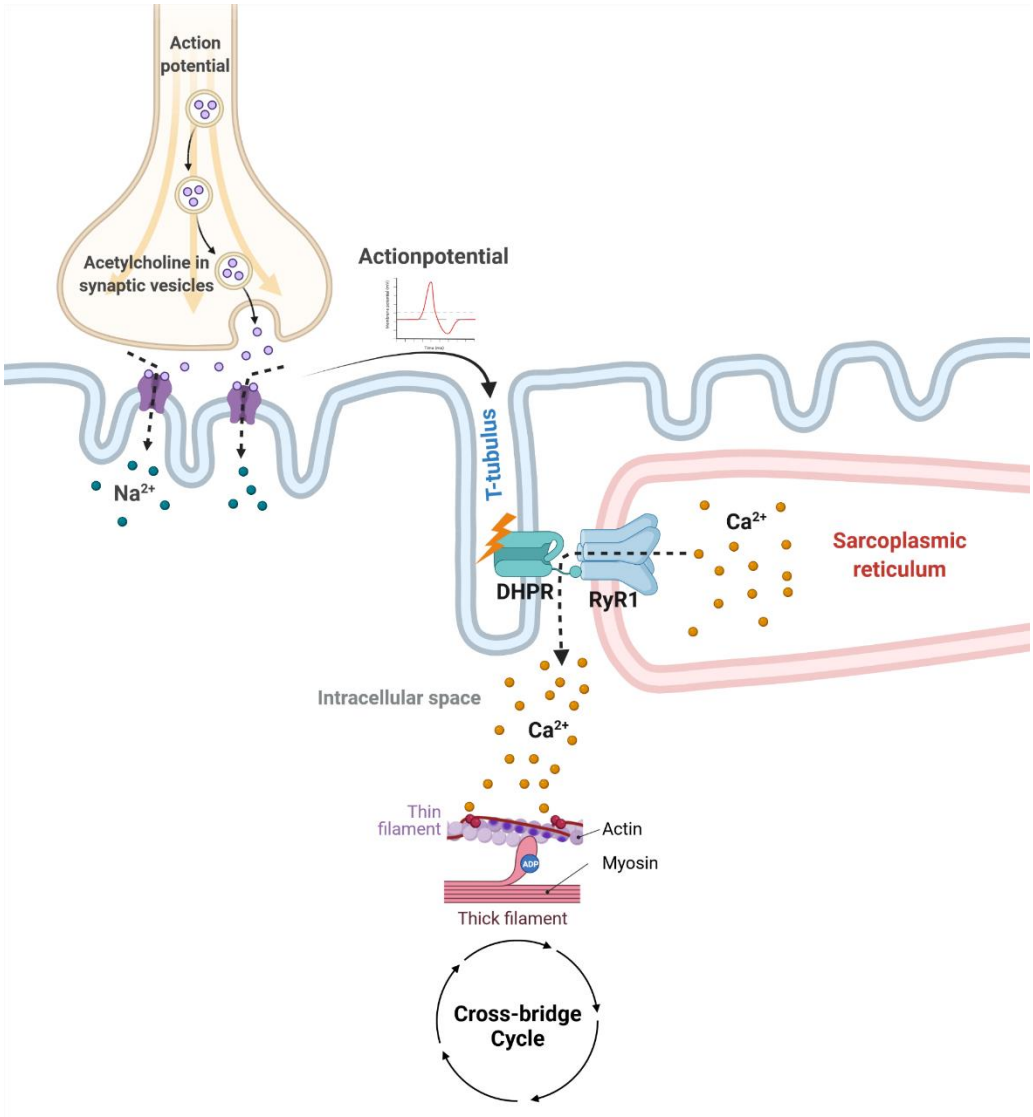

Figure 2: Excitation-contraction coupling in skeletal muscle from action potential at the neuromuscular junction till the cross-bridge cycle. Figure created with BioRender.com.

1.1.4 Skeletal muscle energy sources

Skeletal muscle is an important metabolic organ in the body, since muscle contractions require ATP as energy source. ATP is used for muscle contraction and is mainly produced in three ways: from creatine phosphate (CrP) storage, through anaerobic glycolysis and oxidative phosphorylation pathways [13]. ATP and CrP storage sustain high intensity during short duration activities or sudden bursts of exercise (seconds). Intramuscular glycogen storages provide the substrate for anaerobic glycolysis, which is a fast course for ATP production, but results in lactate accumulation which will not allow for long durations (minutes). Oxidative phosphorylation (aerobic respiration) inside the mitochondria is used for activities with longer duration time (minutes to hours) [9, 13]. However, these pathways for energy utilization are not used exclusively, but rather overlap to ensure a wide range of skeletal muscle performance.

1.2 METABOLISM

1.2.1 Glucose uptake and storage

Glucose is an important energy source. Upon glucose intake of the body, the blood sugar level rises, and insulin is secreted by the pancreas to facilitate glucose uptake into tissue e.g., skeletal muscle. Through the binding of insulin to its receptor at the sarcolemma of the skeletal muscle, an intracellular signaling cascade is initiated, including PI3 kinase activation and Akt phosphorylation. This activates the translocation of glucose transporter 4 (GLUT4) from intracellular storage organelles to the sarcolemma which facilitates glucose uptake into skeletal muscle [17]. In skeletal muscle GLUT1 and GLUT4 are the most abundant isoforms [18]. GLUT1 is also localized at the sarcolemma and responsible for the basal glucose uptake into skeletal muscle [19, 20].

1.2.1.1 Glycogen storage

In abundancy, glucose is stored as glycogen (glycogenesis) in muscle and in the liver, a process mainly catalyzed by glycogen synthase. In the fasting state or during energy demand, glycogen storage units can be broken down into glucose (glycogenolysis) by glycogen phosphorylase, to provide immediate energy [21]. The glycogen phosphorylase exists in two forms, as the less active, non-phosphorylated form phosphorylase b, and as the more active, phosphorylated form phosphorylase a. By phosphorylation, phosphorylase b can be converted to phosphorylase a through phosphorylase kinase [22]. In addition to phosphorylation, phosphorylase can also be allosterically regulated through molecule binding. Activating molecules include glycogen, AMP and inorganic phosphate (P_i) whereas ATP, glucose-6-phosphate (glucose-6-$P_)$ and glucose inhibit the glycogen phosphorylase in the skeletal muscle [22]. Furthermore, glycogen phosphorylase can also be regulated through reactive nitrogen species (RNS), where peroxynitrite ($ONOO^-$) drastically inhibits phosphorylase and thus glycogenolysis [23].

1.2.2 TCA cycle and the electron transport chain

Mitochondria are the powerhouse of the cell and generate most of the cellular energy in the form of ATP. They are harboring the citric acid cycle (TCA cycle) as well as the oxidative phosphorylation.

In the TCA cycle, acetyl-coenzyme A (CoA), generated through fatty acid metabolism (β-oxidation, see **1.2.3**) or from pyruvate (glycolysis), is converted into oxaloacetate in a series of chemical reactions. Oxaloacetate can further be converted into citrate by the enzyme citrate synthase (CS) to continue the cycle. The electrons from NADH and $FADH_2$, produced in the TCA cycle and/or by β-oxidation in the mitochondrial matrix, travel through a series of enzymatic complexes (I-V) in the inner mitochondrial membrane. Briefly, NADH and $FADH_2$ are oxidized to NAD^+ and FAD^+ in complex I (NADH ubiquinone oxidoreductase) and II (succinate dehydrogenase), initiating an electron and proton flux across the inner mitochondrial membrane. The electrons produced in CI and CII are transferred to ubiquinone (Q) reducing it to ubiquinol (QH_2). Subsequently, electrons are transferred from QH_2 to cytochrome c thereby reducing it at the complex III (cytochrome bc1 complex) and oxidizing QH_2 back to Q, and subsequently contributing to forming a proton (H+) gradient across mitochondrial intermembrane. In Complex IV (cytochrome c oxidase), electrons are transferred from cytochrome c to the final electron acceptor oxygen, which is in turn reduced to water and further establishes the H+ gradient. Finally, the electrochemical (proton) gradient generated is used by the ATP synthase (complex V) to produce ATP [9, 24].

As described in further detail below (see chapter **1.4.1.1**) mitochondria are also the main site of cellular ROS production, especially superoxide ($O_2^{-\bullet}$), as well as ROS scavenging (indicated in **Figure 3**). Mitochondria have high plasticity and capacity to adapt to different stimuli. During endurance exercise the mitochondrial content is highly increased in skeletal muscle, leading to an enhanced oxidative capacity [25]. The peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1 alpha (PGC 1α) is important for mitochondrial biogenesis

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1 alpha (PGC-1α) is an important regulator of mitochondrial biogenesis, driving the organism's adaptation to meet diverse energy demands [26, 27]. In skeletal muscle, PGC-1α is mainly activated through exercise and induces exercise-adaptative effects, including angiogenesis and mitochondrial oxidative metabolism [28]. Through PGC-1α mediated co-activation of the transcription factors NRF-1/-2 or PPARγ nuclear-encoded mitochondrial genes, relevant for essential mitochondrial functions, are expressed [27]. Additionally, PGC-1α is regulated by the stress-activated p38 mitogen-activated protein kinase (MAPK) and AMPK, the central regulator of cellular energy homeostasis.

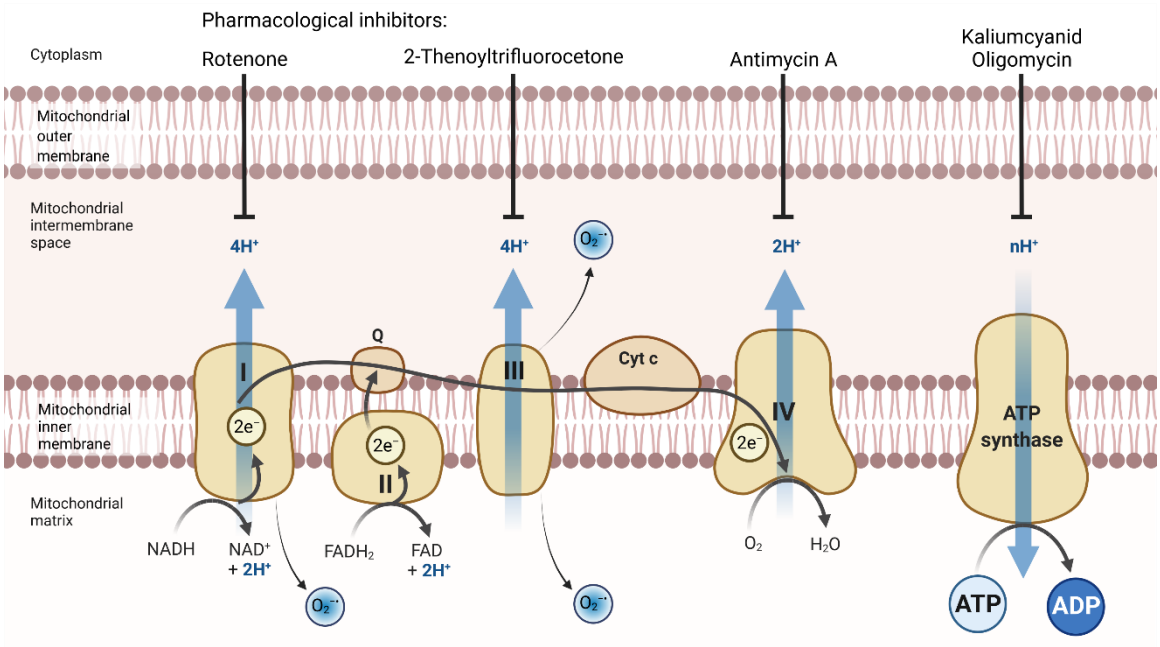

Figure 3: The mitochondrial electron transport chain (ETC). Electrons travel through a series of enzymatic complexes (I-IV) in the inner mitochondrial membrane in the mitochondrial membrane to the final electron acceptor oxygen. The hereby created electrochemical proton (H^+) gradient.is used by the ATP synthase (complex V) to generate ATP. Indicated are the generation of superoxide ($O_2^{-\bullet}$) through incomplete reduction of oxygen, and pharmacological inhibitors used to inhibit separate complexes. Figure created with BioRender.com.

1.2.3 Fatty acid metabolism

Apart from glucose, cells can also generate energy from metabolizing fatty acids. Fatty acids enter the cell through fatty acids transporters, such as the fatty acid translocase (CD36) or the fatty acid transport proteins (SLC27 family) [29]. In skeletal muscle, fatty acids can also be generated from triglycerides stored in lipid droplets by the process of hydrolysis (lipolysis) through the rate-determining enzymes adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) [30]. From the cytosol, fatty acids are transported into the mitochondria by the carnitine palmitoyl transferase (CPT). In the mitochondrial matrix, β-oxidation occurs, catalyzed by the enzyme β-hydroxyacyl-CoA dehydrogenase (β-HAD), where two carbons are removed from the fatty acid to form Acetyl-CoA, which can be used in the TCA cycle (see chapter **1.2.2**) [29].

1.3 CANCER

1.3.1 Breast cancer

Breast cancer is the most common malignancy in women and overall, one of the most common cancers, with 19.3 million new cancer cases diagnosed yearly worldwide [31] and the incidence rates are increasing [32]. Cancer is diagnosed at different stages. At onset, the increase of cell numbers in an organ is called hyperplasia or neoplasia, depending on a physiological or non-physiological stimuli. An adenoma or fibroadenoma is a benign tumor of the epithelial and stromal breast tissue. Benign tumors have a clear border and are not invasive. Carcinomas are defined as abnormal cell proliferation which might develop the ability to invade into nearby

tissue and eventually metastasize, thereby the cancer is spreading to other organs than where it originated from. The different types of breast cancer (neoplasia, adenoma, carcinoma) are morphologically assessed by hematoxylin, eosin and immunohistochemistry stains.

1.3.2 Muscle weakness as comorbidity in breast cancer

Patients with breast cancer commonly report muscle weakness and fatigue as a secondary condition to their disease [16]. They frequently experience problems with physical strength and fatigue, which reduces their quality of life [33-38]. Cancer-related muscle dysfunction was apparent in patients regardless of tumor stage or nutritional state and was already observed in patients with early-stage breast cancer [39, 40]. For instance, about 25% lower strength in lower extremities have been reported in patients with breast cancer [41]. Traditionally, muscle weakness in cancer has been associated with loss of muscle mass and muscle atrophy [40, 42, 43]. However, the prevalence for patients with breast cancer losing muscle mass (more than 5% of their body mass) is only ~3% [44], and patients with breast cancer do not show significant weight changes and were comparable to controls [45]. The loss of muscle strength was even prevalent before anti-cancer treatment but was most impaired in patients who completed chemotherapy, especially for anthracycline (doxorubicin) treatment [41, 46, 47], hence both the disease itself and the anti-tumor treatment contributes to the changes in muscle function

1.3.3 Interventions

1.3.3.1 Breast cancer treatment

The treatment of breast cancer depends on its progression stage and molecular profile. Surgical resection and removal of the axillary lymph nodes together with a postoperative radiation therapy are the most likely course of action taken against primary, non-metastatic breast cancer tumors. The systemic therapy is determined by the molecular subtype of the breast cancer. Generally, hormone or endocrine therapy (e.g. tamoxifen and aromatase inhibitors) is suggested for estrogen receptor (ER+) and progesterone receptor positive (PR+) tumors. Antibody therapy (e.g. trastuzumab) is used to treat human epidermal growth factor positive (HER2+) tumors and chemotherapy (e.g. taxanes as docetaxel and/or anthracyclines as doxorubicin) for triple-negative (ER-, PR-, HER2-) tumors. For metastatic breast cancer the patients undergo mostly life-prolonging and symptom-relieving therapy [48-50].

In this thesis the effect of a novel anti-tumorigenic compound CX-5461 on tumor progression and muscle function was investigated. CX-5461 selectively inhibits the assembly of the pre-initiation complex (PIC) of the RNA polymerase I (Pol I) on the 45S ribosomal DNA (rDNA), without effecting the mRNA transcription by Pol II [51] (see **Figure 4**), subsequently inhibiting the transcription of ribosomal RNA (rRNA). Normally, rRNAs, together with ribosomal proteins, form the two subunits of the ribosome in a process called ribosomal biogenesis (see **Figure** 4). In cancer, the regulation of ribosomal biogenesis can be disrupted, as cancerous cells upregulate the production of ribosomes to maintain increased protein synthesis during cancer growth [52-54]. In a panel of 50 human cancer cell lines and 5 non-transformed cell

lines it was shown that CX-5461 has anti-proliferative activity selectively for the tumor cells [51]. Overall, the available data indicates a high efficiency of CX-5461 in a wide range of cancers. CX-5461 is currently in two phase 1 clinical trials, one for patients with solid tumors [55, 56] and another for haematologic malignancies [57, 58].

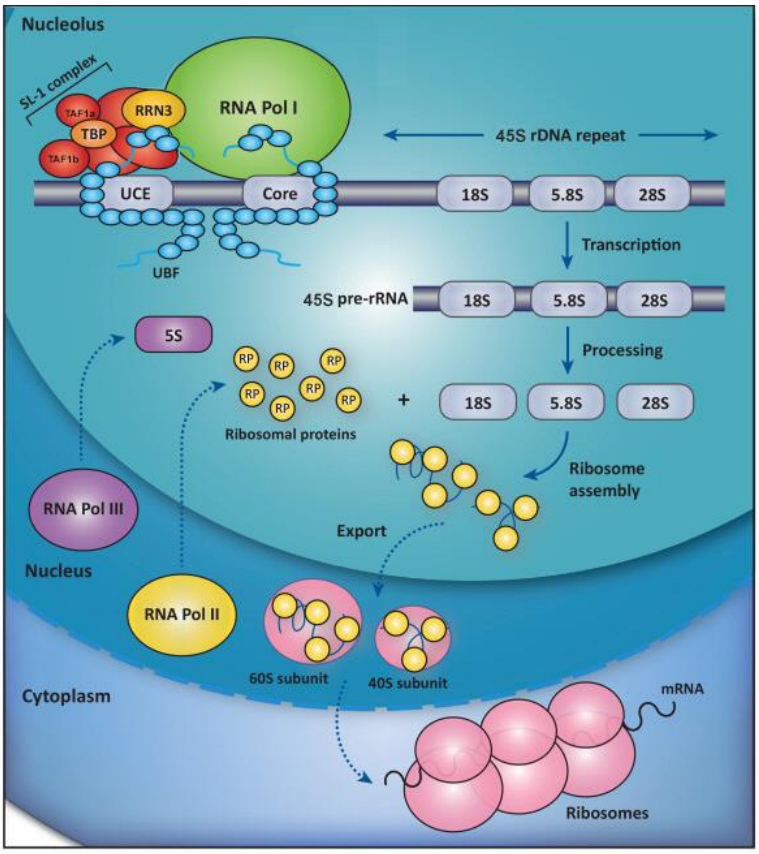

Figure 4: Transcription of 45S rDNA by RNA polymerase 1 (Pol I) as the first step of ribosome biogenesis. The pre-initiation complex, including upstream-binding factor (UBF), SL-1 (compromising TBP (TATA box-binding protein)) and RNA polymerase I (Pol I)-specific TBP-associated factors (TAFs) are recruited to the upstream control element (UCE) on the 45S rDNA promoter. Pol I is recruited by the PIC-element RRN3 to the promoter region. After transcription, the 45S pre-RNA is further processed into mature 18S, 5.8S, and 28S rRNAs. Together with the 5S rRNA, which is transcribed by Pol III, and ribosomal proteins, transcribed by Pol II, they form the 40S and 60S ribosomal subunits. Figure modified from [59].

1.3.3.2 Physical exercise in breast cancer

Already early studies of breast cancer found a clear correlation between performance status (ability to perform activities of daily life), treatment response rate and survival [60, 61]. As described in chapter **1.1.4**, skeletal muscle relies on different energy sources depending on the exercise intensity and duration. During intense short-term exercise, ATP is synthesized through the conversion of PCr and glycogen. Endurance exercise on the other hand, requires aerobic ATP synthesize from carbohydrate and fat substrates (as described in chapter **1.2.2** and **1.2.3**), with the mitochondria being the central key player [62].

In pre-clinical mouse models, voluntary exercise has been shown to influence the incidence, multiplicity, and weight of tumors in a wide range of breast cancer models and exercise protocols [63]. In PyMT mice, voluntary access for 10 weeks to a low-profile running wheel

significantly reduced the tumor size in the early stage of development, but not late-stage tumors or metastatic burden. The reduced tumor size correlated with the distance covered on the wheel until 3 weeks of running, but beyond 5 weeks no beneficial effects on tumor growth were observed [64].In patients with breast cancer, physical activity post-diagnosis has been shown to improved overall survival by 44% [65], reduce breast cancer deaths by 34%, and disease recurrence by 24% [66, 67]. Moderate intensity activity (150 min/week) or an equivalent of 3-5 hours of walking per week showed the greatest benefits [65, 68, 69].

1.3.4 Cancer metabolism

It is well-established that in contrast to normal differentiated cells, high proliferative cancer cells utilize anerobic glycolysis instead of oxidative phosphorylation to meet their increased energy demand, also called the "Warburg effect" [70, 71]. The "reverse Warburg effect" was found in cancer-associated stroma cells, where the cells surrounding the tumor also utilize anerobic glycolysis to support the tumor cells [72].

In patients, the increased energy demand by the tumor can be visualized by 18F-deoxyglucose positron emission tomography (FDG-PET) to investigate the glucose uptake into the tumor tissue. I*n vivo* glucose uptake was significantly higher in larger tumors and lymph node metastases of patients with breast cancer [73], which correlates with an unfavorable prognosis and recurrence of the cancer [74, 75]. Alongside upregulated glucose metabolism, cancer cells also showed an upregulated *de novo* fatty acid synthesis [76, 77]. The produced lipids are used by the proliferative tumor for membrane biosynthesis and as substrate for ATP synthesis through mitochondrial β-oxidation [78]. Hence, tumors are highly metabolic flexible and adaptable. Individual cancer cells exhibit a high metabolic heterogeneity, where some cells utilize anaerobic glycolysis, others oxidative phosphorylation or fatty acid oxidation (β-oxidation) [79, 80], depending on glucose and oxygen availability [81].

Although it is known that cancer cells alter their energy metabolism, little is known about the regulating molecular mechanisms. More studies suggest that the development of drug resistance in cancer therapy is associated with dysregulated cancer cell metabolism [80, 82].

1.3.4.1 Breast cancer and diabetes

Several studies suggest an increased insulin resistance and impaired glucose metabolism in patients with breast cancer completing chemotherapy [83].

The correlation is especially strong for diabetes. Not only, has it been shown that type 2 diabetes (T2D) increases the risk of breast cancer by up to 23%, mainly through decreased estrogen levels as a result of insulin resistance [84, 85], but the reverse is also true as breast cancer leads to a higher risk of developing diabetes, specifically it has been reported that breast cancer survivors have an increased risk by 10% of developing T2D over ca. six years after treatment [86, 87]. However, it is difficult to distinguish in patients with breast cancer if the effects on the whole-body metabolism stem from the cancer itself or the anti-tumor treatment.

1.4 STRESSORS

Skeletal muscle contraction and metabolism can be influenced by a multitude of endogenous and exogenous stressors.

1.4.1 Reactive oxygen species in skeletal muscle

An intact reduction-oxidation (redox) homeostasis is important for all cellular functions, as excessive oxidative changes (i.e., oxidative stress) can cause molecular damages [88].

ROS are essential molecular signals in intracellular pathways in response to physiological stimuli, e.g., inflammation and exercise adaptation. An example of ROS is the superoxide radical ($O_2^{-\bullet}$), which can be converted into hydrogen peroxide (H_2O_2). Further they include reactive nitrogen species (RNS), such as nitric oxide (nitrogen oxide, $NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) which can be converted into nitrite (NO_2^-), or peroxynitrite ($ONOO^-$). The antioxidant scavenging system includes enzymes such as, SOD, catalase (CAT), glutathione peroxidase (GPx) and Peroxiredoxins (PRx) [88, 89]. A simplified overview of the redox system is given in **Figure 6**.

Oxidative stress describes excessive production of ROS which is not normalized by the antioxidant scavenging system (i.e. the endogenous antioxidant system) (see **Figure 5**).

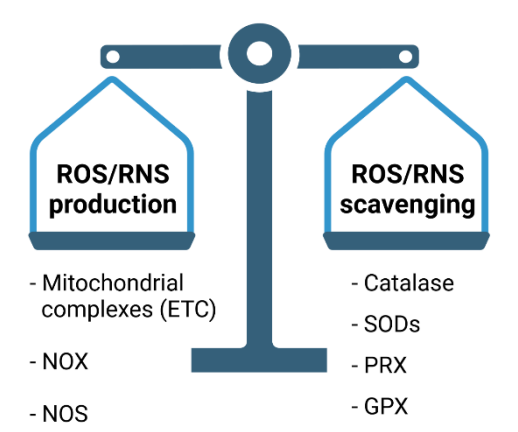

Figure 5: The balance between the generated ROS/ RNS and ROS/RNS scavenging enzymes (antioxidants) creates the redox homeostasis within the skeletal muscle. Figure created with BioRender.com.

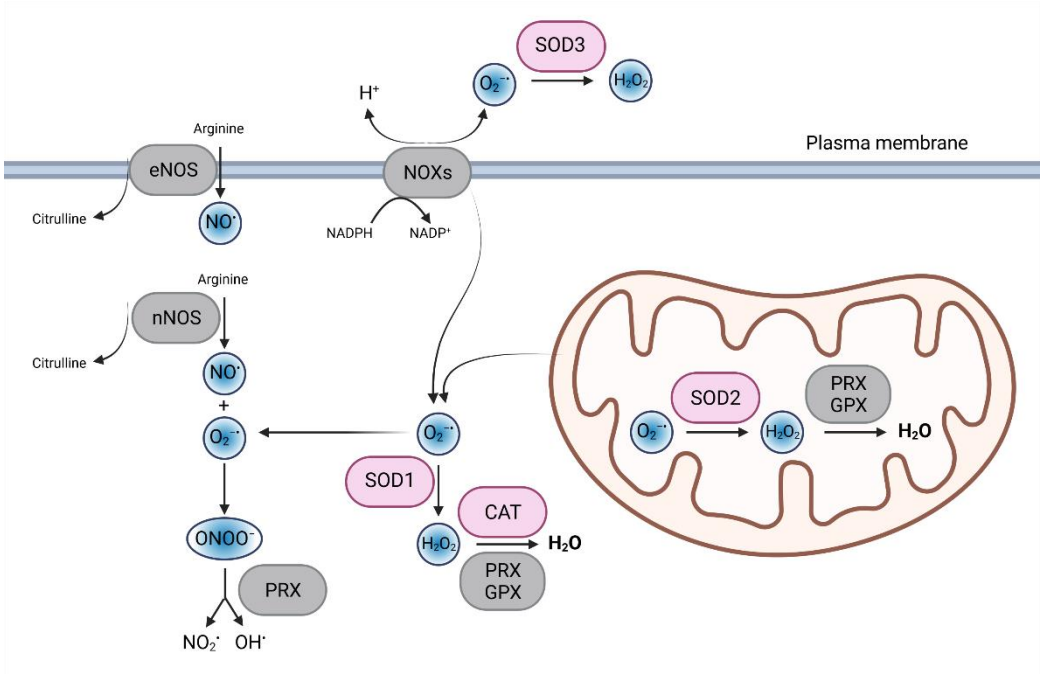

Figure 6: Reactive oxygen species (ROS) and the cellular ROS scavenging system. Enzymes in red are studied in this thesis. Figure created with BioRender.com.

1.4.1.1 Sources of reactive species and the antioxidant scavenging system

Incomplete reduction of oxygen in the mitochondrial complex chain, primarily from electron transport chain (ETC) complexes CI and CIII (indicated in **Figure 3**) as well as from NADPH oxidase (NOX) 2 and 4 in the cytosol are the two main source of superoxide ($O_2^{-\bullet}$) in skeletal muscle [90, 91]. The free radical $O_2^{-\bullet}$ is rapidly dismutated either spontaneously or catalyzed by SODs to H_2O_2 [92]. The three major H_2O_2 scavenging enzymes are Cat, Prx and Gpx.

Apart from ROS, contribute RNS to oxidative stress in skeletal muscle. The nitric oxide synthase (NOS) generates RNS through the catalyzation of $NO^\bullet$ and citrulline from L-Arginine, NADPH and O_2. Two isoforms, neuronal (nNOS) and endothelial NOS (eNOS) are constitutively expressed in skeletal muscle and can be activated by high free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Under a high production rate of $NO^\bullet$ the radical can react with $O_2^{-\bullet}$ to form $ONOO^-$ [90]. $ONOO^-$ can cause a post-translational modification in proteins, called 3-NT (3-nitrotyrosine). In the diseased state, muscle weakness can be caused by oxidative modifications (malondialdehyde adducts (MDA) and 3-NT) on the RyR1 complex as well as 3-NT formation on actin, mediating a mechanical contractile dysfunction as shown in mice with inflammatory arthritis [93-96]. In patients of rheumatoid arthritis, three specific hotspots on α-actin targeted by 3-NT and MDA were identified to directly contribute to the muscle weakness [94].

1.4.1.2 Oxidative stress and mitochondria in breast cancer

Various cancer, including breast cancer, have reported mitochondria DNA (mtDNA) mutations [97-99]. Alterations in mtDNA increase mitochondrial ROS generation, which induce tumor-promoting effects. Those were shown to be reversed by catalase overexpression in a mouse model of intestinal tumors [100]. Similarly, PyMT mice expressing mitochondrial catalase had a lowered tumor invasiveness together with a decreased activation of p38 MAPK [101]. Some studies indicate that ROS produced by mitochondria, induced genomic damage and by that further promotes oncogenic transformation [102]. On the other hand, oncogenic transformation, e.g., ectopic expression oncogene K-ras, can in return increase mitochondrial ROS production [103, 104]. Contrarily, overexpression of transformed endogenous oncoproteins as K-Ras, B-Raf and Myc suppresses ROS production by increasing Nrf2 transcription contributing to tumorigenesis [105]. This suggests an important regulatory role of oncogenes on the redox balance [106].

1.4.2 Inflammation

Generally, inflammation is described as the body's response to stressors, such as infections or toxins. Inflammation is modulated in the body by small signaling proteins released by the cell, so called pro-inflammatory and anti-inflammatory cytokines [107]. As signaling proteins, cytokines can modulate the maturation, growth, and responsiveness of cells or tissues. When binding to the cellular surface receptors, cytokines can activate the stress-activated p38 MAPK. The p38 MAPK is a major key player in inflammation and stress response, which apart from cytokines, is also activated by ROS, RNS osmotic stress or radiation.

1.4.2.1 Inflammation in cancer

The role of inflammation in cancer is complex and poorly understood. The tumor microenvironment is known to secrete cytokines and through that drive the proliferation, progression and invasion of the tumor itself [108, 109]. Not only does the inflammation create a tumor supportive environment, but also promotes metastasis by increasing cytokine levels in the circulation [108, 109]. (**Figure 7**).

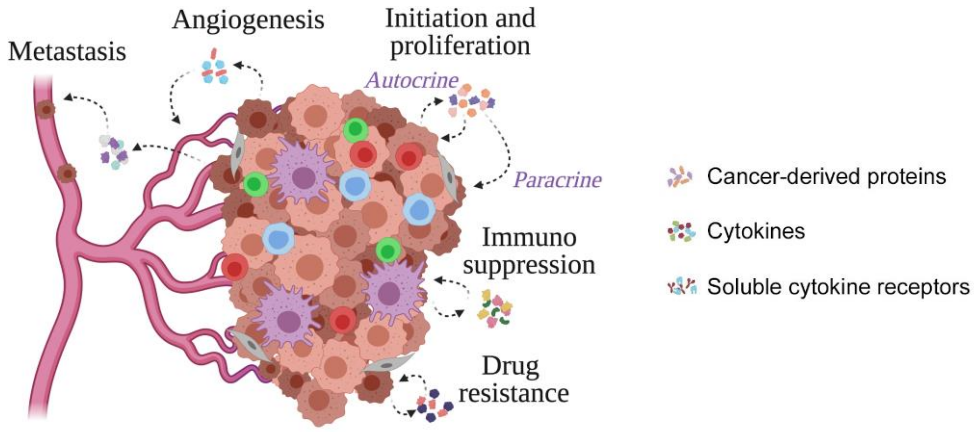

Figure 7: Cytokines secreted by the tumor create tumor-supportive microenvironments both locally and distantly. The tumor microenvironment consists of cytokine-secreting cancer cells, stromal cells and immune cells, which together support tumor progression. Illustration from [108].

To date, several different cytokines have been reported in association with breast cancer progression and metastasis [110]. Kawaguchi et al. reported distinctive cytokine-signatures in serum of metastatic vs. non-metastatic patients with breast cancer which correlated with clinical outcomes and could predict disease prognosis and therapeutic effects [111]. Among them interleukin-17 (IL-17), which is thought to be important for establishing a pre-metastatic niche for breast cancer [112], as well as tumor necrosis factor α (TNF-α). The pro-inflammatory cytokine TNF-α has a generally elevated expression in breast cancer and has been shown to correlate with increased tumor cell proliferation, higher malignancy grade, increased metastasis occurrence, and a mostly poor prognosis for the patient [113].

1.4.2.2 Inflammation in skeletal muscle

Inflammation has an ambiguous role in skeletal muscle. On one side, the energy deficit of an acute bout or short-term exercise leads to elevated oxidative stress and cytokine levels, e.g., interleukin-6 (IL-6) in skeletal muscle [114]. Similarly, TNF-α has been reported to be significantly increased in serum after a marathon race [115].The cytokine increase is thought to be beneficial as it leads to the adaptation of skeletal muscle to acute stress [116]. More recent studies suggested IL-6 role in modulating skeletal muscle metabolism [117]. This is in accordance with the wide evidence that regular exercise reduces pro-inflammatory cytokines and increases anti-inflammatory markers [118, 119].

On the other side, chronic inflammation and exposure to extended levels of pro-inflammatory cytokines have a negative impact on skeletal muscle. This can lead to loss of skeletal muscle mass [120], impair the regenerative capacity of skeletal muscle [121] but also cause exercise intolerance and weakness without muscle atrophy [122].TNF-α in particular, has been shown to promote muscle weakness through atrophy and induced contractile dysfunction, mediated through ROS and RNS [123, 124]. Furthermore, TNF-α activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway, contributing to mitochondrial dysfunction and increase in cellular ROS in skeletal muscle [125] potentially leading to force-

reducing capacity of the muscle fibers. In agreement, the muscle-specific inhibition of IκB kinase (IKK), a downstream target of TNF-α, resulted in enhanced regeneration and improved muscle force [126]. TNF-α also activates the central stress-response p38 MAPK pathway in skeletal muscle [127, 128], consequently regulating the expression of PGC-1α thus further modulating mitochondrial biogenesis. This regulation has also been shown *vice versa* were when muscle-specific ablation of PGC-1α in mice lead to a significant increase in gene expression of IL-6 and TNF-α in muscle [129]. Overall, the dynamic signaling pathways between cytokines such as TNF-α, and p38 MAPK as well as PGC-1α in the inflammatory state are under continuous investigation.

2 RESEARCH AIMS

The overall aim of this thesis is to study the role of different physiological and pathophysiological stressors, such as breast cancer, anti-tumorigenic treatment and oxidative stress on the function of skeletal muscle. Therefore, the thesis is divided into 3 specific sub-aims:

1. Identify the underlying molecular mechanisms of breast cancer-induced muscle weakness and the beneficial effect of physical exercise.

2. Elucidate the affects of the novel anti-tumorigenic compound CX-5461 on the whole-body and muscle-specific metabolism.

3. Clarify the role of nitration on the function of glycogen phopshorylase and glycogenolysis in skeletal muscle.

3 MATERIALS AND METHODS

3.1 ANIMAL MODEL

3.1.1 Ethical permits

All studies involving animals were performed in accordance to the Swedish Animal Welfare act, the Swedish Welfare ordinaire and applicable regulations and recommendations from Swedish authorities. The studies were approved by the Stockholm North Ethical Committee on Animal experiments (see respective numbers in the specific papers).

3.1.2 Animal models

All animals used in this thesis were housed at a temperature- and humidity-controlled facility at room temperature (24°C) on a 12:12-h light-dark cycle. Standard chow and water were provided *ad libitum*.

3.1.3 MMTV-PyMT and WT model

Female mice of the breast cancer mouse model FVB/MMTV-PyMT (PyMT) were used in **paper I and II** of this thesis. In this model the mammary gland-specific polyoma middle tumor-antigen (PyMT) is under the control of the mouse mammary tumor virus promotor (MMTV) [130, 131]. The PyMT mice had an FVB (Friend leukemia virus B strain) strain background [132], therefore FVB/NRj mice were used as wildtype (WT, Janvier Labs, France) control mice. The model was chosen, as it resembles the human tumor progression, especially the luminal B subtype of human breast cancer [133]. First hyperplasic lesions develop spontaneously in female PyMT mice at the age of ~4 weeks. At ~8-9 weeks of age PyMT mice develop adenomas. By around 10 weeks of age carcinomas have formed in the female PyMT mice. Mice were monitored weekly and tumor measurements were taken under isoflurane anesthesia with standard calibers. The total tumor volume is calculated in mm^3. All PyMT and WT mice were 8 weeks of age at the start of all experiments and were sacrificed at 12 weeks of age with CO_2 or cervical dislocation after isoflurane anesthesia for collection of skeletal muscles.

3.1.4 C57BL/6

Adult C57Bl/6JOlaHSd male mice 8–12weeks (~ 25 g) were used in **paper III** of this thesis. The mice were sacrificed by cervical dislocation.

3.1.5 Ethical consideration

Ethical consideration and animal care were carefully considered when planning and performing all experiments in this PhD thesis. During the course "Philosophy of science and research ethics" this project was presented, and awareness and understanding of the important ethical aspects of this research was achieved. All animal experiments were performed under the ethical permits N19/15 (amendment 3067-18), B 6847-2020, 2010/63/EU and IL-149-01-18. The experiments were performed to meet the “3 R principle” in order to reduce, replace and refine

the work with mice involved in this study. This includes careful planning of each experiment and using several muscles from the same animal to reduce the mice numbers. As refinement, the mice involved in the studies were carefully monitored and their bodyweight as well as tumor volumes were measured weekly to minimize potential suffering from the tumor. Mice which reached the humane endpoint of tumor volume earlier than the experimental endpoints were sacrificed.

3.2 INTERVENTIONS

3.2.1 Voluntary wheel running

Individually housed mice were given free access to either a counting (active) or locked (inactive) low-profile wireless running wheel (ENV-047 or ENV-044-02, Med Asscoiates Inc.). The wheel access was limited to four weeks. The wheels were wireless connected to a software recording the distance and time run by each mouse and by that confirming their actual training.

3.2.2 Treadmill exhaustion run

The mice were adapted to the six-lane treadmill (Columbus Instruments) for four days and run to exhaustion at the fifth. The mice run individually and were separated by partitions. Each day of the adaptation training the mice were subjected to 10 min of running with a 10% inclination. On the first day the speed was kept at 5 m/min, on the second day it increased from 6 to 9 m/min, up to 12 m/min on the third and 15 m/min at the fourth day. The exhaustion test was performed with the speed increasing from 6 m/min to maximal 33 m/min by 3 m/min every 3 minutes. The speed of 33 m/min was kept till exhaustion. The mice were motivated by slight manual pushing by hand and if they withstand 3 manual pushes, the mice would be graded exhausted. To finally access the mice exhaustion status, mice which refused to run further were taken out from the treadmill and into a new environment.

3.2.3 CX-5461

CX-5461 and its mechanism of action was defined in chapter **1.3.3.1**. PyMT and WT mice were intraperitoneal (i.p.) injected with 250 µl of 20, 50 or 75mg/kg CX-5461 (CX, dissolved in NaH_2PO_4, Selleckchem) or 250 µl of 50 mM NaH_2PO_4 as Vehicle (Veh) once per week from the age of 8 till 12 weeks.

3.3 *EX VIVO* FORCE MEASUREMENT

3.3.1 Whole muscle

The protocol for the force measurement was followed as previously described [134]. During dissection and force measurement, the SOL and EDL muscles were kept in in a Tyrode solution containing (in mmol/L): 121 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 24 $NaHCO_3$, 0.1 EDTA, and 5.5 glucose, at 31°C gassed with 95% O2, 5%CO_2 to achieve a pH 7.4.

The proximal and distal ends of the fibers were tied with nylon thread and mounted between a force transducer and adjustable holder (World precision Instrument). The fiber length was corrected obtaining the maximal tetanic force (L_0). Fibers were left to recover for 15 minutes. Parallel arranged platinum electrodes delivered the stimulation of supramaximal electrical pulses (0.5 ms in duration). The following stimulus frequencies were used to determine the force-frequency relationship: 1 (twitch stimulus), 10, 15, 20, 30, 50, 70, 100, and 120 Hz for SOL muscles (1000 msec tetanic duration); 1, 20, 30, 40, 50, 70, 100, 120, and 150 Hz for EDL muscles (300 msec tetanic duration). In between each stimulation the recovery was one minute. In between each stimulation, the recovery was one minute. Electrically stimulated force production was expressed as absolute force (mN) and as specific force (kN/m^2). After the experiment, the tendons were cut to determine the muscle mass. The muscle CSA was calculated by dividing the muscle mass by the L_0 and muscle density (1.06 g/cm^3).

3.3.2 Single fibers

Isolated, intact single muscle fibers were mechanically dissected from slow-twitch SOL muscles of PyMT and control littermate mice and simultaneous quantification of force and $[Ca^{2+}]_i$ in living single fibers with a completely intact intracellular milieu were performed as previously described in detail [16]. Some fibers were exposed to caffeine (5mM), a potent RyR1 agonist [17], and stimulated at 120 Hz.

3.4 CALCIUM MEASUREMENT

Intracellular calcium $[Ca^{2+}]_i$ was measured with the fluorescent radiometric Ca^{2+} indicator Indo-1 (Thermo Fisher Scientific) intact single muscle fibers from SOL muscle. An intracellular calibration curved was used to convert the fluorescence of Indo-1 at rest and during tetanic contractions to $[Ca^{2+}]_i$ [135]. Electrically stimulated force production was expressed as absolute force (mN) and as specific force (kN/m^2). After the experiment, the tendons were cut to determine the muscle mass. The muscle fiber CSA was calculated by dividing the muscle mass by the muscle length and muscle density (1.06 g/cm^3).

3.5 MOLECULAR BIOLOGY

3.5.1 RNA isolation, reverse transcription and real-time quantitative PCR (RT qPCR)

Total RNA was isolated from Tibialis anterior (TA), gastrocnemius (GAS), EDL or SOL muscle by homogenizing the sample with TRIzol® Reagent (ThermoFisher). After adding chloroform, RNA is precipitated from the upper aqueous phase with 2-Propanol and washed with 70% ethanol. RNA was treated with DNase I using the TURBO DNA-free™ Kit (ThermoFisher). 500 ng of RNA were used for cDNA preparation using the SuperScript™ IV Reverse Transcriptase Kit and Oligo (dT) primers (both ThermoFisher). Quantitative Real-Time PCR was performed with SYBR Green PCR Master Mix in a the CFX96 Real-Time PCR Detection System (both BioRad). The analysis of gene expression was performed with the ΔΔCt method and gene expression normalized to ribosomal protein lateral stalk subunit

p0(RPLP0) mRNA levels. Gene expression analyses are expressed as mRNA levels relative to the wildtype controls.

3.5.2 Protein isolation and western blot

Approximately 20 mg of TA or GAS muscles were homogenized in 20 volumes of the following extraction buffer containing (in mM): 10 Tris-maleate, 100 KCl, 2 $MgCl_2$, 2 EGTA, 2 Na_4P2O_7, 1 $NaVO_4$, 25 KF, and protease inhibitor (Roche, 1 tablet/50 mL). Centrifuging at 700xg for 10 min at 4°C cleared the lysates. The protein concentration was quantified with the Bradford assay (Bio-Rad). Equal amounts of protein (20 µg/well) were electrophoresed on tris-glycine protein gels and transferred to PDVF membranes (Immobilon-FL, Millipore). Membranes were incubated with primary antibodies (phospho-p38 MAPK (#9211, Thr180/Tyr182, Cell Signaling), p38 MAPK (#9212, Cell Signaling), RyR1 (#ab2868), CSQ1,2 (#ab3516) and DHPR (#ab2864) in Odyssey blocking buffer (LI-COR Bioscience) overnight at 4°C. Infrared-labeled secondary antibodies (IRDye 680, IRDye 800, LI-COR Biosciences) were incubated for 1 h at room temperature. Detection and analyses were performed with the LI-COR infrared imaging system, quantified with Image Studio Lite version 5.2. and normalized against total protein or DHPR. Total protein was determined by Ponceau S or Coomassie staining.

3.5.3 Immunoprecipitation

EDL or SOL muscles were homogenized and incubated for 45 min on ice in buffer containing (in mM): 10 Tris-maleate, 100 KCl, 2 $MgCl_2$, 2 EGTA, 2 $Na_4P_2O_7$, 1 $NaVO_4$, 25 KF, and protease inhibitor [Roche, 1 tablet/50 mL], pH 7.4). The supernatant was collected after centrifugation at 1,000 g for 10 min and protein concentration measured with the Bradford assay (Bio-rad). Equal amounts of protein were incubated with 3-nitrotyrosine (3-NT) antibody overnight at 4°C. Afterwards magnetic Dynabeads protein G beads (ThermoFisher Scientific, 10003D) were added and incubated for 2h at 4°C. Samples were separated by gel electrophoresis and transferred onto a PVDF membrane (Immobilon-FL, Millipore). Membranes were incubated with the primary anti-phosphorylase antibody at 4C overnight and incubated with secondary infrared-labelled secondary antibodies to be scanned in the LI-COR Odyssey Infrared Imaging System.

3.5.4 Immunohistofluorescence analysis and FCSA (muscle sections)

For immunohistochemical analysis, fresh samples of SOL muscle were frozen in ice-cold isopentane and stored in -80°C until further staining. Serial transverse sections were obtained with a cryostat at a thickness of 7-10 µm and were blocked with MOM blocking regent (Vector, #MKB-2231) for 60 min at room temperature. The sections were incubated with primary antibodies against myosin heavy chain (MHC) isoforms (MHC1, BA-D5; MHC2a, SC.71. Hybridoma Bank) in PBST overnight at 4°C. Secondary antibodies (IgG2b Alexa Flour 350, A21140; IgG1 Alexa Flour 488, A21121, Invitrogen) in PBST were incubated for 1h at room temperature, followed by co-staining of the sarcolemma with Wheat Germ Agglutinin (WGA) conjugated with TX-Red (1:50, Invitrogen) for 1h at room temperature. Nuclei were stained by

10 min incubation with DAPI solution (1:10000, Invitrogen) and mounted with fluorescent mounting medium (Vectashield). Slides were imaged and photographed with a Leica microscope. Fiber type distribution was determined based on the expression of MHC1 (type-1 fibers), MHC2a (type-2a fibers) in fibers, and unstained fibers (type 2x/2b fibers). Fiber-type, FCSA and FCSA distribution were analyzed from 251 ± 23 fibers per SOL muscle on average.

3.5.5 Immunohistochemical analysis (tumor sections)

For immunohistochemical analysis, tumor tissue was fixed overnight in 4% PFA and kept in 70% ethanol until further processing. De-hydration series was run by the Histological Core Facility at Karolinska institutet, followed by embedding the samples in paraffin. Paraffin sections of 10 μm were de-paraffinised, re-hydrated and antigen retrieval was performed with warm Citrate Buffer (Sigma). The endogenous peroxidase was blocked with 3% H_2O_2 in Methanol for 10 min, followed by blocking with BSA for 1h at room temperature. The sections were stained with the primary antibody for CK8 (ab59400) and CK14 (ab181595) diluted in 0.1% BSA in PBS overnight at 4°C as previously described [136]. The signal was visualized with the ImmPRESS® HRP Horse Anti-Rabbit IgG Polymer Detection Kit (Vector laboratories), according to the manufacturer's instructions. Counter-staining was performed with Harris hematoxylin (Sigma) and slides mounted with Pertex mounting media (Histolab). The quantification of oxidized hydrogen peroxide catalyzed by HRP (DAB, brown) was analyzed in 16-20 visual fields/tumor by ImageJ analysis software at a magnification of x40. Intensity corresponds to the mean intensity quantified ImageJ, after subtracted background. Equal color threshold was used for all conditions compared.

3.6 METABOLIC MEASUREMENTS

3.6.1 Dual energy X-ray Absorptiometry (DXA)

WT and PyMT with CX-5461 or vehicle treatment were anaesthetized with light isoflurane-anesthesia at 12-weeks of age and imaged by Dual-energy X-ray absorptiometry (DXA; Lunar PIXImus densitometer; GE Medical-Lunar, Madison, WI, USA) to assess their body composition.

3.6.2 Comprehensive Lab Animal Monitoring System (CLAMS)

Both WT and PyMT mice treated with CX-5461 or vehicle (see above) were individually housed and monitored in the comprehensive lab animal monitoring system (CLAMS; Columbus Instruments, Columbus, OH) from the age of 11 weeks for 5 days, on a 12-hour light/dark cycle and provided with standard rodent chow and water *ad libitum*. The calorimetry system simultaneously measures locomotion, food intake, heat, O_2 consumption, CO_2 production. From there energy expenditure (kcal/hr/body weight) and respiratory exchange ratio (VCO_2/VO_2) can be calculated. To adjust for the acclimatization of the mice in the CLAMS cages, the first 24 h were excluded and the following 48 h (two dark and two light cycles) were analyzed.

3.6.3 Glucose tolerance

Both WT and PyMT mice treated with CX-5461 or vehicle (see above) were fasted for 4 h at 11 weeks of age, and a blood sample taken via the tail vein (0 min). Afterwards glucose (2 g/kg of body weight) was intraperitoneal injected and blood samples taken 15, 30, 60, and 120 min via the tail vein after glucose injection for measurement of glucose concentration (One Touch Basic glucose meter; Lifescan). Blood samples taken at 0 min and 15 min were processed for measuring insulin concentration as described below.

3.6.4 Glucose uptake

The glucose uptake was experiments were performed as previously described [137, 138]. After 4 h of fasting, both SOL and EDL muscles were dissected from anesthetized WT and PyMT mice. Muscles were incubated 30 min in oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit bicarbonate (KHB) containing (in mM) 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 25 $NaHCO_3$, pH 7.4, supplemented with 5 mmol/L glucose and 15 mmol/L mannitol and bubbled with 95% O_2 and 5% CO_2, giving a final pH of 7.4 for a recovery phase. Afterwards, muscles were incubated for 30 min in KHB buffer with three insulin conditions: (i) without insulin (basal), (ii) with 0.36nM (sub-maximal insulin stimulation) or (iii) with 120nM insulin (maximal insulin stimulation). Then the muscles were incubated in glucose-free KHB buffer for 10 min with 20 mmol/L mannitol with and without or the above insulin concentrations. Muscles were then transferred to KHB buffer supplemented with 1 mmol/L 2-deoxy-d-glucose, 19 mmol/L mannitol, 2.5 μCi/mL [$^{1,2\text{-}3}$H]-2-deoxy-d-glucose, and 0.7 μCi/mL [^{14}C] mannitol with the above insulin conditions and incubated for 20 min, before snap-frozen in liquid nitrogen. To determine the glucose uptake the frozen muscles were weighted, digested for 1 h in 0.5 M NaOH at 60ºC while constantly shaken at 800rpm and the resulting lysate centrifuged for 3 min at 13000 rpm. Radioactivity was determined by liquid scintillation counting (WinSpectral "1414 Liquid Scintillation counter", Wallace) and normalized to protein content (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific).

3.6.5 Citrate synthase and β-hydroxyacyl-CoA activity assay

SOL muscles were homogenized in ice-cold buffer containing (in mM): KH2PO4, 50; EDTA, 1; and 0.05% Triton X-100. After centrifugation centrifuged at 1400 g for 1 min at 4°C, the resulting supernatant was analyses at room temperature for citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) activities using standard spectrophotometric techniques [139]. Activities were adjusted for protein concentration using the Bio-Rad Protein Assay (#500-0006, Bio-Rad).

3.6.6 SOD activity assay

GAS muscles were homogenized in ice cold 0.1M Tris/HCl, pH 7.4 buffer containing 0.5% Triton X-100, 5mM β-ME, 0.1 mg/ml PMSF. The activity of SOD was determined with the colorimetric kit (#ab65354) according to the manufacturer's instructions and values were normalized to muscle weight (mg).

3.6.7 Oxidants and Phosphorylase Activity

EDL and SOL muscles were dissected and incubated for 30 min in an oxygenated Tyrode solution containing (in mmol/L): 121 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 24 $NaHCO_3$, 0.1 EDTA, and 5.5 glucose, at 31°C, blotted and snap-frozen in liquid nitrogen. The muscles were homogenized in buffer containing (in mM): 10 EDTA, 50 KF, and 30% glycerol (v/v), pH 7.0, and centrifuged at 4°C (10,000 g). The samples were incubated for 30 min at 30°C and thereafter the phosphorylase activity with the incorporation of D-[U-^{14}C] glucose 1-P into glycogen was assayed at 30°C, as described previously [140]. In the reaction mixture with glycogen (6.7 mg/mL) and glucose 1-P (66 mM) Phos a (phosphorylated enzyme) was determined in the absence and of 3.3mM AMP, whereas Phos a + b (total, phosphorylated and non-phosphorylated) was determined in the presence of 3.3mM AMP.

3.7 STRESS MEASURMENT

3.7.1 Serum Cytokine Array

Serum was collected from WT and PyMT mice and the Mouse Cytokine Antibody Array (30 Cytokines RayBio®) was performed according to the manufacturer's protocol.

3.7.2 TNF-α levels

Serum (5 µl) or SOL muscle (10 µg) were homogenized using an ELISA kit according to the manufacturer's instructions (BMS607, ThermoFisher) to quantify TNF-a levels.

3.8 STRESS INDUCTION

3.8.1 $ONOO^-$ treatment

Similarly, to the whole muscle *ex vivo* force measurement EDL and SOL muscles were tied with silk threads to the tendons and incubated in Tyrode solution. The optimal length was determined, and after a 30 min recovery period, isometric tetanic contractions were performed for 300ms at a frequency of 70Hz in EDL and 600ms at a frequency of 50Hz in SOL. Muscles were incubated in Tyrode buffer containing (in mM): 2 $ONOO^-$ for 30 min, or 10 DTT, for 30 min or 20 NAC for 60 min or with diluent (5.25 NaOH, 5 or 10 NaCl) as controls for 30 min. After the incubation muscles were directly snap-frozen (basal) or a series of 10 repeated contractions was performed (stimulated) whereafter muscles were snap-frozen.

3.9 STATISTICAL ANALYSES

Data are mostly presented as mean ± SEM or as mean ± SD. When presented as box- and whiskers-plot the median of the distribution is indicated as horizontal line, the boundaries indicate the medians of the first and third quartiles and the error bars extend to the extremes of the observation. Two groups were analyzed by 2-tailed unpaired or paired student's t-test. Multiple comparisons were analyzed by analysis of variance (ANOVA). P values less than 0.05 were considered significant (*P<0.05; **P<0.01; ***P<0.001; **** P<0.0001). All statistical analysis were carried out with Prism 7.0 (GraphPad).

4 RESULTS AND DISCUSSION

4.1 MUSCLE WEAKNESS AND THE EFFECTS OF EXERCISE IN A MODEL OF BREAST CANCER (PAPER I)

Clinical data has shown that breast cancer affects muscle strength and fitness of human patients [40, 41]. In order to understand, how breast cancer exerts these effects on the muscle, we investigated the skeletal muscle performance and intrinsic properties in the breast cancer mouse model MMTV-PyMT (PyMT). Additionally, this study investigated the effects of non-pharmacological intervention., i.e., moderate exercise on muscle function and its potential to counteract cancer-induced effects on skeletal muscle.

4.1.1 Decreased physical performance and muscle force

In **Paper I**, PyMT mice were studied between the age of 8 and 12 weeks of age. In line with clinical data, PyMT mice displayed poor physical performance as they ran ~20% shorter and covered ~30% less distance, compared to their WT littermates (WT), during a treadmill exhaustion run at 12 weeks of age (**Paper I; Fig. 1 A-B**). Similarly, the muscles of PyMT mice, especially the slow-twitch SOL muscle (**Paper I; Fig. 1 D-E**), produced significantly less force when *ex vivo* isometric force was measured.

Decreased physical performance could also stem from altered skeletal muscle function, morphological changes and/or intrinsic muscle dysfunction. Here, no difference could be found in the muscle mass, the size or distribution of muscle fiber cross-sectional area (FCSA), the fiber type composition based on myosin heavy chain (MHC) isoforms, or Ca^{2+} sensitivity between PyMT and WT mice (**Paper I; Fig. 1 C, F-L**). These results suggested that the weakness is rather caused by intrinsic contractile dysfunction. This is agreement with studies in human breast cancer, were the prevalence of developing cachexia (weight loss and muscle atrophy) is only ~3% [44], but patients nevertheless report a loss in muscle strength and reduced physical fitness [40, 41].

That the reduced force was observed in oxidative SOL muscle rather than glycolytic EDL muscle could be due to that a higher number of slow-twitch fibers are activated during everyday movement, and thus glycolytic muscles are not challenged to the same extent and might be protected.

4.1.2 Exercise diminishes the invasive capacity of the tumor

Moderate exercise has been recommended for patients both pre-diagnosis and post-diagnosis, as it has shown to drastically reduce the risk of breast cancer-related deaths and recurrence [65-67]. However, the molecular reasons of the benefit of exercise on whole body and skeletal muscle level in patients with breast cancer are poorly understood. This is partly explained by that this cannot be addressed in clinical studies as the effect of cancer itself or treatment cannot be looked upon separately. Thus, to investigate the effect of breast cancer tumors and to mimic post-diagnosis exercise, PyMT and WT mice were given *ad libitum*

access to running wheels, after the onset of the tumor. The exercise behavior was monitored over the four weeks, showing that PyMT and WT mice ran the same time and distance on the wheel (**Paper I; Fig. 2 A-B**). As expected, the exercise did not affect the tumor volume per se, but it induced a more favorable molecular profile of the tumor, with the invasive basal pathological marker CK14 being decreased (**Paper I; Fig. 2 C-E**). These results are in agreement with other data reporting no effect on PyMT mice tumor size after four weeks of voluntary running, but a beneficial increase of the immune system infiltration at the tumor site was observed [141]. However, an early onset of the exercise at 6 weeks of age and a longer duration of 10 weeks attenuated early tumor growth in PyMT mice, without an increased the immune cell infiltration into the tumor microenvironment [64, 141].

4.1.3 Exercise counteracts the decreased physical performance of PyMT mice

As mentioned, WT and PyMT mice covered the same distance and time on the voluntary running wheels **(Paper I; Fig. 2 A, B)**, but when performing a treadmill exhaustion test after four weeks of voluntary running a greater physical improvement was observed in WT mice than in PyMT mice. This shows that the breast cancer attenuates the body's response to exercise (**Paper I; Fig. 2 F-G** and **Figure 8**). Nevertheless, impressively, the voluntary running exercise counteracted the breast cancer-induced muscle weakness in SOL muscle of PyMT mice, and the muscles from PyMT mice could generate similar forces as muscle of WT mice (**Paper I; Fig. 2 I-J**)

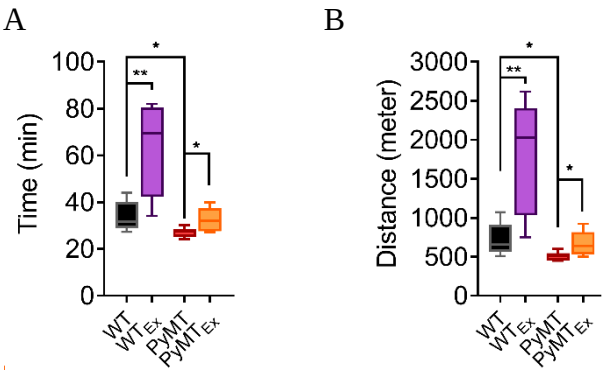

Figure 8: Four weeks of voluntary running counteracted the breast-cancer induced muscle weakness. Time (A, min) and distance (B, meter) recordings of mice which underwent a treadmill exhaustion test after voluntary in-cage running wheels for four weeks (WT_{Ex}, $PyMT_{Ex}$) compared to inactive mice (WT, PyMT).

4.1.4 Afflicted mitochondria gene expression and activity in skeletal muscles of PyMT mice is improved by exercise

It has been hypothesized that the recommended non-anabolic physical activity for patients with breast cancer, protects muscle fiber area and mitochondrial oxidative capacity. We detected a reduced expression in 6 out of 10 representative mitochondrial electron transport chain (ETC) genes (CI—IV, ATPase), as well as decreased enzymatic activity of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) in skeletal muscles of PyMT as compared with WT mice. However, gene expression and enzyme activities were improved by the voluntary

exercise (**Paper I; Fig. 3 A-E, Suppl. Fig. 1 E-H**). For instance, the mitochondrial enzymes, CS and β-HAD (function described in chapters **1.2.2** and **1.2.3**), showed reduced activities in skeletal muscles of PyMT mice, indicating a reduced mitochondrial function. Both enzyme activities improved after four weeks of voluntary exercise, indicating an exercise adaptation of skeletal muscle of both WT and PyMT mice (**Paper I; Fig. 3 F-G**). In healthy individuals, mitochondrial biogenesis, content and density is increased in healthy skeletal muscle through endurance exercise [79, 80]. However, the CS and β-HAD activities in muscle of PyMT mice were still compromised as compared to muscles of WT mice after the four weeks running. This further shows that the presence of a tumorigenic disease interferes with the beneficial responses to physical exercise.

In clinical studies, the effect of exercise can only be observed in cancer patients undergoing treatment, as the cancer itself and the treatment cannot be looked upon separately. Studies on breast cancer patients have shown that exercise regimens during anthracyclines or taxane chemotherapeutical treatment increased the CS activity, and especially aerobic training interventions lowered all mitochondrial complex I-IV protein levels compared to sedentary patients [142].

4.1.5 Stress-response pathways are underlying the breast-cancer induced muscle weakness

The next aim was to identify the causing factors of the muscle weakness and altered mitochondrial activity. It is widely recognized that tumor cells themselves or tumor infiltrating cells of the immune system, create enhanced intrinsic stress in the form of pro-inflammatory cytokines [113]. For instance, increased levels of tumor necrosis factor α (TNF-α) have been linked to mitochondrial dysfunction [125] as well as to induce negative effects on muscle force [123]. Therefore, we hypothesized that the impaired mitochondrial function in skeletal muscle of PyMT mice stems from a chronic intrinsic stress-response, caused by pro-inflammatory markers, originating from the breast tumor.

A serum cytokine antibody array of ~30 targets showed overall higher levels of interleukins and chemokines in serum from PyMT mice than WT mice **(Paper I; Suppl. Fig. 2 A**). TNF-α levels were also specifically measured in serum and skeletal muscle and both were higher in PyMT than WT mice. The 4-week voluntary exercise significantly reduced the TNF-α levels as well as the expression of several TNF-α target genes, including TNF receptor-associated factor 2 (*Traf2*), inhibitor of kB-α (*IκB-α*) ankyrin repeat domain 1 (*Ank1*) and nuclear factor-κB1 (*NFκB1*) in GAS and SOL muscles of PyMT mice (**Paper I; Fig. 4 A-G**).The p38 mitogen-activated protein kinase (MAPK) is the key player in controlling stress-depending cellular responses and can be activated through pro-inflammatory cytokines [143] . The phosphorylated and active form of p38, in ratio to total p38 expression, was significantly higher in muscles of PyMT mice than WT mice, and levels in PyMT mice were normalized and more similar WT levels after the exercise intervention **(Paper I; Fig. 4 H-I)**.

The initiation of intramuscular stress is also associated with oxidative stress caused by excessive amounts of ROS and/or reduced capacity of the antioxidant defense system [90, 94]. Skeletal muscle of PyMT mice has generally a lower expression of endogenous ROS scavengers than WT mice, but the exercise intervention was able to counteract the expression pattern of superoxide dismutase (*Sod1*, *Sod2*) and catalase (*Cat*) as well as the SOD activity in GAS muscle of PyMT mice (**Paper I; Fig. 4 J-M**). Overall, four weeks of voluntary running where sufficient to reduce the systemic and intramuscular stress response in skeletal muscle of PyMT mice.

Other studies have shown that treadmill exercise of intraperitoneal (i.p.) doxorubicin treated and untreated WT rats was able to increased levels of ROS scavengers, such as superoxide dismutase 1 and 2 (SOD1 and SOD2) and glutathione peroxidase (GPx) in skeletal muscles [144].

4.1.6 PGC-1α exercise-activated expression in skeletal muscle impacted by breast cancer

The cellular response to exercise is modulated by the transcriptional co-activator PGC-1α, which regulates the mitochondrial biogenesis [28, 145]. PGC-1α is regulated by NF-κB, p38 MAPK and TNF-α [146], which all were observed to be elevated in skeletal muscle of PyMT mice as compared to WT mice (**Paper II; Fig. 4 A- I**).

PGC-1α is commonly found increased after acute exercise rather than after prolonged endurance exercise [147, 148] and accordingly, no increased expression of PGC-1α was observed in skeletal muscle after four weeks of voluntary running exercise. However, PGC-1α gene expression was significantly increased in exercised WT mice after the treadmill exhaustion run (**compare Paper I, Fig.2 F-G**), but not in PyMT mice which underwent the same exercise protocols (**Paper I; Fig. 4 N-O**). This shows that the breast cancer exhibits a repressing effect on the muscular responses to exercise.

In summary, the presented results in **Paper I** show that breast cancer in PyMT mice leads to a skeletal muscle dysfunction displayed as reduced physical performance and muscular weakness. This seems to originate from altered mitochondrial transcriptional as well as activity changes, together with high levels of intramuscular stress-related pathways, and impairment in the transcription and enzymatic activity of the antioxidant scavenging system. Four weeks of voluntary running counteracted most of the breast cancer inflicted effects and restored the skeletal muscle force and physical performance of PyMT mice. See also **Figure 9** for a graphical summary.

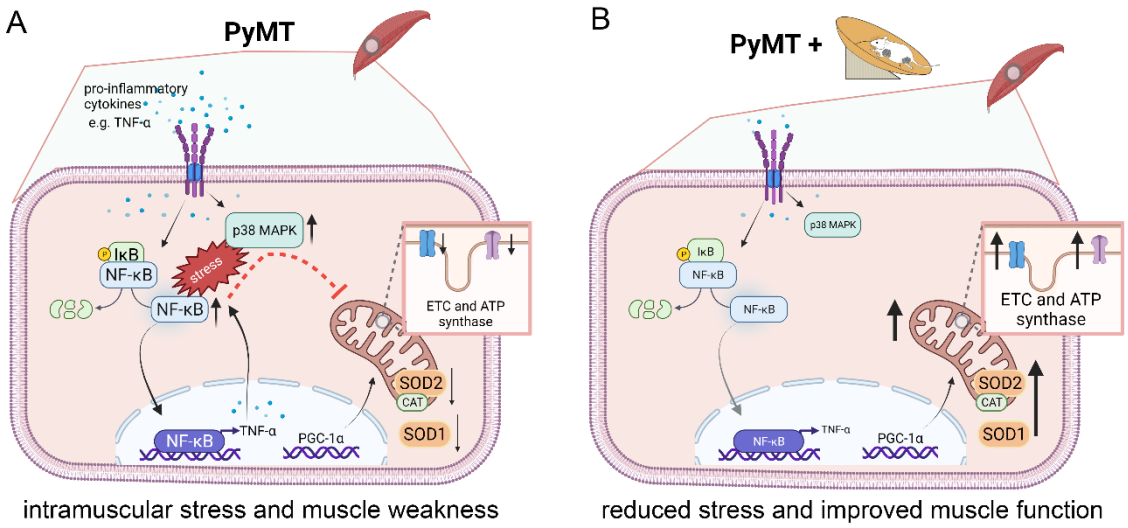

Figure 9: Schematic view on the findings presented in paper I. Describing (A) how the breast cancer in PyMT mice leads to a reduced skeletal muscle force production and (B) how 4-weeeks of voluntary exercise counteracts the tumor-induced muscle weakness through decreased intramuscular stress-related pathways. Figure from [149], created with BioRender.com.

4.1.7 Future considerations (Paper I)

Due to the animal welfare matters, our experiments had to be concluded at 12 weeks of age of PyMT mice. A longer duration of the disease might lead to intensified mitochondrial dysfunction and consequently further elevated ROS and oxidative damage to proteins.

To further elucidate the mitochondrial dysfunction in mice with breast cancer and its consequences for skeletal muscle function, the lab would like to obtain muscle biopsies from patients with breast cancer, as well as other type of cancers, to be used for global analyses, e.g., RNA-sequencing to further disentangle cancer-induced muscle dysfunction.

ROS signaling, including the source of ROS as well as the cellular scavenging system is another avenue to further investigate to obtain more in-depth knowledge about breast cancer induced muscle dysfunction. For instance, the ROS signaling and oxidative stress should be explored in a time-dependent manner to the exercise, for example through isolation of fibers at different timepoints post-exercise and detection of cellular oxidative stress with fluorogenic probe, e.g., mitosox or other redox probes.

Another aspect that would be intriguing to explore further is trying to understand why different fiber-types are affected differently by the disease, in this case breast cancer. A better knowledge concerning fiber-type specific changes could have significant impact for future interventions trying to counteract muscle dysfunction and weakness as a comorbidity.

4.2 NOVEL ANTI-TUMORIGENIC COMPOUND AFFLICTS WHOLE BODY AND MUSCLE METABOLISM IN A MODEL OF BREAST CANCER (PAPER II)

As described in **Paper I**, exercise is beneficial for breast cancer related disease outcomes. Nevertheless, breast cancers therapy primarily relies on surgical resection together with postoperative radiation and chemotherapy, depending on the progression state and molecular profile of the tumor. Since there are long-term effects of the anti-cancer treatment and hence implications on the quality-of-life of patients in remission these are areas more and more under investigation [150]. In **Paper II**, we investigate the effects that the novel anti-tumorigenic compound CX-5461 exhibited on the whole-body as well as on the skeletal muscle metabolism.

4.2.1 CX-5461 shows efficient tumor-reducing capabilities in PyMT mice

Cancer cells often show a dysregulated ribosome biogenesis and larger nucleoli, the location of ribosome biogenesis, have been observed [52-54]. This has also been proven to be true for the tumors of PyMT mice, which exhibit an accelerated protein synthesis [151]. The novel RNA-Polymerase I (Pol I) assembly inhibitor CX-5461 acts through inhibition of the of the Pol I pre-initiation complex (PIC) assembly. CX-5461 has a selective anti-proliferative activity for multiple human cancer cell lines [51, 152, 153] and is currently under investigation in two Phase I trials [55, 57, 58] (further discussed in chapter **1.3.3.1**).

Here, WT and PyMT mice were treated 1/week with 75mg/kg CX-5461 (or vehicle) by intraperitoneal injection (i.p.) for four weeks. The CX-5461 treatment showed comparable effectiveness in reducing the tumor volume (by ~18-fold) as compared with the established drug and mitotic cell division inhibitor Docetaxel (**Paper II; Fig. 1 A-B, Suppl. Fig.1 A**). The reduction of breast tumor mass was measured with calipers and confirmed by dual-energy X-ray absorptiometry (DXA) measurements of PyMT at the age of week 12. Treated PyMT mice displayed a significant reduction of the whole-body mass and lean body mass, which was not an effect of reduced muscle mass, as muscle weight was stable among the groups (**Paper II; Fig. 1 C-E**, **Suppl, Fig. 1 F-G**).

4.2.2 CX-5461 induced alterations in the whole-body energy utilization

About half of all patients with patients with breast cancer report severe or very severe side-effects when undergoing treatment [154]. The effect of cancer-treatment on body composition is widely accepted [155], but little is known of their extended effect on metabolism.

In **paper II** we investigated the effect of CX-5461 on the whole-body metabolism after treatment of WT and PyMT mice. PyMT and WT mice treated with CX-5461 (or vehicle) for four weeks were monitored for 5 days in the comprehensive lab animal monitoring system (CLAMS). The movement was markedly reduced in untreated PyMT mice by ~40%, likely caused by mobility hindrance by the tumor, which significantly improved after CX-5461 treatment, likely being a result of the tumor volume reduction (**Paper II; Fig. 2 A-C,** compare **Paper II; Fig. 1 A**).

The results also showed a strong effect of CX-5461 on key metabolic parameters not only in PyMT, but as well in WT mice. Total food intake, energy expenditure, VO_2 consumption and VCO_2 production were significantly increased in both groups treated with CX-5461 (**Paper II; Fig. 2 D-E**). The respiratory exchange ratio (RER, VCO_2/VO_2) calculates the relationship between utilizing carbohydrates (RER=1) or fat (RER=0.7) as energy and with increasing workload the proportion of utilized carbohydrates will increase (>1.0 indicate accumulation of lactate). Here CX-5461 treatment significantly increased the RER for both WT and PyMT mice, reflecting an increased carbohydrate utilization by the body (**Paper II; Fig. 2 L-N**). This was observed even though treated WT mice moved less than untreated WT mice and although treated PyMT mice moved more than untreated PyMT mice but they still didn't move as much as untreated WT mice (**Paper II; Fig. 2 A-C**).

Traditionally, cancer research has focused on the energy requirement of cancer cells and how that exerts an effect on the surrounding tissue. Cancer cells alter their own metabolism to promote survival, growth and invasion contributing to the tumor's malignancy and development [81, 156-158]. In tumors of untreated PyMT mice a lower glucose concentration and increased glycerol-3-phophase levels have been observed, indicative of aerobic glycolysis (Warburg effect) [151].

Breast cancer survivors have an increased risk of developing type 2 diabetes (T2D) with an incidence of 10% over arounds six years after treatment [86, 87] and thus recent clinical studies have focused on the effect the cancer and anti-tumorigenic treatments have on the whole-body metabolism. For instance, estrogen receptor modulator treatment with tamoxifen seems to be associated with and increased risk of developing T2D in breast cancer survivors [159], but it is still difficult to distinguish which effects stem from the tumor itself and which from the anti-tumor treatment.

4.2.3 CX-5461 induces hyperglycemia in WT and PyMT mice

To continue this line of thought and based on the CLAMS results showing a shift of the whole-body metabolism towards carbohydrate utilization (**Paper II; Fig. 2 L-N**), we took a closer look on the blood glucose level in both untreated and CX-5461 treated WT and PyMT mice. Blood glucose was measured after fasting and followed up after a 2 g/kg bw i.p. glucose injection. Untreated WT and PyMT mice showed no difference in baseline glucose levels or glucose clearance.

However, the blood glucose was significantly higher in CX 5461 treated mice, compared to untreated or vehicle-treated mice at baseline level. After a 2 g/kg i.p. glucose injection, the treated mice showed a slower glucose clearance and the plasma glucose concentration remained elevated even after 120 min (**Paper II; Fig. 3 A-D** and **Figure 10 A-C**). No change in plasma insulin levels was observed between the treated and untreated mice and hence seems unlikely that CX-5461 affects the insulin secretion from pancreas (**Paper II; Fig. 3 E**).

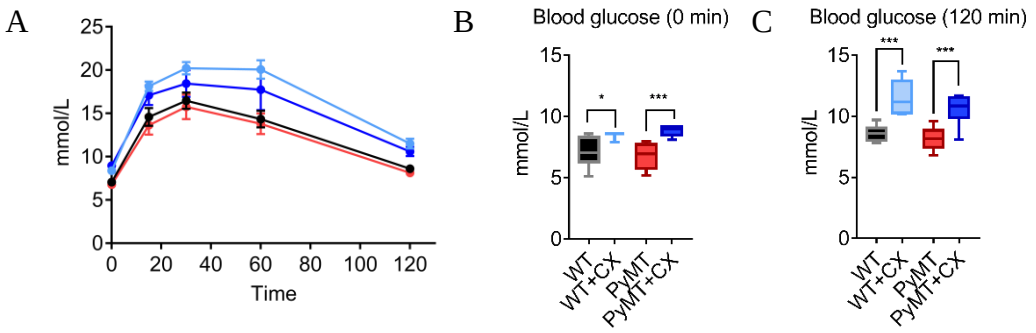

Figure 10: CX 5461 increases blood glucose levels in WT and PyMT mice. Glucose tolerance test (GTT) curve (A) after intraperitoneal (i.p.) glucose injection (2 mg/g). Blood glucose level (mmol/L) at baseline (C; 0 min) and after 120 min (D). Data are mean ± SEM or presented as box-plot and whiskers blot with the median indicated as horizontal line, the boundaries extend from the 25th to the 75th percentiles, and the error bars cover the extremes of the observation. (A-C n=8 per group). ***p<0.001.

4.2.4 Alterations on glucose uptake by CX-5461 in skeletal muscle

Skeletal muscle is a key organ for the body's metabolism and also a major site for postprandial glucose uptake and storage [1, 2]. To further investigate the metabolic effects of CX-5461, we conducted a 2-deoxyglucose (2-DOG) uptake experiments in slow-twitch SOL and fast-twitch digitorum longus muscle (EDL) skeletal muscle of both untreated and treated WT and PyMT mice. The glucose uptake was measured in the absence (basal) and presence of insulin (0.36 nM, sub-maximal). Interestingly, CX-5461 exerted different effect in WT and PyMT mice. In WT mice, CX-5461 reduced the basal glucose uptake with no significant effect on insulin-stimulated glucose uptake. Whereas in PyMT mice, CX-5461 treatment blunted the insulin-stimulated glucose uptake but had no effect on basal glucose uptake (**Paper II; Fig. 4 A-D**). Thus, it appears as CX-5461 exerts different effects on healthy and diseased mice which may be related to that the breast cancer itself attenuates the metabolic response to the drug in PyMT. The CX-5461 effects were observed in glycolytic EDL muscles but not in oxidative SOL muscle. The cause of the different response between different muscle fiber types remains unresolved, but in line with **paper I** it shows that there are indeed differences between the metabolic type of muscle fibers that should be further evaluated in the future.

4.2.5 Direct inhibitory effects of CX-5461 on skeletal muscle

The previous results suggested a direct effect of CX-5461 on skeletal muscle. Therefore, we investigated the Pol I transcript levels as well as relevant genes of the Pol I pre-initiation complex (PIC) in mammary and tumor tissue, as well as skeletal muscle.

Tumor tissue of PyMT mice showed a significant increase in the pre-rRNA transcript 45S as well as other relevant PIC genes, compared to mammary tissue from WT mice, as shown before [52-54, 151]. As predicted from CX-5461 reported target-specificity [160], a decrease in gene expression of the 45S pre-rRNA and its processed rRNA transcripts (18S, 5.8S and 28S rRNA) as well as selected PIC genes was detected in tumor tissue in CX-5461 treated PyMT mice. Mammary tissue of treated WT mice showed no changes in gene expression (**Paper II;**

Fig 5 A-K). Muscle from WT mice treated with CX-5461 showed no change in the expression of rRNA transcript, but significantly decreased expression of the PIC genes (**Paper II; Fig. 5 L-V**). Additionally, reduced gene expression levels of the same genes were observed in skeletal muscles which had been had been treated acutely with CX-5461 (*ex vivo* incubation for 6 hours) (**Paper II; Fig. 5 W-AG**). Thus, the results confirmed the hypothesis about CX-5461 exerting off-target effects in skeletal muscle.

4.2.6 CX-5461 disrupts key metabolic regulators

We initiated the investigation into the molecular pathways underlying the CX-5461-induced metabolic effects. A broader screen of gene expression of rate-limiting enzymes in EDL muscle involved in both glycolysis (see **Figure 11**, **Paper II; Fig. 6 A-D**) and fat metabolism (see **Figure 12**, **Paper II; Fig. 6 E-J**), revealed an opposing pattern. Muscle of WT mice treated with CX-5461 had a mainly increased gene expression of these markers, including pyruvate dehydrogenase kinase 4 (*Pdk4*; **Paper II; Fig. 6 C**), fatty acid translocase (*Cd36)*, fatty acid transport protein 1 (*Slc27a1*) and hormone-sensitive lipase (*Lipe*; **Paper II; Fig. 6 E-J**). Whereas, in the same muscle a mostly decreased expression was observed in PyMT mice, including genes for Pdk4, Lipe and cytidine triphosphate synthase 1 (*Ctps1*; **Paper II; Fig. 6 A-J**). At this point we can only be speculated how the gene transcription possibly translates into the enzyme activities. However, reduction in enzymes such as the hormone-sensitive lipase (*Lipe*), are often found in adipose tissue of diabetic patients [161] and is linked to defective insulin signaling in skeletal muscle [162]. Contrary, the increase of those markers in skeletal muscle of treated WT mice, is in contrast to the previously observed increased whole-body RER after CX-5461 treatment. But the skeletal muscle metabolism in CX-5461 treated PyMT mice with a decrease of fatty acid metabolism markers (**Paper II; Fig. 6 E-J**), could be extrapolated to be in line with the previously observed whole-body higher RER, indicating utilization of carbohydrates (**Paper II; Fig. 2 L-N**).

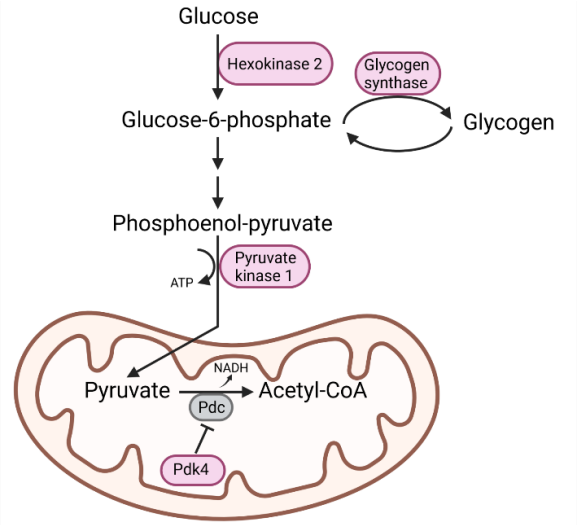

Figure 11: Selected markers (red) of glycolysis to screen for CX-5461 related effects on cellular metabolism. Enzymes in red are studied in this thesis. Image created with BioRender.

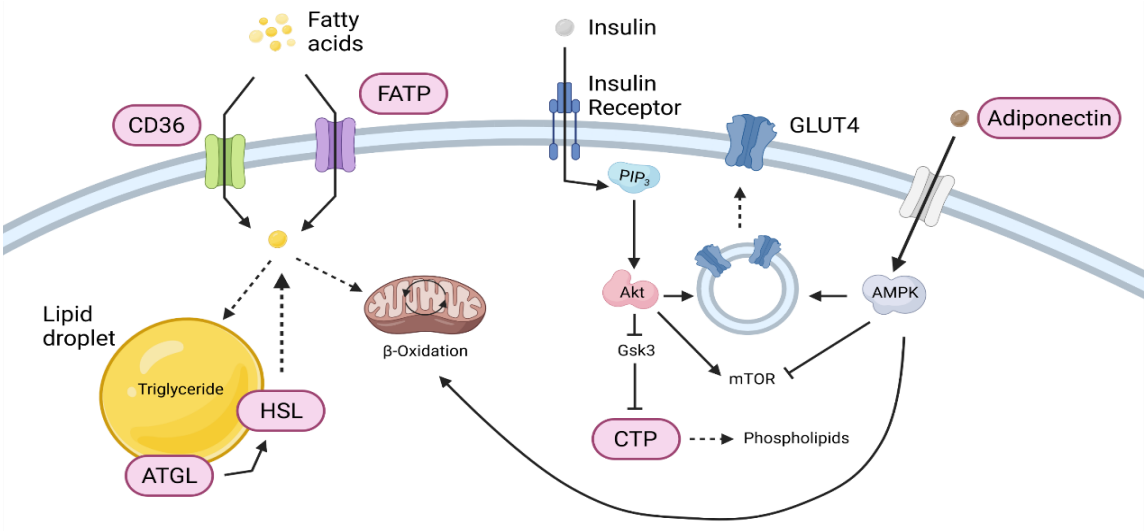

Figure 12: Selected markers (red) to screen for CX-5461 related effects on cellular fatty acid metabolism. Enzymes in red are studied in this thesis. Figure created with BioRender.com.

In summary, the data collected in **Paper II** shows that although the novel anti-tumorigenic compound CX-5461 effectively reduced the breast tumor in PyMT mice after just four weeks of injection, the treatment leads to elevated levels of basal blood glucose in mice and a dysregulated glucose uptake in skeletal muscles. Interestingly, CX-5461 had to some extent different effects on WT and PyMT mice, a phenomenon which we currently cannot explain. A better understanding of the metabolic impact of anti-tumorigenic drugs might lead to different treatment regimen and prevent patients with breast cancer from developing T2D, thus improving their quality of life during remission. The findings of this manuscript are summarized in **Figure 13**.

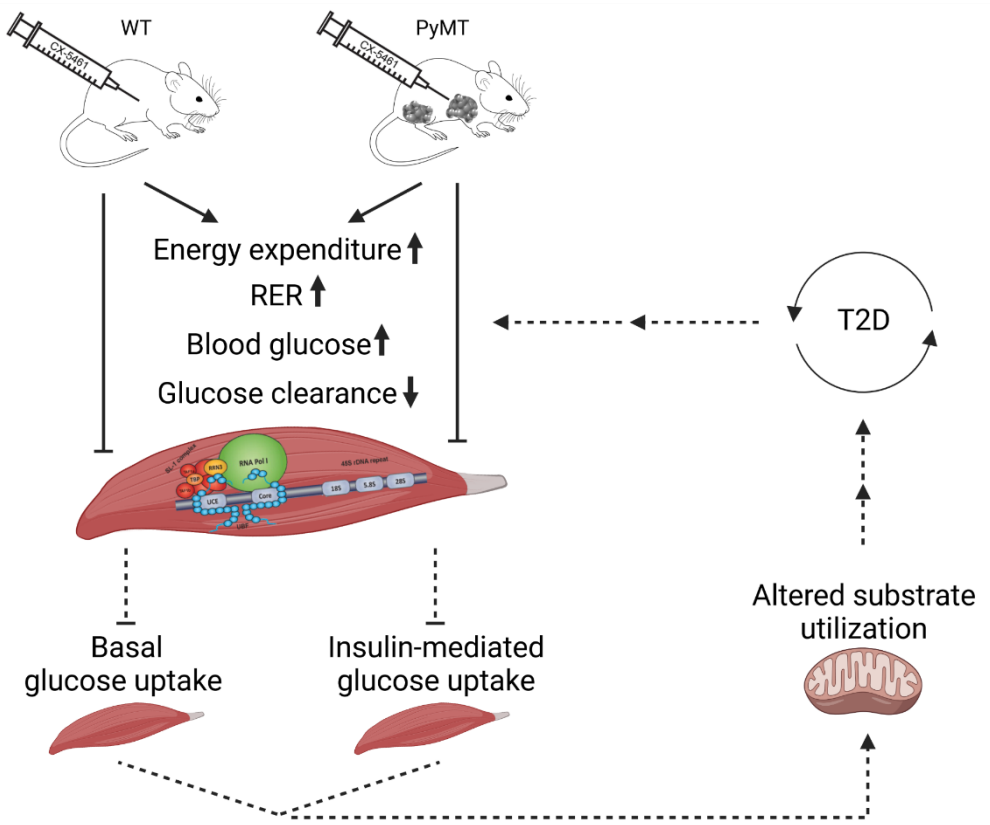

Figure 13: Graphic summary of the results presented in paper II. Describing the effects of CX-5461 on the whole-body as well as on skeletal muscle-specific metabolism. Figure created with BioRender.com.

4.2.7 Future considerations (Paper II)

The different metabolic responses of WT and PyMT mice to the anti-tumorigenic compound CX-5461 should be looked upon more closely. To investigate the whole-body effect of CX-5461 a metabolomic study could give indications of altered metabolites between tumor, mammary tissue and skeletal muscle.

Sequencing of skeletal muscles from treated and untreated mice might give a better understanding of the differentially expressed genes and responses to the treatment. Additionally, non-coding small nucleolar RNAs (snoRNAs) have been found to be responsible for posttranscriptional modification and maturation of rRNA as well as being overexpressed in both murine and human breast cancer [163, 164]. For a better understanding how CX-5461 treatment might affect the posttranslational modification, e.g., methylation, sequencing could be used to measure individual RNA modifications.

More research is needed to distinguish the difference CX-5461 has on slow-twitch and fast-twitch skeletal muscle metabolism. An analysis into proteins involved in the glucose transport and fractionation of vesicle- and membrane bound GLUT1 and 4 could give a better understanding how the glucose uptake is impacted by CX-5461 in skeletal muscle of treated

and untreated WT as well as PyMT mice. The impact of CX-5461 on the activity of central metabolic enzymes such as AMPK or Akt in skeletal muscle should be examined.

To investigate the metabolic response of WT and PyMT mice to the novel anti-tumorigenic treatment CX-5461, a comparison to the traditionally used drug Docetaxel should be done. These experiments should be complemented with a clinical study involving patients with breast cancer on different treatments and healthy controls, were muscles biopsies and blood samples are taken to investigate the treatment- induced alterations in energy utilization.

4.3 REACTIVE OXYGEN SPECIES AS STRESSOR OF SKELETAL MSUCLE FUCNTION AND METABOLISM (PAPER III)

In **paper I** the disease breast cancer and in **paper II** the breast cancer treatment were described as stressors to the skeletal muscle function and metabolism. In **paper III** we investigated how stress in the form of reactive oxygen species (ROS) affects the skeletal muscle endogenous glycogen phosphorylase (phosphorylase) and thus glycogenolysis.

4.3.1 Regulation of glycogen phosphorylase

Glycogen is the form in which glucose is stored in skeletal muscle cells. The breakdown of glycogen into glucose molecules (glycogenolysis) thus providing cells with energy is catalyzed by the rate-limiting enzyme phosphorylase. Although the phosphorylase was identified almost 100 years ago [165], the regulation of phosphorylase is still debated. For instance, muscle contractions can lead to an increase of AMP which is thought to activate phosphorylase [140]. On the other hand, phosphorylase can be inhibited through ROS [23, 166], such as nitric oxide ($NO^{\bullet}$) which also impacts muscle contractile proteins and metabolism [90, 167].

In **Paper III** the hypothesis is examined whether the redox state alters phosphorylase activity and glycogenolysis in contracting skeletal muscle.

4.3.2 Glycogen phosphorylase is strongly inhibited by $ONOO^-$, but not by H_2O_2 in muscle extracts

The two ROS molecules hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) were examined in muscle extracts of both SOL and EDL muscle. At the highest concentration of 1 mM H_2O_2 only inhibited the phosphorylase activity by ~20%, which was reversed by the reducing agent dithiothreitol (DTT) (**Paper III; Fig.2 A-D**). The inhibition might be different for tissue-specific phosphorylase isoforms (muscles, liver and brain), as it has been shown that H_2O_2 inhibits phosphorylase activity in brain tissue, but to lesser extent in muscle tissue, and not at all in liver tissue [166]. In contrast, 1 mM $ONOO^-$ completely inhibited the phosphorylase activity in SOL and EDL muscle extracts (**Paper III; Fig.3 A-F** and **Figure 14**). Similarly, to H_2O_2, DTT was able to fully reverse the inhibition by $ONOO^-$ (**Paper III; Fig.3 A-F** and **Figure 14**).

The physiological concentration of ONOO- is unknown, but thought to reach micromolar levels in order to nitrate tyrosine residues [168, 169]. We have previously shown that the contractile protein actin is nitrated in skeletal muscle from patients with rheumatoid arthritis, who also exhibited muscle weakness, which indicates that indeed ONOO- can reach endogenously micromolar levels to induce nitration [94]. Moreover, ONOO- has also been directly linked to muscle weakness [94, 170]. Those findings are in line with other studies that found ONOO- inhibits purified phosphorylase by nitrating Tyr-613 as well as glycogenolysis in C1C12 myotubes [23, 166].

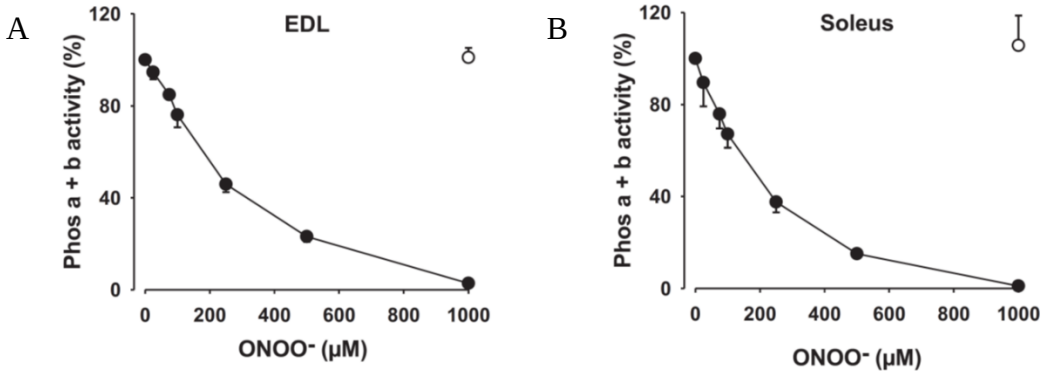

Figure 14: Full inhibition of phosphorylase activity by $ONOO^-$ in muscle extracts. Phosphorylase activity was measured both in EDL and SOL muscle after 30min exposure to increasing concentrations of $ONOO^-$.

4.3.3 $ONOO^-$ induces metabolic stress in intact muscles

After the inhibition of phosphorylase through $ONOO^-$ has been shown in muscle extracts, the next experiments were to determine the effect on glycogenolysis in dissected, intact muscles, which have an intact physiology and can be exercised by contraction.

As expected, incubation with ONOO- lead to an inhibition of glycogenolysis in intact skeletal muscle in the basal (non-contracted) state, indicated by an increase in glucose-6-P together with a marked reduction of ATP and PCr (**Paper III; Tab. 1**). The unexpected decreases of lactate and malate might be due to the added potential of $ONOO^-$ to inhibit glycolysis and mitochondrial respiration [171, 172].

A series of ten contractions was chosen to allow for activation of glycogenolysis, but without exerting strong effects on the force in SOL and EDL muscles. The contractions lead to a decrease of PCr, but an increase of glucose-6-P, lactate as well as malate, whereas the concentration of ATP remained the same. $ONOO^-$ incubation of the contracted muscle reduced almost all metabolites (**Paper III; Tab. 1**).

4.3.4 $ONOO^-$ reduces skeletal muscle force of EDL and SOL, but does not affect glycogen phosphorylase activity in intact muscles

During the series of ten contractions, the forces of both EDL and SOL remained mainly stable. However, incubation of the contracting muscle with $ONOO^-$ significantly reduced the force. The two different muscle types responded differently to $ONOO^-$. Both EDL and SOL showed significant decreases in the force already with the first contraction, but thereafter the force of SOL did not further decrease, whereas the force of EDL continued to deteriorate over the series of contractions (**Paper III; Fig. 4**). To estimate glycogenolysis more accurately and to exclude the effect of mitochondrial respiration, muscles were incubated with a low concentration of sodium cyanide (NaCN) to inhibit cytochrome c oxidase. The chosen NaCN concentration did not alter key metabolites, including lactate (**Paper III; Tab. 2**), but effectively inhibited

mitochondrial respiration, as seen by the remaining stable level of malate after repeated contractions (**Paper III; Tab. 1, Tab. 3, Tab. 4**).

Contrary to the inhibitory effects on muscle extracts, $ONOO^-$ did not affect phosphorylase or glycogen synthase activity in intact skeletal muscles (**Paper III; Suppl. Tab. 1-5**). It is unlikely that the used concentration of $ONOO^-$ (2 mM) is inhibiting other key metabolic enzymes, without also inhibiting phosphorylase. A possible explanation is that the $ONNO^-$ concentration in the assay mixture was lower than 1 µM and thereby not sufficient to suppress enzyme activity (compare **Paper III; Fig. 3**). For instance, if $ONOO^-$ inhibition would only target e.g., aldolase, and through that inhibit glycogenolysis, the abolished accumulation of lactate would go together with an increased accumulation of glucose-6-P [173], contrary what we have reported. Other studies shown a similar effect where a higher concentration of $ONOO^-$ was needed to inhibit phosphorylase in intact C2C12 myotubes in contrast to a lower $ONOO^-$ concentration needed to inhibit purified phosphorylase [23].

4.3.5 DTT lowers SOL force but only minorly affects metabolite profile

To examine endogenously produced ROS regulating phosphorylase and glycogenolysis after contraction, muscles were incubated with antioxidants or ROS scavengers. Incubation with one common ROS scavenger, i.e., DTT, induced small decreases in lactate and malate in both EDL and SOL in the basal state. After repeated contractions, DTT incubation did not induce significant differences in metabolite levels in EDL muscles (**Paper III; Tab. 3**). SOL muscles showed a minor elevation of glucose-6-P after repeated contractions. Overall, DTT treatment did not induce major changes in the levels of the measured metabolites. In regards to the force production, DTT incubation significantly reduced SOL force throughout the contraction series, but not EDL muscles (**Paper III; Fig. 5**). The difference in inhibition is likely due to the slow-twitch SOL muscle relying on oxidative phosphorylation for energy production, contrary to the glycolytic fast-twitch EDL muscle. DTT might increase reductive stress with elevated levels of NADH, and NADPH inducing mitochondrial dysfunction [174].

4.3.6 NAC reduces the force of SOL and EDL muscles, similar to DTT

In addition to DTT, another common ROS scavenger was used., i.e., N-acetylcysteine (NAC) to further determine the role of endogenous ROS in skeletal muscle. In EDL muscle, NAC treatment led to a significant reduction in PCr in the basal state, but no changes occurred in the metabolite profile after repeated contraction. No notable changes were detected in the SOL muscle (**Paper III; Tab. 4**) in either basal state or after contraction. The incubation with DTT or NAC did not alter the activity of both total phosphorylase and glycogen synthase (**Paper III; Suppl. Tab. 1-5**). The force of both EDL and SOL significantly decreased during the first three contraction in the series of 10 contractions after DTT or NAC incubation (**Paper III; Fig. 6**). Altogether, the force profiles look similar for both muscles when treated with DTT or NAC.

Overall, the impact of ROS and antioxidants on muscle force is still not entirely understood, mainly due to the interplay of many different factors e.g., concentration, duration, stimulation protocol, fiber type etc. [175-177]. For example, short-term H_2O_2 exposure can increase muscle

force production, whereas prolonged exposure to H_2O_2 decrease the force [178]. It can be hypothesized that under basal conditions, muscles fibers are slightly reduced [176] and moderate ROS levels would increase the oxidative state of force-generating proteins, leading to an overall increase of force. Here, the inhibition of mitochondrial respiration by DTT or NAC might have led to an inhibition of superoxide $O_2^{\cdot -}$ production, which normally accumulates after repeated contractions. Suggesting, that the addition of the antioxidants NAC and DTT convert the muscle fiber to a reduced state and thereby decreasing the force [179].

4.3.7 $ONOO^-$ induced 3-nitrotyrosine on glycogen phosphorylase

To prove our hypothesis that phosphorylase can be nitrated, immunoprecipitation experimented were performed. Intact EDL muscles, incubated with $ONOO^-$ showed indeed a higher nitration of phosphorylase (**Paper III; Fig. 7** and **Figure 15**). No change in nitration could be detected in SOL muscle. Repeated contraction did not alter the nitration levels of phosphorylase, as it was expected from the little effect antioxidant (DTT, NAC) exerted on glycogenolysis during contraction. This indicates that sufficient high levels of $ONOO^-$ are able to induce phosphorylase nitration, in turn inhibiting phosphorylase activity and resulting in compromised glycogenolysis during repeated contractions. The inhibition of glycogenolysis through $ONOO^-$ is thus likely a result of the inhibition of phosphorylase.

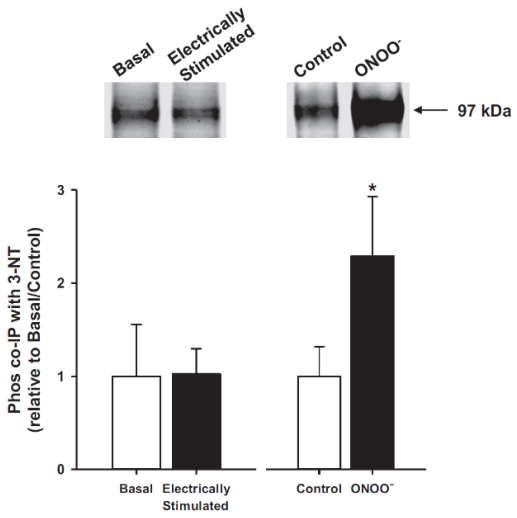

Figure 15: Induction of post-translational modification 3-nitrotyrosine on phosphorylase after $ONOO^-$ treatment in EDL. Repeated contraction did not change the nitration of phosphorylase.

In summary, **paper III** shows that exogenous $ONOO^-$ inhibits phosphorylase activity in muscle extracts and glycogenolysis in intact contracted muscles. However, the exposure of contracting muscles to exogenous antioxidants, such as DTT and NAC, lead to a force reduction, but only played a minor role in regulating phosphorylase activity and glycogenolysis. A visual summary of the results is presented in **Figure 16**.

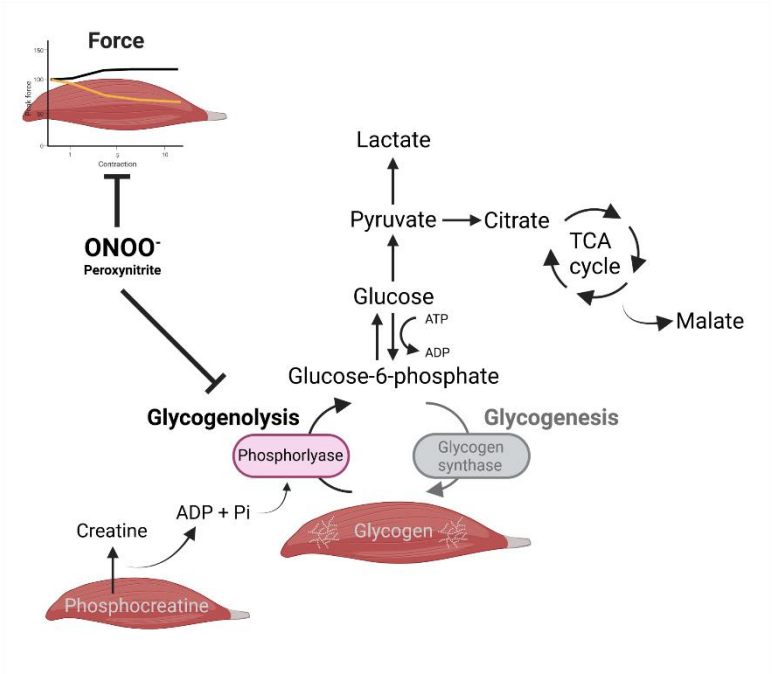

Figure 16: Graphical summary of the results presented in paper III. Reporting the inhibitory effect of exogenous $ONOO^-$ on phosphorylase and glycogenolysis activity, as well as skeletal muscle force. Figure created with BioRender.com.

4.3.8 Future considerations (Paper III)

The current concentrations of $ONOO^-$ were not enough to inhibit phosphorylase in intact skeletal muscle. The experiments should be continued with a sufficient concentration of $ONOO^-$ to impact phosphorylase.

Both antioxidants NAC and DTT affected the force of fast-twitch and slow-twitch muscle to a similar degree. However, the difference in force reduction between the muscle types is poorly understood. The hypothesized mechanism should be further investigated with a deeper analysis of mitochondrial enzyme activity in skeletal muscle after exposure to DTT and NAC.

In the same manner, future studies should focus of understanding the temporal and spatial responses of the endogenous oxidative signaling and its scavenging system.

5 CONCLUSIONS

Skeletal muscle is an important organ for all everyday movements. Additionally, it is one of the main metabolic organs in the body. Weakened or dysfunctional skeletal muscle has a great impact on the quality of life of afflicted patients.

Different stressors, from disease to endogenous ROS, can affect the performance, function and metabolic capacity of the skeletal muscle. To resemble the systemic stress and its impact on skeletal muscle, mouse models mimicking a certain disease can provide important data to be translated to human patients in the future.

Paper I showed that the PyMT mouse model of breast cancer mimics the observed non-cachexic muscle weakness in human patients. With no changes in skeletal muscle mass, morphology or fiber type distribution, PyMT mice exhibited a force reduction and overall reduced physical performance compared to WT mice. For the first time, a potential underlying molecular mechanism has been shown. The results obtained indicated that this is caused by elevated levels of pro-inflammatory cytokines which in turn activate intramuscular stress response pathways, such as p38 MAPK and TNF-α, further afflicting the mitochondrial gene expression and enzyme activity in skeletal muscle of PyMT mice. With moderate exercise, four weeks of voluntary running, the cancer-inflicted muscle weakness was counteracted, and the physical performance improved. The exercise intervention led to a reduced intrinsic stress profile and normalized mitochondrial gene expression as well as enzyme activities to comparable WT levels. I can be hypothesized that a similar dysfunction and mechanism can be detected for other cancers types not prone to muscle atrophy, e.g., lymphomas. Further studies should investigate the proposed mechanisms in human patients with cancer. Above all, gathering a better perception of the molecular processes involved in the beneficial effect of moderate exercise on cancer-induced muscle weakness, lays the foundation for translational research and improving the quality of life for breast cancer patients.

Paper II demonstrated that the novel anti-tumorigenic Pol-I-assembly inhibitor CX-5461 effectively reduces the tumor burden in PyMT mice. However, our data showed for the first time that CX-5461 has off-target effects on skeletal muscle. Through that the whole-body metabolism as well as both the glucose and lipid metabolism of skeletal muscle are affected. CX-5461 distinctly induced hyperglycemia and decreased the glucose uptake into skeletal muscle in the treated mice. The effect was different in WT and tumor-bearing PyMT mice, indicating an additional effect of the breast cancer on the skeletal muscle metabolism. Future studies are needed to clearly distinguish the effect of CX-5461 on the whole body and muscle-specific metabolism, especially in comparison to commonly used cancer treatments. Altogether, the study issues a caution that patients with breast cancer in remission might have to be continuously monitored as they could suffer from long-term metabolic treatment effects.

Paper III presented the different roles the oxidative system plays in inhibiting phosphorylase, a key enzyme of glycogenolysis. Incubation with ONOO- was shown to significantly inhibited the phosphorylase activity and glycogenolysis during repeated contractions. Increased levels of tyrosine nitration on phosphorylase were thought to inhibit its enzymatic activity. When the endogenous ROS system was challenged by exogenously introduced antioxidants, the effect on the phosphorylase activity was only minor. Nevertheless, the antioxidant treatment (DTT, NAC) led to a reduction of muscle force. This indicates that the endogenous redox signaling is delicately regulated and the duration and concentration of ROS exposure is crucial for exerting the desired action.

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