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*FELLOWSHIP FINAL REPORT*

# Restoring essential fatty acid balance improves muscle condition through mitochondria-associated mechanisms

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REPORT INFO

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ABSTRACT

*Muscle loss (atrophy) and fatty infiltration of muscle (myosteatosis) are prevalent in people with cancer and are exacerbated during chemotherapy treatment. Each of these features are independently prognostic for survival in cancer patients. Ongoing work in the applicant's laboratory has revealed an improvement in tumor response and reduced muscle loss when eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) were provided to patients and in a pre-clinical model (rodents) undergoing cytotoxic treatment for cancer. The objective of this research is to develop an in vitro model to determine mechanisms by which EPA+DHA act on the mitochondria to protect muscle from development of atrophy and myosteatosis during exposure to chemotherapy. Human skeletal muscle cells are cultured with and without exposure to chemotherapeutic agents to establish the timeline and characteristics of myosteatosis. Cells cultured with or without EPA+DHA (at physiological levels) will be compared for triglyceride-fatty acid content, lipid droplet content and size, mitochondrial number, oxidative capacity and function. This information is required to develop or refine therapeutics directed at muscle wasting in cancer patients and contributes to collaborative efforts focused on improving prognosis of cancer patients.*

## 1- Introduction

Muscle is essential for movement and vitality, but becomes perturbed in disease states such as cancer. Muscle loss (atrophy) and fatty infiltration of muscle (myosteatosis) are prevalent in people with cancer and are exacerbated during treatment with anti-neoplastic drugs (chemotherapy). Both atrophy and myosteatosis are independently prognostic for survival in cancer patients (e.g., 1-7)

Mitochondria convert food sources of fuel into energy that is used by the cell. Muscle loss may be evoked by disruptions in normal mitochondrial function. In non-cancer conditions, such as obesity and aging, the dysfunction of mitochondria has been linked to myosteatosis (reviewed by 8, 9). The importance of mitochondria in atrophy and myosteatosis is only emerging in the cancer setting.

In both clinical and experimental studies, the essential fatty acids, EPA (C20 :5n-3) and DHA (C22 :6n-3) have been shown to be beneficial for muscle health, preventing muscle loss and myosteatosis, although the underlying reasons for these beneficial effects remain incompletely characterized. Many beneficial effects of EPA and DHA on mitochondrial content and oxidative capacity have been reported including the stimulation of mitochondrial biogenesis through activation of PGC-1α in skeletal muscle cells and increased function of the major enzymes in the electron transport chain [reviewed by (10)]. However, the role of EPA and DHA on mitochondrial density, as well as oxidative

capacity, has not been investigated in relation to myosteatosis in any neoplastic condition.

As most experimental models of cancer focus on muscle wasting in response to the tumor alone, little work has focused on the mechanisms by which chemotherapy molecules induce skeletal muscle wasting. Those that do exist suggest an important role played by alterations in mitochondrial bioenergetics.

The focus of the fellowship was to develop methods to study myosteatosis upon exposure to common chemotherapy agents. Further, modification of these pathways by EPA and DHA with an emphasis on mitochondrial function will be determined. This work is premised on the concept that myosteatosis develops as a result of altered mitochondrial function in muscle cells, and these underlying pathways are further exacerbated by exposure to chemotherapy agents. Chemotherapy induces alterations in mitochondrial bioenergetics at the muscle level, reducing lipid oxidation capacity, promoting intramuscular lipid storage (myosteatosis), and, muscle atrophy. EPA and DHA supplementation reduce myosteatosis and atrophy in part by limiting alterations in mitochondrial bioenergetics via changes in the fatty acid composition of cell and mitochondrial lipids.

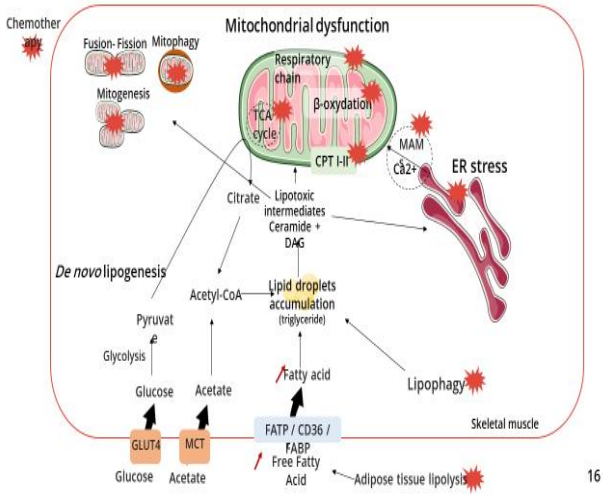
**Objectives:** *The objective of this research is to determine the effects of EPA+DHA on the mitochondria during the development of atrophy and myosteatosis that occurs during exposure to chemotherapy.* This objective is achieved by utilizing an *in vitro* model of human skeletal muscle cells as well as a rodent model of chemotherapy induced myosteatosis.

**Hypothesis:** *The hypothesis of this research is that muscle cells exposed to chemotherapy agents exhibit perturbations in mitochondria. Providing EPA+DHA corrects an imbalance of fatty acids in membrane phospholipids of myocytes and mitochondria to restore homeostasis to muscle. Increasing EPA and DHA in the cell membranes and mitochondria of muscle cells activates cellular respiration, mitochondrial biogenesis and mitochondrial quality. Collectively, these simultaneous events mitigate pathways evoking development of myosteatosis.*

## 2- Experimental details

A major focus of the research conducted in the host lab (Dr. Servais) was to establish an *in vitro* model to acheive the objectives.

**FIGURE 1**
**Overall concept of Experimental Plan**




Human muscle cells (HSMM) are differentiated into myotubes which are then exposed to chemotherapeutic agents (5-fluorouracil, and Irinotecan, either alone or combined). Measures between cells exposed to chemotherapy or not, and those exposed to EPA and DHA or not are assessed for the measures listed in Table 1.

Human skeletal muscle cells are exposed to chemotherapy agents using primary culture methods to induce atrophy and myosteatosis in a background of physiological levels of fatty acids modified only in the content of EPA+DHA, which are added to culture at levels we have identified as being physiologically relevant in people undergoing treatment for cancer (11). The agents proposed to study in this proposal, 5-Fluorouracil, with or without platin based agents, reduce the amount of intracellular ATP (12), and reduce mitochondrial biogenesis (13).

**FIGURE 2**
**Development of Myosteatosis in Human Skeletal Muscle Cells**

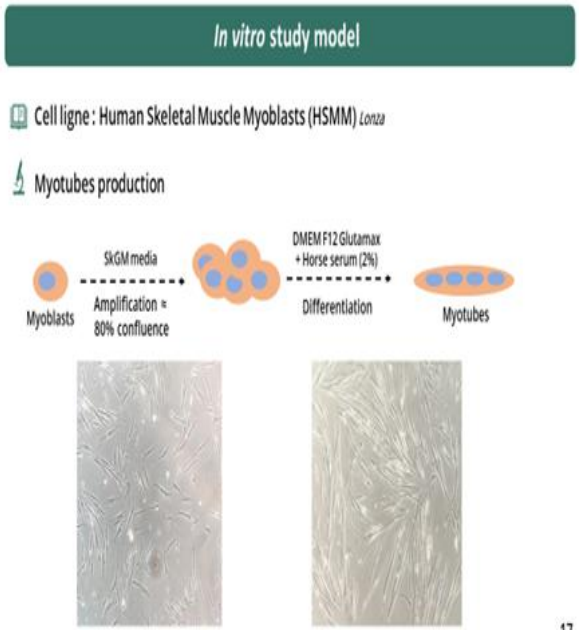



**TABLE 1**
**Measures performed on Human Skeletal Muscle Cells Exposed to Chemotherapy and EPA+DHA**

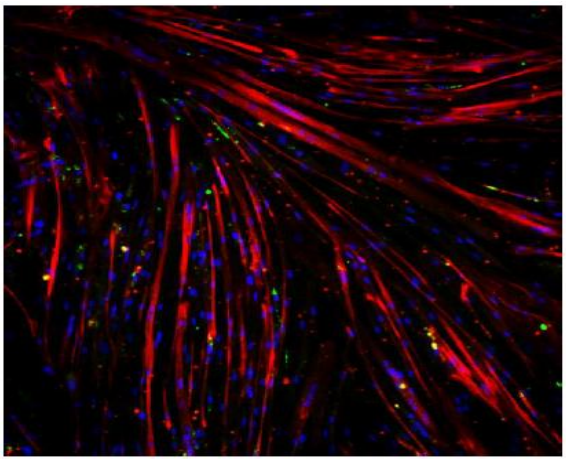
| | |
|---|---|
| Lipid Storage | Time course of appearance of lipid droplets<br>Fatty acid transport<br>Lipophagy |
| Lipogenesis | Key Enzymes<br>Transcription factors |
| Mitochondrial Dysfunction | B-oxidation and oxidative phosphorylation<br>Mitochondrial quality |
| Endoplamic Reticulum Stress | BiP/GRP78<br>isitol-requiring enzyme 1(IRE1) Protein kinase RNA-like ER kinase (PERK)<br>Activation transcription factor 6 (ATF6)<br>C/EBP homologous protein (CHOP) X-box binding protein 1 (XBP-1) |
| Mitochondria-Associated Membrane proteins | Voltage-dependent ion channel (VADC)<br>Mitofusion-2 |

Triglyceride Quantification and Phospholipid Assessment (to confirm myosteatosis and fatty acid incorporation): Total triglyceride is quantitatively assessed (14). Fatty acid content and composition of each of the lipid species in muscle (free fatty acids, phospholipid, cardiolipin, ceramides and diacylglycerol) are quantitatively assessed by established techniques in LC-MSMSMS (15, 16) to completely characterize lipid components and their metabolites. This analysis will enable matching of measures to changes in fatty acids within lipid components of the muscle, and mitochondria including cardiolipin. Determination of mitochondrial membrane lipid composition will be performed using by LC-MS/MS/MS, with particular focus on cardiolipins and ceramide species (15, 16).

Mitochondrial Bioenergetics: Measurements of oxygen consumption and mitochondrial reactive oxygen species production will be performed by high-resolution oxygraphy (OROBOROS O2K®). Data will be analyzed using DatLab 6® (Oroboros Instrument) software (17,18).

Mitochondrial Network: Fibers are fixed with paraformaldehyde (4%). The mitochondrial network will be updated with the use of the Red CMXros Mitotracker marker accompanied by DAPI labeling. Observations and images will be made by confocal microscopy. The images will then be analyzed by the Imaris® software, which will allow a 3D reconstruction of the network in the muscle fibers (17,18). This will be

**FIGURE 3**
**Myosteatosis in HSMM cells**



Red: Myosin, myotubes marker; Blue: nucleus; Green: lipid droplet (BodiPy 493/503). Lipid droplets are localized in myotubes.

complemented by the analysis of the expression of genes encoding proteins involved in fission (Fis1, Mff), and mitochondrial fusion (Mitofusin 1 and 2, OPA1). An integrative analysis of the genome is planned using the RNA-Seq approach to establish the transcriptomic profile with high-throughput sequencing (NGS).]

## 3- Results and discussion

Main culture parameters of skeletal muscle primary cells (HSMM) as myoblast doubling time, myoblast to myotubes differentiation and cell viability over time and passages have been established.

To date, we have confirmed the development of myosteatosis when myotubes are cultured 24h and 48h with conditioned media from colorectal cancer cells (HCT116). We have determined the optimal concentrations of the drugs (chemotherapy: 5-fluorouracil and Irinotecan) promoting myosteatosis without considerable cell death.

The methods employed are technically challenging and several conditions were tested to determine the optimal conditions for the experiments. The experimental aspects are ongoing and will continue as the foundation for the PhD project of a co-supervised student, Anais Pascal.

## 4- Conclusion

These initial measures are embedded into the larger experimental series being carried out within the host and home Principal Investigators' laboratories. The completed objectives and subsequent work in progress addresses many gaps in the literature. Myosteatosis is a novel marker of poor prognosis in cancer, and underlying mechanisms have not been characterized. Mechanisms for the combined effects of tumor plus treatment remain elusive. This research focusses on mechanisms related to cancer therapy that affect mitochondria which then alters the cellular bioenergetics. If so demonstrated, this work holds significant potential to impact the lives of people diagnosed with cancer, the majority of whom undergo treatment with chemotherapeutic agents.

A strength of our study is the use of human skeletal muscle as well as exposure to physiological concentrations of EPA and DHA (informed by plasma analysis of cancer patients). This approach ensures the translatability of the findings which then can be recapitulated in established animal models and clinical settings. The methods are technically challenging, including the determination of the kinetics of events (lipid droplet accumulation, mitochondrial function changes) and are foundational for further exploration informed by the results of the ongoing work. This data when combined with data collected at the home institution on human biopsies provide a comprehensive evaluation of mechanisms evoking protection of muscle from development of atrophy and myosteatosis using translational approaches.

## 5- Perspectives of future collaborations with the host laboratory

Following the cell culture experiments, the co-supervised PhD student will perform a short term research stay in Edmonton, Alberta where primary cells from human cancer patients will be used to assess similar measures explored on the culture model in progress. To enhance the translatability of the ongoing work, the PhD candidate will apply similar methods to study the behaviour of primary culture of human muscle, derived from patients who have cancer and for whom muscle mass is known. We are currently using this approach to delineate potential disruptions in the myogenic program, and anabolic capacity in the muscle of people with cancer. The added value of the planned experiments will be functional assessment of mitochondria. The next step in this broader line of questioning is how n-6 and n-3 PUFAs modify these responses to potentially enhance muscle function and restore homeostasis.

## 6- Articles published in the framework of the fellowship

*Mechanisms underlying the development of myosteatosis: Emerging Concepts*. To better understand the state of the literature that has emerged regarding the etiology and causes of myosteatosis, one of the main objectives of this work was to perform a scoping review. Scoping reviews are a robust way to map the literature to address a broader research question and identify heterogeneity in approaches. As the biological understanding of myosteatosis is limited in any human condition, we sought to better define mechanisms underlying myosteatosis

development. Over 9000 articles were screened and the manuscript is in progress.

*Mitochondrial Dysfunction and restoration by EPA and DHA is fibre type specific*: We have evaluated two muscle groups that represent the spectrum of fibre type distribution in a rat model of chemotherapy induced myosteatosis. The aim of this study is to assess the effects of tumor, chemotherapy, and EPA+DHA on TG deposition and mitochondiral function in the different muscle fiber types. The hypothesis is that muscle fiber types' response will differ based on their different characteristics and mitochondrial content.

*Dietary EPA and DHA restore disruptions in mitochondria*: This publication explores the mitochondria related pathways from a pre-clinical rodent model that develops myosteatosis upon exposure to the same chemotherapy agents. Using a proteomics approach, we have revealed several mitochondria-related pathways to be markedly affected by administration of chemotherapy. These pathways are completely reversed when fish oil is provided in the diets of the animals initiated at the start of treatment. These pathways are being interrogated and will direct the functional assessments that will be conducted during the cell culture experiments. Using in iterative process, the same functional measures are applied to muscle samples already collected from the animal model alongside the cell culture experiments in progress. Mitochondrial function must be assessed in fresh tissue, methodologies which are enabled by the cell culture experiments. The cell culture approach also enables the sequence of events to be established.

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