THE EFFECT OF MELATONIN TREATMENT ON DOXORUBICIN-INDUCED SKELETAL MUSCLE ATROPHY WITHIN A CANCER MODEL

by
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Dissertation presented for the degree of Doctor of Physiological Sciences in the Faculty of Science at Stellenbosch University

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December 2018

Declaration

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Summary

Background and Aim: Skeletal muscle atrophy is a major concern in patients suffering with malignancy. Chemotherapeutic agents, such as doxorubicin (DOX), can further exacerbate this loss of skeletal muscle. Although many cancer patients on chemotherapeutic agents suffer from this condition, there are no therapies routinely used to moderate muscle atrophy. The aim of the study was to investigate whether melatonin (MLT) can attenuate doxorubicin-induced skeletal muscle and myotube atrophy in an *in vivo* rodent model of breast cancer as well as in an *in vitro* model of DOX-induced myotoxicity respectively. The safe and cost-effective role of melatonin as a possible therapy to limit the burden of doxorubicin-induced muscle toxicity in cancer patients serves as rationale for the *in vivo* study and the *in vitro* study allows for the exploration of more invasive mechanistic aspects using the cell lines, which would not be possible when viewing excised tissue.

Methods: Female Sprague-Dawley rats were inoculated with LA7 cancer cells and were randomly assigned to six groups: Control, Tumour control (TCON), Vehicle control (VEH), MLT, DOX and DOX + MLT (DM). Prophylactic treatment of MLT (6 mg/kg) was administered in drinking water daily and rats received three intraperitoneal injections of DOX (4 mg/kg, 3 times at 3-day intervals). Following sacrifice blood samples (whole blood counts) and skeletal muscle tissue were collected for histological, immunoblot, antioxidant capacity and immunofluorescence analyses. Furthermore, C2C12 myoblasts grown to confluency and differentiated into myotubes were pretreated with MLT (50 nM) for 48h followed by DOX treatment (0.8 μM) for 24h. The effect of MLT treatment on C2C12 myotube diameter, mitochondrial reactive oxygen species (mtROS) production, sirtuin levels and autophagy activity was then assessed.

Results: DOX treatment significantly reduced animal weight (279.1 ± 21.34 g vs. 222.2 ± 20.40 g, $p<0.0001$) compared to DM weight (281.5 ± 7.11 g vs. 284.0 ± 6.53 g) and *gastrocnemius* muscle weight (1.4 ± 0.13 g vs. 0.99 ± 0.076 g, $p<0.0001$) and cross sectional area (CSA), while increasing

markers of muscle degradation compared to MLT treated groups. Serum myoglobin levels were significantly elevated in the DOX group compared to the DM group (572.6 ± 444.19 ng/mL vs. 218.2 ± 83.66 ng/mL, p<0.0001); while, white & red blood cell counts (WBC & RBC) were significantly decreased in the DOX group compared to the MLT treated groups respectively (2.06 ± 1.59 x 10^9L^{-1} vs. 4.13 ± 1.56 x 10^9L^{-1} & 4.00 ± 1.52 x $10^{12}L^{-1}$ vs. 5.66 ± 1.03 x $10^{12}L^1$, p<0.0001). Furthermore, MLT treatment significantly increased intramuscular antioxidant capacity, mitochondrial biogenesis and satellite cell number. *In vitro* DOX treatment resulted in increased myotube atrophy, mitochondrial ROS levels and these effects were significantly reduced with MLT pre-treatment.

Discussion: The improvement in animal weight, muscle to body weight ratio, muscle CSA as well as the reduction in myoglobin levels in the treatment groups compared to the DOX group indicate that MLT protects against DOX-induced atrophy. Moreover, MLT pre-treatment improved circulating levels of WBC & RBC compared to the DOX only group and attenuated skeletal muscle atrophy by reducing cell apoptosis and increasing satellite cell number suggesting that MLT assists with muscle repair. The *in vitro* study indicated that DOX-induced myotube atrophy was preceded by increases in mitochondrial ROS.

Conclusion: Results indicate that pre-treatment with exogenous MLT protects against skeletal muscle wasting induced by DOX in a pre-cachectic tumour-bearing rat model.

Opsomming

Agtergrond en Doel: Skeletspier atrofie is 'n groot bekommernis van pasiënte wat aan kanker ly. Chemoterapie soos doxorubicin (DOX) kan skeletspierverlies nog verder vererger. Alhoewel heelwat kankerpasiënte wat chemoterapie kry, hierdie toestand onder lede het, is daar nog geen terapie wat normaalweg toegedien word om dit te verlig nie. Die doel van hierdie studie was dus om te bepaal of melatonien (MLT) doxorubicin-geïnduseerde skeletspieratrofie in 'n *in vivo* rotmodel van borskanker sowel as in 'n *in vitro* model van DOX-geïnduseerde miotoksisiteit kan teenwerk. Die veilige en koste effektiewe rol van melatonien as 'n moontlik terapeutiese middel om doxorubicin-geïnduseerde spiertoksisiteit in kankerpasiënte te verminder, dien as rasionaal vir die *in vivo* studie en die *in vitro* studie is gedoen om meganistiese aspekte van die werking van die terapie meer in diepte te ondersoek.

Metodes: Vroulike Sprague-Dawley rotte is met LA7 selle geïnokuleer en is in ses groepe verdeel: Kontrole, Tumor Kontrole (TCON), Oplosmiddel Kontrole (VEH), MLT, DOX en DOX + MLT (DM). Die rotte is profilakties met MLT (6 mg/kg), wat daagliks in hul drinkwater toegedien is, behandel. Die rotte het ook drie intraperitoneale DOX (4 mg/kg, 3 keer met 3 dae intervalle) inspuitings ontvang. Na die eksperimentele tydperk, is bloedmonsters (heelbloedtellings) en skeletspier versamel vir histologie, immunoblot, antioksidant kapasiteit en immunofluoresensie bepalings. Verder is C2C12 mioblaste gedifferensieer in miobuise en met MLT (50 nM) behandel vir 48 uur voordat 0.8 μM DOX toegedien is vir 24 uur. Die effek van MLT in C2C12 miobuise is bepaal deur die miobuis deursnit, mitokondriale reaktiewe suurstof spesie (mtROS) produksie, sirtuinvlakke en autofagie aktiwiteit te bestudeer.

Resultate: DOX behandeling het die gewig van die diere insiggewend verminder (279.1 ± 21.34 g vs. 222.2 ± 20.40 g, p<0.0001) asook die *gastrocnemius* spiermassa (1.4 ± 0.13 g vs. 0.99 ± 0.076 g, p<0.0001) en deursnitarea (CSA) in vergelyking met die DM groep (281.5 ± 7.11 g vs. 284.0 ± 6.53 g), terwyl merkers van atrofie verhoog is in vergelyking met die MLT groepe. Serum myoglobien vlakke is

insiggewend in die DOX groep verhoog in vergelyking met die DM groep (572.6 ± 444.19 ng/mL vs. 218.2 ± 83.66 ng/mL, p<0.0001); terwyl die wit- en rooibloedseltellings (WBS & RBS) weer insiggewend verlaag is wanneer dit met die MLT groepe vergelyk is (2.06 ± 1.59 x 10^9L^{-1} vs. 4.13 ± 1.56 x 10^9L^{-1} & 4.00 ± 1.52 x 10^{12}L^{-1} vs. 5.66 ± 1.03 x 10^{12}L^{-1}, p<0.0001). Verder het MLT behandeling die intramuskulêre antioksidant kapasiteit, mitokondriale biogenese en aantal satellietselle insiggewend verhoog. *In vitro* DOX behandeling het miobuis atrofie asook mitokondriale ROS vlakke verhoog, terwyl hierdie effekte insiggewend verlaag is in die groepe wat vooraf met melatonien behandel is.

Bespreking: Die toename in die diere se gewig, spier tot liggaamsgewig ratio en spierdeursnitarea asook die verlaging in mioglobienvlakke in die MLT behandelde groepe in vergelyking met die DOX groepe, bewys dat MLT die liggaam teen DOX-geïnduseerde atrofie beskerm. Verder het MLT behandeling ook die sirkulerende vlakke van WBS & RBS verhoog in vergelyking met die DOX behandeling, terwyl apoptose verminder het en satellietselle toegeneem het, wat aanduidend is van MLT se rol in spierherstel. Die in vitro studie dui aan dat DOX-geïnduseerde miobuis atrofie voorafgegaan is deur 'n toename in mitokondriale ROS.

Gevolgtrekking: Resultate dui aan dat vooraf behandeling met eksogene MLT, DOX-geïnduseerde skeletspieratrofie teenwerk in 'n tumor-draende rot model.

Acknowledgements

I would like to express my sincere gratitude to the following people for their help and support for the duration of this study and the completion of my PhD degree:

To my supervisors, Professors Anna-Mart Engelbrecht; Kathy Myburgh and Ben Loos: your incredible influence as role models, supervisors and colleagues have largely shaped my personal and professional life. I thank you for your leadership, always by example, guidance and constructive criticism when necessary. Thank you for your commitment towards the completion of this thesis and aid in achieving my goals. I hope that I have garnered some of your qualities, both professionally and personally and know that I appreciate everything.

Thanks to all the staff and post-graduate students at the Department of Physiological Sciences Stellenbosch, my home away from home, for creating a friendly, positive and professional environment.

Thank you to the DSG and MRG groups for the friendships, teamwork and insightful discussions. Thanks to Jenelle, Daleen, Megan and Tanja for your contribution towards this thesis, I am forever grateful.

Also thanks to Theo, Balindiwe and Danzil for the morning coffee breaks and interesting discussions and advice.

Lastly, a special thanks to my friends and family, especially my mother Carol Scheepers and fiancé Madelaine Swartz for their love, support and encouragement. Working fulltime and completing a PhD part-time was no easy feat but you kept me sane, I love you.

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Abbreviations

02- - superoxide anions

AIDS, acquired immune deficiency syndrome

Akt, protein kinase b

ANOVA - analysis of variance

CA2+, calcium

cAMP - cyclic adenosine monophosphatase

cCaspase 3 - cleaved caspae 3

CK - creatine kinase

CON, control

cPARP- cleaved Poly (ADP-ribose) polymerases

CRP - C-reactive protein

CSA, cross-sectional area

Cu - copper

DM -Doxorubicin + Melatonin

DMI - Doxorubicin + Melatonin + Luzindole

DNA - deoxyribonucleic acid

DOX, doxorubicin

DRP1, dynamin related protein 1

e- - electron

ELISA - enzyme-linked immunosorbent assay

ERK - extracellular signal-regulated kinase

FOXO, forkhead box

GSH - glutathione

GSK3b, glycogen synthase kinase 3b

GSSG - glutathione, oxidised form

GSTα1 - glutathione-S-transferase α1

H&E - haematoxylin and eosin

H+ - hydrogen ion

H2O2 - hydrogen peroxide

H2O2 - Hydrogen peroxide

HGF - hepatocyte growth factor

HO- - hydroxyl radicals

HRP - Horse radish peroxidase

i.p. - intraperitoneal

IFN - interferon

IGF - insulin-like growth factor

IGF-1, insulin-like growth factor-1

IL, interleukin

i-NOS - inducible nitric oxide synthase

iNOS, nitric oxide synthase

IRS, insulin receptor substrate

JAK - janus activating kinase

JNK, jun N-terminal kinase

KO - knock out

LUZ - Luzindole

MAFbx, atrogin-1

MAPK - mitogen activating protein kinase

Mb - Myoglobin

M-cad - M-cadherin

Mfn1 - mitofusin 1

Mfn2 - mitofusin 2

MI - Melatonin + Luzindole

mins - minutes

MLT, melatonin

MRFs - myogenic regulatory factors

m-RNA - messenger ribonucleic acid

MSC - mesenchymal stem cells

MSC - mesenchymal stem cells

mTOR, mammalian target of rapamycin

MuRF1, muscle ring-fingre-1

NADH-TR, nicotinamide adenine dinucleotide tetrazolium reductase

N-cad - neural cadherin

NFκB - nuclear factor-kappa B

NK - natural killer cell

nNOS - neuronal nitric oxide synthase

NO - nitric oxide

NO, nitric oxide

NOS - nitric oxide synthase

NOx - nitrite/nitrate

NSAIDs - non-steroidal anti-inflammatory drugs

OH, hydroxide

ORAC - oxygen radical absorbance capacity

PARP, Poly (ADP-ribose) polymerases

PBS - phosphate-buffered saline

PI3-K - phosphatidylinositol-3-kinase

Pi3k, phosphatidylinositol-3-kinase

PVDF - polyvinylidine fluoride

RBC - red blood cell

RNA - ribonucleic acid

RNS - reactive nitrogen species

ROS - reactive oxygen species

SCs - satellite cells

SD - standard deviation

SDS-PAGE - Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Electrotransfer

and Immuno-detection

SEM - standard error of the mean

SIRT1 - sirtuin 1

SIRT3 - sirtuin 3

SOD - superoxide dismutase

TA - tibialis anterior

TBS-T - TRIS-buffered saline-Tween Solution

TCON, tumour control

TNFa, tumor-necrosis factor alpha

TNF-α - tumour necrosis factor alpha

TWEAK, tnf-related weak inducer of apoptosis

UPP - ubiquitin proteasome pathway

UPR, unfolded protein response

UPS, ubiquitin proteasome system

VCAM - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor

VEH, vehicle control

WBC - white blood cell

Zn - zinc

Units of measurement

%	percent/percentage
A	ampere
cm	centimetre
cm2	centimetres squared
g	gram
hr/s	hour/s
kD	kilodalton
l/L	litre
M	molar
mg	milligram
mg/kg	milligram per kilogram
mg/m2	milligram per metre squared
mins	minutes
mL	millilitres
ml/min	millilitre per minute
mm	millimetre
mM	millimolar
mmol/l	millimoles per litres
mm2	millimetre squared
nm	nanometre
nM	nanomolar
ºC	degrees Celsius
RLU	relative light units
rpm	revolutions per minute

sec	seconds
V	volt
µg	microgram
µg/ml	microgram per millilitre
µmol/L	micromoles per litre
µl	microlitre
µm	micrometre
µM	micromolar
µm2	micrometre squared

Preface

This study was presented at:

- The Physiology Society of Southern Africa in Cape Town, South Africa during August 2016

- 38th World Congress of The International Union of Physiological Sciences in Rio de Janeiro, Brazil during August 2017

Chapter 1:

1.1 Introduction

Cancer cachexia is a major concern in patients suffering from malignancy. It is reported that up to 50% of cancer patients experience a progressive loss of skeletal muscle (Tisdale 2009), which occurs due to the depression of muscle protein synthesis combined with an increase in protein degradation (Lorite et al. 1998). This reduction in muscle mass affects various aspects of muscle function and is associated with muscle weakness and fatigue (Roberts et al. 2013), with consequently reduced quality of life (Aversa et al. 2017, Penna et al. 2018). Loss of muscle mass is even associated with mortality, emphasizing the clinical significance of this condition (Morrow et al. 2002, Roscoe et al. 2002, and van Norren et al. 2009). Although many cancer patients suffer from this condition, there are no available therapies routinely used to reduce muscle loss.

In addition to the disease mechanisms, chemotherapeutic agents, such as doxorubicin, can further exacerbate the loss of skeletal muscle (Morrow et al. 2002, Burckart et al. 2010, Gilliam and St Clair 2011). Doxorubicin, a standard antineoplastic anthracycline, has shown great success in the treatment of several malignant and solid tumours such as breast cancer (Koka et al. 2010, Gilliam et al. 2011, Wergeland et al. 2011), however, its benefits are limited due to its cytotoxic side effects on non-targeted tissue, such as skeletal muscle (Burckart et al. 2010, Gilliam and St Clair 2011, Gilliam et al. 2012).

Loss of skeletal muscle is characterised by a shift towards protein degradation. Two highly conserved pathways, namely the ubiquitin-proteasomal pathway and the autophagy pathway mediate protein degradation in skeletal muscle (Bonaldo and Sandri 2013). In normal skeletal muscle the ubiquitin-proteasomal pathway is

constitutively active and is responsible for the turnover of most soluble and myofibrillar proteins (Solomon and Goldberg 1996). During muscle atrophy the ubiquitin-proteasomal pathway activity is significantly elevated through the transcriptional activation of ubiquitin; various proteasomal subunit genes; and muscle specific ubiquitin ligases, MAFbx and MuRF1 (Mammucari et al. 2007).

The mechanism by which doxorubicin induces toxicity in skeletal muscle remains largely unexplored. However, it was demonstrated in a recent study that a reduction in differentiated C2C12 myotube width occurred following doxorubicin treatment, which was accompanied by increased expression of markers of apoptosis (cleaved caspase3) and atrophy (atrogin-1/MAFbx) (Gilliam et al. 2012). The authors further demonstrated that the inhibition of reactive oxygen species (ROS) preserved myotube size and concluded that doxorubicin acts via mitochondrial ROS to stimulate catabolism of myotubes.

Furthermore, Smuder et al. (2011), provided evidence that systemic doxorubicin administration in rats increased the expression of various autophagy markers in *soleus* muscle. During the autophagic process, portions of the cytoplasm and cell organelles are isolated into vacuoles, termed autophagosomes which then fuse with lysosomes where autophagosome contents are digested by lysosomal hydrolases (Mizushima et al. 2008).

Various strategies have been explored in attempts to reduce doxorubicin-induced muscle atrophy. These include physical activity (Smuder et al. 2011), synthesis of alternative anthracycline analogues (Petrioli et al. 2008), as well as the use of nanoparticles (Dong et al. 2009) and liposomes (Mrozek et al. 2005) as novel, more targeted drug delivery systems. Nevertheless, the use of doxorubicin with its

associated negative side effects persists (Hayward et al. 2013). Therefore, it is of utmost importance to identify an adjuvant therapy capable of alleviating the effects of doxorubicin on skeletal muscle. Ideally, such an adjuvant should be multi-acting to alleviate multiple mechanisms inducing side effects.

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring pleiotropic hormone primarily secreted by the pineal gland (Tasdemir et al. 2012). Its initial function is hypothesised to serve as antioxidant in Cyanobacteria which first appeared on earth approximately 3.5 billion years ago (Manchester et al. 2015). This hormone influences almost all cell processes and has secondary functions which include oncostatic properties (Proietti et al. 2013), anti-inflammatory (Laste et al. 2012), analgesic (Laste et al. 2012), and neuroprotective characteristics (Srinivasan et al. 2008, Robertson et al. 2013). Numerous studies have revealed the efficacy of melatonin in the suppression of neoplastic growth in a variety of tumours (Proietti et al. 2013). Additionally, it has been found to be beneficial as adjuvant therapy when used in conjunction with chemotherapeutic agents in patients suffering from breast cancer and melanoma (Kilic et al. 2013).

Interestingly, a literature search revealed only three *in vivo* studies investigating the effects of melatonin treatment in the prevention of skeletal muscle atrophy. The first study showed that melatonin treatment maintained *soleus* muscle fibre diameter in an *in vivo* rat castration model (Oner et al. 2008). It was found in two more recent studies that melatonin treatment prevented skeletal muscle atrophy in a contusion spinal cord injury model (Park et al. 2012) and in a stroke-induced skeletal muscle atrophy model (Lee et al. 2012). The mechanisms of action uncovered by these studies will be addressed later in section 2.7.6.

1.2 Significance of the study

Evidence from observational data over the years alludes to a potential role for melatonin in cancer prevention. Furthermore, melatonin has previously been found to prevent muscle wasting both *in vitro* (Park et al. 2013) and *in vivo* (Oner et al. 2008, Lee et al. 2012) in various atrophy models. Therefore, it could in future play a dual role by allowing for reduction of the chemotherapeutic agent dose and reducing some of the side effects. However, to the best of our knowledge this is the first study to investigate the effects of melatonin on skeletal muscle in a cancer model.

1.3 Hypothesis

Based on previous research, we hypothesise, that melatonin treatment will prevent doxorubicin-induced skeletal muscle atrophy in a rodent tumour-bearing model.

Chapter 2: Literature Review

2.1 Cancer cachexia

2.1.1 What is cancer cachexia?

The word cachexia originates from the Greek words 'kakos' meaning bad and 'hexis' meaning condition. The Greek, Hippocrates, described the condition in the third century before Christ (BC) and associated it with a poor prognosis. The cachectic condition is characterised by loss of appetite, anorexia, oedema, anaemia, weakness, inflammation and gradual atrophy of adipose tissue and skeletal muscle (Tisdale 2009, Fearon et al. 2012, Chen et al. 2015, Xi et al. 2016), all of which cannot be reversed with nutritional intervention in the face of continuation of the disease process.

Cachexia is seen in patients with various chronic diseases, such as diabetes, multiple sclerosis, chronic obstructive pulmonary disease, congestive heart failure, tuberculosis, acquired immune deficiency syndrome (AIDS), and cancer (Graul et al. 2016, von Haehling et al. 2016). The condition is easily recognisable in its advanced stage but is much more difficult to predict for the purposes of early intervention. In the past various definitions of cachexia were used in different studies in the literature. The more general classification of cachectic patients is a 10% weight-loss from pre-illness weight (Feliu et al. 1992). Cancer cachexia as defined by McDonald et al. (2003) is a syndrome of muscle and fat wasting as a direct consequence of tumour factors and has been described by Fearon et al. (2012) as a state of 'autocannibalism' in which the tumour survives at the expense of the host.

2.1.2 Skeletal muscle atrophy as a consequence of cancer cachexia

Cancer is a leading cause of morbidity and mortality globally, with approximately 14 million new cases diagnosed and cancer related deaths in 2012 totalling 8.2 million

(Sorensen et al. 2016). It is reported that up to 50% of all cancer patients, as well as the majority of patients with advanced diseases, experience cachexia (Tisdale 2009; Fearon et al. 2012); furthermore approximately 30% of cancer patients die as a direct result of cancer cachexia (Mu et al. 2016). Cancer cachexia affects both physiological and pathological conditions which dictate variation of skeletal muscle mass and individual muscle fibre size (Schiaffino et al. 2013).

Skeletal muscle represents more or less 40% of the total body weight, making it the most abundant tissue of the human body, regulating both energy availability and expenditure (Frontera and Ochala 2015). During the physiological state there exists homeostatic balance between protein synthesis and degradation (Rothman 2010). Skeletal muscle atrophy as consequence of cancer cachexia occurs when protein degradation exceeds protein synthesis resulting in the net loss of protein. Patients experience subsequent unexplained weight loss, muscle weakness, fatigue, decreased mobility and quality of life, which eventually leads to mortality (Argiles et al. 2015, Mueller et al. 2016). The unexplained weight loss and fatigue are often the primary reasons patients seek medical consultation, since nutritional interventions have been shown to be ineffective at combating the cachectic state. Current therapies used to treat cachexia are also ineffective and the main reasons (Burckart et al. 2010) reside in the facts that the disease state mechanisms are multimodal and not clearly defined. Several mediators have been identified, including pro-inflammatory cytokines (TNF-α, IFNγ, IL-6) as well as the tumour-secreted proteolysis-inducing factor (PIF), which enhance both autophagic-lysosomal and proteasome-mediated muscle catabolism (Noguchi et al. 1996, Tisdale 2008, Fearon et al. 2012). Up-regulation of muscle specific ubiquitin-ligases, namely muscle RING finger-containing protein 1 (MuRF1) and muscle atrophy Fbox protein (MAFbx), alter the balance between

synthesis and degradation of muscle protein (Costelli et al. 2006). Several transcription factors (FoxO, NF-kB and AP-1) have been implicated in muscle wasting (Hasselgren 2007). Reduced testosterone concentrations have also been reported in cachectic patients (Feld and Hirschberg 1996).

Increased protein degradation may be due to both an increase in ubiquitin-proteasome pathway and lysosomal activity, while protein synthesis mechanisms are decreased. It is important to note that factors and processes contributing to muscle synthesis or degradation may differ depending on cancer type and model used for scientific investigation. Furthermore, human biology in health/disease states is complex and multiple signalling pathways are involved in muscle wasting which is evident in cancer cachexia. Herewith certain mechanisms which may contribute to muscle tissue degradation in cancer cachexia are described below.

2.2 Mechanisms of cancer cachexia

Tisdale 2009, describes three main proteolytic pathways responsible for the degradation of skeletal muscle proteins, which include the: i) ubiquitin-proteasome pathway (UUP), ii) lysosomal/autophagy system, and iii) calcium-activated system. In conjunction with these three pathways, some of the main molecular mechanisms were described: iv) caspases, v) Forkhead box (FoxO) of transcription factors and vi) proteolysis-inducing factor (PIF).

2.2.1 The ubiquitin-proteasome pathway (UPP)

The ubiquitin-proteasome pathway, is an ATP-dependent system working in co-ordination with the calpain enzyme to degrade muscle myofilaments (Hasselgren and Fischer 2001). The UPP catalyses the breakdown of most long-lived proteins and removes proteins which have been damaged by genetic alterations or by thermal or

oxidative stress (Kedar et al. 2004). This system is characterized by the polyubiquitin chain of three enzymes: E1 - Ub-activating enzyme; E2 - Ub-conjugating enzyme and E3 - Ub-protein ligase.

The protein substrate is tagged by the ubiquitin chain via a reaction sequence consisting of a series of enzymes involving E1 (ubiquitin activation). This system has been shown to be active in various *in vivo* models of atrophy such as denervation, immobilization, muscle unloading (Bodine et al. 2001), and cancer (MacDonald 2003). In fact, *in vivo* cancer cachexia model studies along with studies of cancer patients, advocate that the UPP may have the biggest role to play in myofibrillar protein degradation (Khal et al. 2005). The muscle-specific E3 ubiquitin ligases, namely, MuRF1 and MAFBx/atrogin-1 were shown to be highly upregulated in many animal models of muscle wasting, including cancer cachexia (Bodine et al. 2001, Gomes et al. 2001). MAFBx promotes degradation of a vital muscle transcription factor, regulating muscle differentiation, MyoD (Tintignac et al. 2005), while MuRF1 can interact with and control important structural proteins, including myosin heavy chain (Clarke et al. 2007), troponin-1 (Kedar et al. 2004), myosin light chain and myosin binding protein C (Cohen et al. 2009). The overexpression of MAFbx in myotubes resulted in reduced myotube size *in vitro*, while mice lacking either MAFbx or MuRF1 were resistant to atrophy (Bodine et al. 2001). Furthermore, knockdown of MAFBx has been shown to attenuate muscle loss during fasting (Cong et al. 2010).

However, literature on the contribution of E3 ligases to muscle atrophy has been controversial. Investigations in patients presenting with atrophy caused by a myriad of diseases have demonstrated both increased and decreased expression of MuRF1 and MAFBx/atrogin-1 (Edstrom et al. 2006, Leger et al. 2006, Doucet et al. 2007, de Palma et al. 2008, Fredriksson et al. 2008, Salanova et al. 2008). Furthermore, investigations

of UPP activity have demonstrated that lung cancer patients presenting with cachexia had similar levels of UPP activity compared to controls (Op den Kamp et al. 2012). Although the evidence on UPP activity in cancer cachexia seems to be conflicting, the timing of investigation, stage of cachectic progression and tumour type are factors that need to be taken into consideration to clearly establish the role of the UPP in cancer cachexia.

2.2.2 The lysosomal/autophagy system

Macroautophagy/autophagy is a highly conserved process occurring at basal levels of all eukaryotes (Masiero et al. 2009) executing the degradation of intracellular proteins and cell receptors (Hasselgren and Fischer 2001), damaged organelles and intracellular pathogens (Mizushima et al. 2008). Under physiological conditions, basal autophagy is responsible for the degradation of redundant cellular constituents which are then used as additional energy sources for different metabolic processes (Uchiyama et al. 2008) and thus protects against the aggregation of components which may become cytotoxic (Button et al. 2017 and Inguscio et al. 2012). However, under conditions of cellular stress, such as hypoxia and nutrient deprivation, autophagy activity is enhanced and non-essential cell components may be broken down to fuel essential metabolic processes as a mechanism of protection (Inguscio et al. 2012). The protein Beclin 1 is a central regulator of autophagy and acts during the initiation stage of autophagy by manufacturing a double membrane structure to form the autophagosome, which surrounds cytoplasmic components targeted for degradation (Mizushima et al. 2008). Autophagosomes fuse with the lysosomes, whereafter the membrane and content of the autophagosome vesicles are degraded. There are more than 30 known genes regulating autophagy in yeast, many of which are shared commonly with mammals (Atg genes) (Ravikumar et al. 2010), whilst expressed

autophagy specific proteins (Atg proteins) regulate the different processes of autophagy (Mizushima et al. 2010).

This process follows a distinct sequence of events, namely:

1) **Induction of autophagy**: This step includes the formation of phagophores which involves the specific recruitment of proteins into forming autophagosomal membranes from sources such as endoplasmic-reticulum or golgi complexes. Recruited proteins include p62, an ubiquitin cargo-binding protein, which acts as a partial selective receptor for different substrates (Lippai and Lőw 2014). The most common triggers of autophagy are nutrient starvation and hypoxia.

2) **Autophagosome expansion and formation:** This includes the elongation of formed phagophores and is mediated by a collection of autophagy specific genes (Pyo et al. 2012). During autophagosome formation, LC3-I is converted to LC3-II; LC3-II promotes membrane tethering and thus associates with the autophagosome, which is typically a double-membraned organelle. Activated Atg12 covalently binds to Atg5 and this formed complex then conjugates with Atg16L1. Next, LC3 is cleaved to form LC3-I in the cytosol which then conjugates to phosphatidylethanolamine (PE) to form LC3-II via the actions of Atg3 and Atg7. LC3-II remains associated with the autophagosomal membrane, and as such is a valuable molecular marker for autophagy (Ravikumar et al.2010). The protein, p62, another molecular marker for autophagy, is involved in the targeting of poly-ubiquitinated proteins towards autolyosomes for degradation (Barth et al. 2010).

3) **Fusion**: The autophagosomal membrane is formed from intracellular membranous structures such as the endoplasmic reticulum (ER), mitochondria and the Golgi

apparatus. The merger of an autophagosome with a lysosome to form an autolysosome which is a degrading structure now separate from the cytoplasm.

4) **Degradation**: In this step, the targeted cytoplasmic components are degraded by acidification, Fig.2.1 (Lee et al. 2012). The inner membrane of the autophagosome and the cytoplasm-derived materials contained in the autophagosome are then degraded by lysosomal/vacuolar hydrolases.
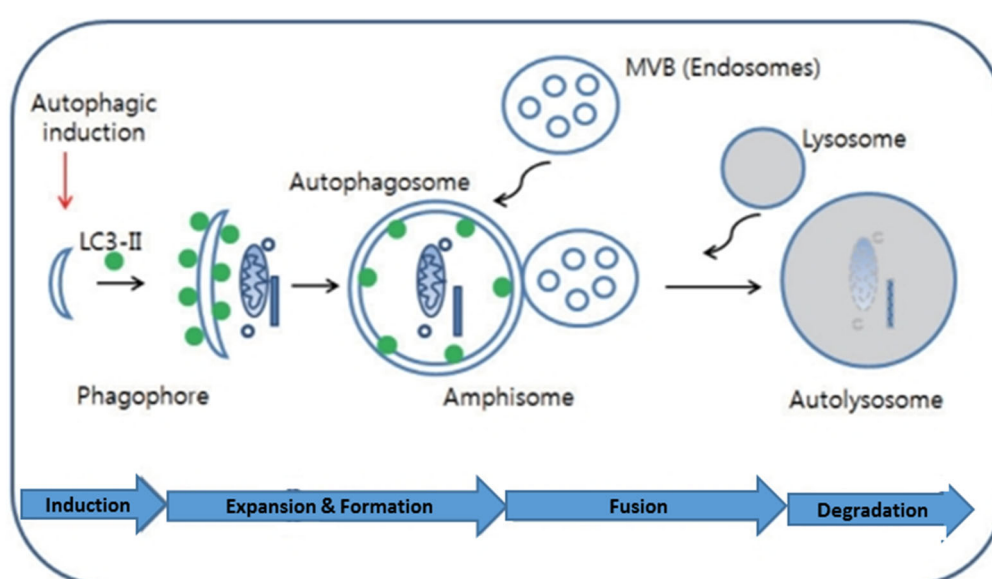
Figure 2.1: The cellular processes during autophagy. Image adapted from Lee et al (2012).

Lysosomes are vitally important in the autophagic process; are comprised of a single-lipid bilayer membrane which encapsulates several types of hydrolases that enable the degradation of variety of substrates (Settembre et al. 2013). Transport proteins residing in the membrane of lysosomes facilitate the passage of both intra- and extracellular proteins as well as acidification of the lumen and fusion with other structures.

Alterations in the autophagy programme in skeletal muscle are associated with a number of myopathies, where the excessive activation of autophagy enhances

skeletal muscle loss (Wang et al. 2005, Mammucari et al. 2007, Zhao et al. 2007, Dobrowolny et al. 2008). Maserio et al. (2009) carried out an *in vivo* "loss of function" study where a muscle-specific autophagy gene, Atg7, was deleted in mice. The investigators found that this deletion resulted in profound muscle loss and muscle weakness. Furthermore, in animal models of cancer, autophagy has been associated with both skeletal muscle and cardiac muscle pathology. An elevation in total lysosomal protease activity has been observed in muscles of tumour bearing rats (Greenbaum and Sutherland 1983). A study by Cosper and Leinwand (2011) demonstrated that autophagy, independent of UPP activity, was associated with cardiac atrophy of myofibrillar and sarcomeric proteins in tumour bearing mice.

Human studies revealed increased cathepsin B (a lysosomal cysteine protease) mRNA in skeletal muscle biopsies from lung cancer patients (Jagoe et al. 2002), with higher expression levels in early versus late stage tumours. UPP activity remained unchanged in these patients, leading investigators to conclude that lysosomal proteolysis may be upregulated early and that the UPP may be activated later in the disease process (Jagoe et al. 2002). A range of autophagy markers at gene and protein level were assessed in this comprehensive study. The authors found Beclin-1 and LC3-IIB protein levels to be increased, suggesting autophagy induction and autophagosome formation or autophagosome turnover.

However, the inhibition of autophagy has also been shown to enhance skeletal muscle loss (Masiero et al. 2009). This demonstrates the homeostatic function of autophagy in maintaining muscle mass. Similarly, suppression of mitochondrial function has been demonstrated to cause significant muscle wasting in adult animals (Romanello et al. 2010). Many cancer cachexia related studies identify mitochondrial dysfunction as a common theme during muscle wasting (Vitorino et al. 2015, Brown et al. 2017).

Mitochondria are packaged densely within myofibres where approximately 20% are associated with the subsarcolemma area and 80% are associated with the intermyofibrillar regions (Vitorino et al. 2015). Therefore, it is perhaps not surprising that myofibrillar loss would affect mitochondrial function. Mitochondrial dysfunction is an early event during cachexia (Vitorino et al. 2015) and is associated with abnormal mitochondrial morphology (Shum et al. 2012), reduced mitochondrial number (Padrao et al. 2013, Antunes et al. 2014) and a decrease in mitochondrial biogenesis regulatory factors (White et al. 2011).

A key feature of cancer cachexia is muscle weakness which may indicate that the mitochondria in the intermyofibrillar regions may be mostly altered, since they supply ATP required for muscle contraction. Calcium ion uptake/regulation by mitochondria is critical for ATP production as demonstrated by Eshima et al. (2017). Therefore, it is conceivable that a low number of mitochondria could potentially lead to increased calcium ion accumulation in the myofibre with subsequent activation of calcium-activated proteases. On the other hand, an overload of calcium ions within mitochondria causes the opening of the permeability transition pore (PTP), the release of cytochrome c and subsequent myofibre apoptosis (Naon and Scorrano 2014).

Furthermore, mitochondrial biogenesis regulatory factor (PGC1-α) coordinates mitochondrial fission (division of a single organelle into two or more independent structures) and fusion (formation of a single structure from two or more independent structures) and is crucial for the maintenance of mitochondrial function, morphology and distribution (Scott and Youle 2010, Romanello and Sandri 2013). It was demonstrated in a recent study that PGC-1α overexpression improves skeletal muscle calcium ion regulation *in vivo* following contractions (Eshima et al. 2017), providing indirect evidence that PGC-1α improves ATP synthesis which was found to be

decreased in *in vivo* models of cancer cachexia (Constantinou et al. 2011, Antunes et al. 2014). However, in transgenic mice over-expressing PGC-1α, it was shown that muscle wasting persisted in a model of cancer cachexia (Wang et al. 2012).

Mitochondrial dynamic processes are essential for the maintenance of mitochondria integrity and efficiency. This is achieved by preserving a balance between mitochondrial fission and fusion. Fusion processes act to extend mitochondrial networks and increase energy production and efficiency (Bordi et al. 2017), whereas fission divorces dysfunctional or damaged mitochondria (Romanello et al. 2010), allowing for their removal through mitophagy (Vitorino et al. 2015). This indicates that crosstalk exists between mitophagy and fission processes to remove mitochondria and also highlights the danger of excessive fission. These processes are ever changing thus allowing for mitochondria to adapt according to the changes in their cellular environment (Rambold et al. 2011).
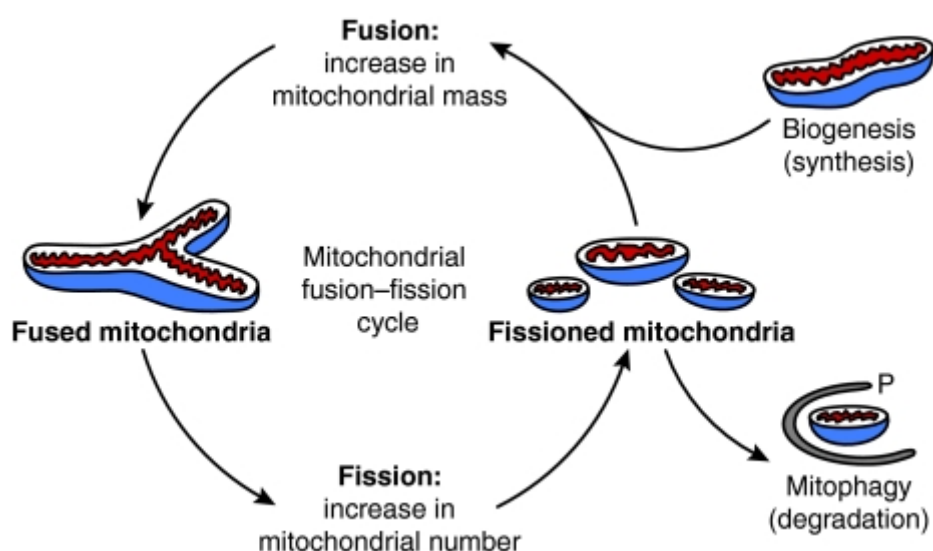
Figure 2.2: Possible relationship between mitochondrial fusion, fission, biogenesis and degradation. Image obtained from Seo et al. (2010).

During prime cellular conditions mitochondria are inclined to form extensive tubules and networks which are characteristic of mitochondrial fusion; however this scenario may also be present during conditions of stress when mitochondria successfully adapt and overcome initial stressors (Rambold et al, 2011). Fusion involves the activity of pro-fusion proteins including mitofusin (Mfn) 1 and 2 at the outer mitochondrial membrane and the optic atrophic protein 1 (OPA1) (Rambold et al, 2011) at the inner mitochondrial membrane. These proteins promote the fusion of membranes of adjacent mitochondria. Mitochondrial fusion is a likely contributor to enhanced respiration and mitochondrial metabolism and may also act to limit mitophagy and apoptosis (Boland, 2013). Alternatively, when mitochondria cannot overcome conditions of stress, they become fragmented which is characteristic of fission (Frank et al. 2001, Karbowski and Youle 2003). The pro-fission protein, dynamin-related protein 1 (Drp1) is recruited to mitochondria where it interacts with a mitochondrial receptor, mitochondrial fission 1 protein (Hfis1), to cut mitochondria into smaller units. When this happens mitochondria depolarize and usually recover; but if restoration of membrane potential fails then the mitochondria may be targeted for degradation by autophagy or otherwise apoptosis is induced through cleavage of OPA-1 (Boland, 2013).

Mitochondrial dysfunction can occur as a result of perturbations in the balance of mitochondrial fission and fusion dynamics, which have been demonstrated in a preclinical model of cancer cachexia (Marzetti et al. 2017). In a multifaceted study by Xi et al. (2016), mitofusin-2 (Mfn2), was assessed *in vitro* in the C2C12 cell line, in an *in vivo* mouse model as well as in *rectus abdominis* muscle tissue from patients who underwent primary surgical resection of gastrointestinal cancer. The *in vitro* model indicated that Mfn2 loss was strongly associated with atrophy of C2C12 myotubes,

while cachexia decreased both *gastrocnemius* muscle mass and Mfn2 expression *in vivo*. By overexpressing Mfn2, a partial attenuation of cachexia-induced *gastrocnemius* muscle loss was observed. Furthermore, Mfn2 protein expression was found to be downregulated in muscle biopsies of cachectic patients. These results suggest that Mfn2 protects against cachexia-induced muscle loss, and that it may be a novel target for the treatment of cachexia in cancer patients.

Mitophagy is a highly selective form of autophagy where mitochondria are targeted for degradation, which may then also serve as a source of nutrients for the cell (Carson et al. 2016). Indeed, generalised autophagy may not result in decreased mitochondrial content as demonstrated by loss-of-function Atg7 (functions in the autophagy pathway) knockout mouse models, where defective mitochondria have been shown to accumulate in certain tissue, including skeletal muscle (Komatsu et al, 2005; Wu et al, 2009). Mitophagy is usually induced to degrade defective mitochondria following damage or stress (Lemasters, 2005). During the mitophagy quality control process, the protein, PINK1, identifies damaged mitochondria for hydrolytic degradation. PINK1 then accumulates and becomes activated on the outer mitochondrial membrane and next phosphorylates the polyubiquitin chain and Parkin. This results in the translocation and ubiquitination of substrates on the outer mitochondrial membrane. Once the outer mitochondrial membrane is tagged by Parkin, the ubiquitinated mitochondria undergoes autophagic degradation (Fig. 2.3). Parkin thus mediates autophagy downstream of PINK1 (Jin et al, 2012), that selectively binds to defective mitochondria. Parkin overexpression had been shown previously to induce mitophagy of damaged mitochondria after membrane potential is lost (Narendra et al, 2008).
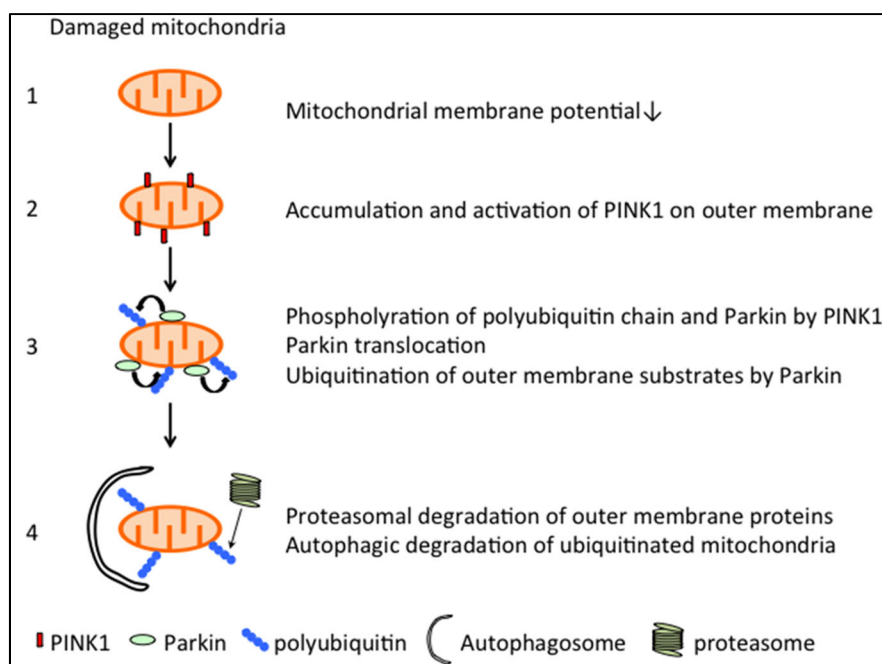
Figure 2.3: PINK1-Parkin-mediated mitophagy. Image modified from Arano and Imai, (2015).

Mitophagy has been shown to be dysregulated in cancer cachexia (Carson et al. 2016). Either a decrease or an increase in the associated processes compared to homeostatic conditions may contribute to the cachectic state. Mitophagic failure can result in an accumulation of impaired mitochondria which can adversely affect metabolism and thus muscle mass, whereas hyperactivation of these processes can also contribute to skeletal muscle mitochondrial dysfunction (Martinez-Outschoorn et al. 2010, Aversa et al. 2016). Recently, it has been shown that mitophagy is induced by tumour-related factors in skeletal muscle through IL-6-dependent signalling (Pettersen et al. 2017) and further evidence also suggests that IL-6, derived from the tumour, is important in regulating mitophagy (VanderVeen et al. 2017). Indeed, targeting mitochondrial dysfunction may be a promising area of research to develop therapeutic interventions to prevent or limit muscle wasting and energy inefficiency.

2.2.3 Calpains system

Ca^{2+}-dependent proteases, namely the ubiquitously expressed calpains, calpain-1 and calpain-2 and the non-ubiquitously expressed calpain-3 which is specific to skeletal muscle fibres, are responsible for the breakdown of myofibrillar proteins (Stolc 1977, Murphy 2010). Defective calpain-3 is associated with limb girdle muscular dystrophy type 2A (Murphy 2010). Calpains have also been implicated in the degradation of other sarcomeric and cytoskeletal proteins *in vitro* (Purintrapiban et al. 2003), where it was demonstrated that inhibition of calpain caused a 20% reduction in protein degradation. This was also confirmed in a murine hindlimb suspension model where sarcomere structure was preserved with calpain inhibition (Salazar et al. 2010). These proteases seem to be the initial trigger for skeletal muscle degradation (Cagan et al. 1991, Huang and Forsberg 1998), as observed by Smith et al. (2011) who demonstrated a 70% increase in calpain activity in gastric cancer patients with minimal weight-loss compared to controls, providing evidence for a role of calpains as an initial trigger, or at least early activity, in the progression of cachexia prior to the appearance of significant clinical changes.

Despite extensive research in identifying potential substrates of calpain-3, its physiological and pathophysiological functions remain poorly defined. Interestingly, since a defect in calpain-3 expression results in limb-girdle muscular dystrophy type 2A (LGMD2A), where affected individuals experience progressive weakness and atrophied trunk muscle (Gallardo et al. 2011), calpain-3 has been suggested to be involved in sarcomeric remodelling and maintenance of muscle integrity rather than its assumed role in muscle degradation (Beckmann and Spencer 2008). However, calpain-3 is thought to have many more functions since it has been observed in the sarcolemma (Taveau et al. 2003), the nucleus (Marcilhac et al. 2006, Murphy et al.

2011) and in triad-associated protein complexes (Kramerova et al. 2008) other than only the sarcomeric cellular compartment alone (Stuelsatz et al. 2010). Calpains may play a critical role in initiating the breakdown of myofibrillar protein, via the release from their tertiary structures of proteins such as desmin, filamen, tropomyosin, troponin T and troponin I; which become suitable for further degradation by proteasomes. Calpains do not work only in concert with the proteasome system, since some evidence supports a role for co-ordination with caspases in muscle wasting (Mellgren et al. 2009). Experimental evidence was provided by Nelson et al. (2012) that calpain can promote caspase-3 activation and active caspase-3 can enhance calpain activity in diaphragm muscle during protracted mechanical ventilation.

2.2.4 Caspases and apoptosis

Activation of the caspases, a set of cysteine proteases, results in cell morphological features such as cytoplasmic contraction, chromatin condensation, plasma membrane blebbing, and DNA fragmentation which are all characteristics of the apoptotic pathway (Sainz et al. 2003). Two major apoptotic pathways of programmed cell death include the intrinsic/mitochondrial and extrinsic/death receptor pathways (Lowe et al. 2004). These pathways intersect at a common juncture encompassing effector caspase activation in the execution pathway of apoptosis (Elmore 2007).
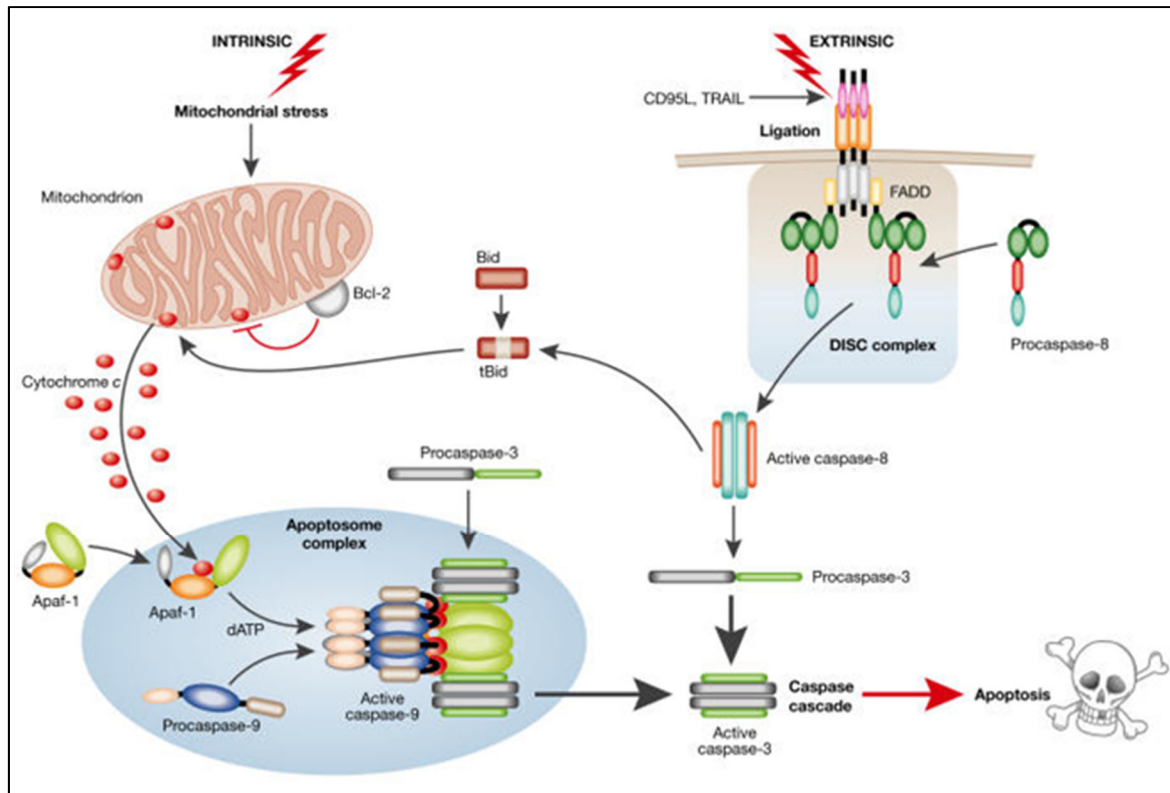
Figure 2.4: Apoptosis: the two major apoptotic pathways leading to caspase activation. Image acquired from MacFarlane and Williams (2004).

The extrinsic apoptotic pathway

The death receptor pathway is activated by TNF-α and other cytokines of the same gene superfamily via TNFR-associated death domain (TRADD), FAS-associated death domain (FADD) and caspase 8 (Marsters et al. 1996). In short, apoptotic cell death is promoted by the binding of death ligands to death receptors on the cellular surface which signals the cleavage of the N-terminal of the prodomain of caspase-8 which activates it (Sainz et al. 2003). After caspase-8 activation a signal is sent to the mitochondria to release cytochrome-c from the inner mitochondrial membrane thereby promoting the formation of the apoptosome via the addition of pro-caspase 9 and Apaf-1. This cascade culminates in the cleavage of caspase 3, also causing its activation, and subsequent apoptotic cell death. Importantly, cytochrome c release is prevented

by Bcl-2 proteins (defined below) and inhibitors of apoptosis protein (IAP) which control the release of cytochrome c from the inner mitochondrial membrane (Sainz et al. 2003). The extrinsic pathway can also indirectly amplify apoptotic signals by triggering the intrinsic pathway (Johnstone et al. 2002).

The intrinsic apoptotic pathway

Non-receptor mediated stimuli such as DNA damage, oxidative stress and chemotherapeutic drugs that result in mitochondrial stress and mitochondrial membrane perturbation trigger the intrinsic apoptotic pathway (Lopez and Tait 2015). This mechanism is in contrast to the extrinsic mediated pathway which requires ligand binding. The resultant mitochondrial membrane perturbations trigger the release of pro-apoptotic proteins, namely cytochrome c and Apaf-1 that activate caspase-9 and further downstream caspase-3, which results in the cleavage and degradation of cytoplasmic structural proteins and chromosomal condensation (Saelens et al. 2004, Putcha et al. 2002, Benn and Woolf 2004). The B-cell lymphoma protein 2 (Bcl2) family of proteins exerts regulatory control over the intrinsic pathway by opposing its actions (Koff et al. 2015). This family of proteins may act by inhibiting pro-apoptotic proteins such as BAX and in so doing prevent cytochrome c release from the mitochondria (Wei et al. 2001).

Caspases are said to be the central executioners of the apoptotic pathway, which act by selective cleaving of specific sets of target proteins leading to the loss of biological activity of that protein (Jin and El-Deiry 2005). A role for caspases in muscle protein degradation was suggested due to the observations of reduced myonuclei numbers during muscle atrophy observed in heart failure and Duchenne muscular dystrophy (Adams et al. 2001, Sandri 2002). Skeletal muscle apoptosis has also been observed

to be increased in motor neuron disorders, denervation, spinal cord injury, muscular dystrophy, and atrophy due to hind limb suspension or immobilization as measured by transferase end-labelling (TUNEL) or by DNA fragmentation in gel electrophoresis (Allen et al. 1997, Tews et al. 1997, Podhorska-Okolow et al. 1998, Adams et al. 1999, Borisov and Carlson 2000, Smith et al. 2000, Dupont-Versteegden 2005). A study by Siu and Alway (2006) demonstrated that apoptosis inhibition, to a certain degree, attenuated muscle atrophy induced by denervation in a *Bax* knockout mouse model. Caspase-3 has also been shown to be involved with actomyosin complex cleavage, while caspase-3 inhibition prevented actin fragment accumulation in skeletal muscle of diabetic and uremic rats; indicating that activation of caspase-3 may play an early role in muscle protein degradation (Du et al. 2004). Other authors disagree with these findings on the premise that non-apoptotic cells are unlikely to activate sufficient caspase-3 to contribute towards myofibrillar protein turnover (Goll et al. 2008). There is evidence of apoptosis in skeletal muscle in the Lewis lung carcinoma mouse and AH-130 hepatoma animal models of cachexia (van Royen et al. 2000), but a role in human cancer cachexia has not been proven. In gastric cancer patients with moderate weight-loss (mean 6%) similar apoptotic myonuclei numbers compared to controls were apparent, accompanied by no increase in caspases (Bossola et al. 2006). Current studies are contradictory; it is thus important to remember that skeletal muscle fibres are multinucleated and apoptosis of a single nucleus will not result in the death of a muscle fibre (Fanzani et al. 2012). Moreover, the transferase end-labelling (TUNEL) technique used to detect DNA fragmentation is not specific and may result in false positive reactions contributing to difficulties in interpretation of cell death data gained from atrophied muscle (Fanzani et al. 2012).

2.2.5 Forkhead box O (FoxO) transcription factors

FoxO3 is the main inducer of autophagy genes such as LC3 and Bnip3 in skeletal muscle (Mammucari et al. 2007). Well-coordinated FoxO3-induced gene expression is crucial to balanced autophagy. Mammucari et al. (2007) suggested that the transcription factor FoxO3 controls both the ubiquitin-proteasome and lysosomal/autophagy pathway in muscle but through different mechanisms. This is interesting, since it is suggested in several studies that the pathways may not run succinctly, as one pathway may be activated and the other remains at basal levels (Zhao et al. 2007). FoxO3 is inactive in its phosphorylated form induced by Akt, where it resides in the cytoplasm (Brunet et al. 1999); upon dephosphorylation, FoxO3 becomes active and translocates to the nucleus (Ramaswamy et al. 2002, Sandri et al. 2004). Activation of FoxO3 is implicated in muscle atrophy (Sandri et al. 2006) and has been shown to activate the E3 ligases MAFBx and MuRF1 (Sandri et al. 2004, Stitt et al. 2004). Zhao et al. (2008) showed that by overexpressing FoxO3 both UPP and autophagy mediated proteolysis were increased. This result suggested that under UPP control, FoxO3 degraded myofibrillar protein and autophagy was responsible for mitochondrial loss. Furthermore, Judge et al. (2014) showed that inhibition of FoxO-dependent transcription in tumour bearing mice prevented both diaphragm and locomotor muscle atrophy and also spared force deficits.

In humans, decreased phosphorylation of FoxO3a has been observed in the *rectus abdominis* muscle of cachectic compared with non-cachectic pancreatic cancer patients (Schmitt et al. 2007). Similarly, FoxO3 was activated in *vastus lateralis* muscle of lung cancer and COPD patients (Puig-Vilanova et al. 2015).

2.2.6 Proteolysis-inducing factor (PIF)

Another catabolic cachectic mediator, proteolysis-inducing factor (PIF), which is released from tumours, has been shown to activate the transcription factor, NF-κB (Whitehouse and Tisdale 2003). Intravenous administration of PIF degraded *gastrocnemius* muscle in mice and increased mRNA levels for ubiquitin (Lorite et al. 2001), providing evidence of a mechanism via which PIF functions. Whitehouse and Tisdale (2003) later provided evidence that PIF-induced upregulation of proteasome expression in C2C12 myotubes may be mediated via NF-κB. NF-κB is known to have oncogenic functions and may also mediate muscle protein breakdown by degradation of specific muscle proteins, inducing inflammation and fibrosis and halting regeneration of myofibres following injury or atrophy (Li et al. 2008).

2.3 Inhibition of muscle regeneration

The imbalance between muscle protein synthesis and degradation is known to contribute to muscle wasting, with a growing amount of literature now suggesting that satellite cell regulation and subsequent regeneration may be impaired in atrophied muscle. This notion stems from various studies reporting a decline in the number of precursor cells (satellite cells) in atrophied muscle (Guo, 2012, Mitchell, 2004, Mozdziak, 2001). Evidence for satellite cell number decline was provided in a study which demonstrated that regeneration from acute cardiotoxin injury was blocked following prior hind-limb suspension and the impaired muscle mass gain lasted for six weeks (Matsuba, 2009). It is yet unclear whether impaired regulation of regeneration contributes to muscle atrophy or whether it is a consequence of muscle atrophy.

2.3.1 Activation of adult muscle satellite cells, a key element in the regulation of muscle regeneration

Satellite cells (SCs), are precursors to skeletal muscle cells and are under normal physiological conditions quiescent and situated in their anatomical position under the basal lamina of myofibres (Bischoff 1997, Grounds 2002). These cells are responsible for the replacement and reparation of damaged myofibres. Induction of stressors such as muscle fibre damage from sarcolemmal wear and tear, acute injuries requiring new fibres to be formed or even hypertrophy, activate SCs. These cells then enter the cell cycle and begin to proliferate under the guidance of cell cycle regulators and then differentiate under the guidance of the myogenic regulatory factors. During proliferation satellite cells express both Pax7 and MyoD and can either self-renew and thus expand the satellite cell pool or exit the cell cycle (Cornelison and Wold 1997, Scime and Rudnicki 2006). Cells exiting the cell cycle produce muscle precursor cells, known as myoblasts which now express higher levels of MyoD and also myogenin (Fig. 2.5). Next, the newly formed myoblasts fuse to form myotubes, which express myogenin and MRF4 and which terminally differentiate into muscle fibres (Tidball et al. 2014).
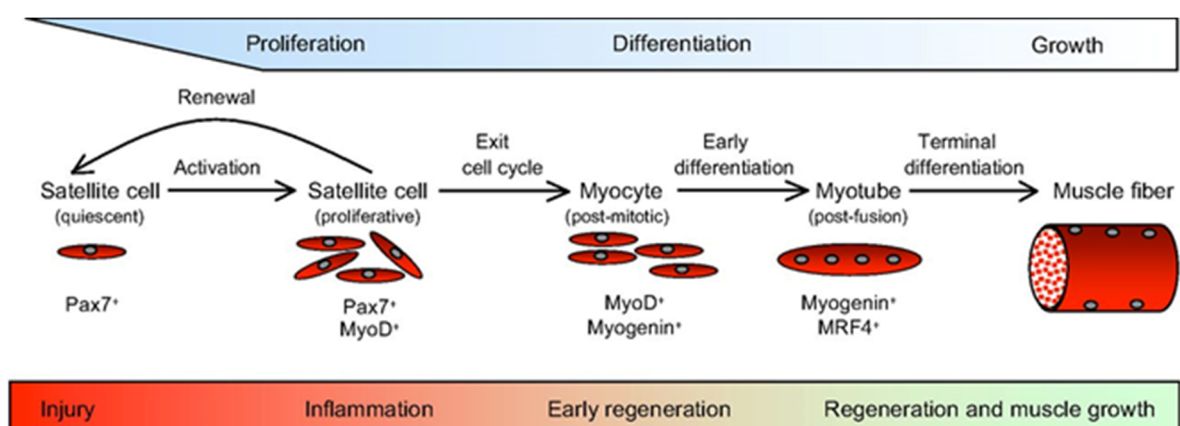
Figure 2.5: Regeneration and muscle growth following injury. Image modified from Tidball et al, (2014).

2.3.2 Impaired remodelling in cancer cachexia

The interaction between the SC and its microenvironment, as well as the interaction between the immune system and skeletal muscle are regarded as important role players in the regulatory process, both modulating events during repair and remodelling.

Basal lamina disruption and membrane damage result in satellite cells' activation. In two separate studies using C-26 tumour bearing mouse models of cancer cachexia, skeletal muscle membrane damage was demonstrated. The more recent study showed that the hindlimbs of these mice contained a pronounced accumulation of immunoglobulins (IgG) not normally present in muscle fibres, together with diffuse laminin staining, increased penetration of Evans blue dye, and reduced expression of extracellular matrix genes (He et al. 2013). In the other study, it was demonstrated that the myofibres of tumour bearing mice had altered sarcolemmas and basal laminas (Acharyya et al. 2005). These data demonstrate sarcolemma and basal lamina perturbations in cachectic muscle, most probably due to tumour related factors in circulation.

He et al. (2013), provided the first clear evidence of impaired regeneration in an *in vivo* model of cancer cachexia. The authors provided evidence that Pax7 is abnormally elevated in cachectic SCs. To prove this was actually part of the mechanism for atrophy, the authors also showed that overexpression of Pax7 (an essential regulator of satellite cell expansion and differentiation during embryonic and fetal myogenesis) induced atrophy in normal muscle. Moreover, they showed that with reduction of Pax7 or exogenous addition of MyoD, during tumour conditions, wasting could be reversed due to restoration of SC differentiation and fusion. They also demonstrated that Pax7 dysregulation was induced by circulating pro-cachectic serum factors from cachectic

mice and patients, in a NF-κB–dependent manner. Damrauer et al. (2008) reported that chemotherapeutic agents such as cisplatin, and doxorubicin may directly cause muscle wasting via activation of the NF-κB pathway, and suggested that these effects were independent of their inhibition of tumour growth.

2.4 Doxorubicin

2.4.1 Structure, function and mechanism of action in tumours

The chemotherapeutic antibiotic, doxorubicin, was first isolated from mutant *Streptomyces peucetiusis* (Arcamone et al. 1969) in the 1960s and found to be highly effective against various solid tumours (Mohammad et al. 2015). The stereochemistry and absolute configuration of doxorubicin have been identified by X-ray crystallographic analysis (Di Marco et al. 1969) which provided detail on the conformation of the molecule in the solid state. This glycoside antibiotic is characterized by a planar four-ring quinone-containing chromophore, which has a high binding affinity for DNA bearing one, two or three sugar moieties attached at the 7-position. It is considered one of the most effective agents in the fight against cancer; however, its clinical value is compromised by toxicity to both the heart and skeletal muscle.
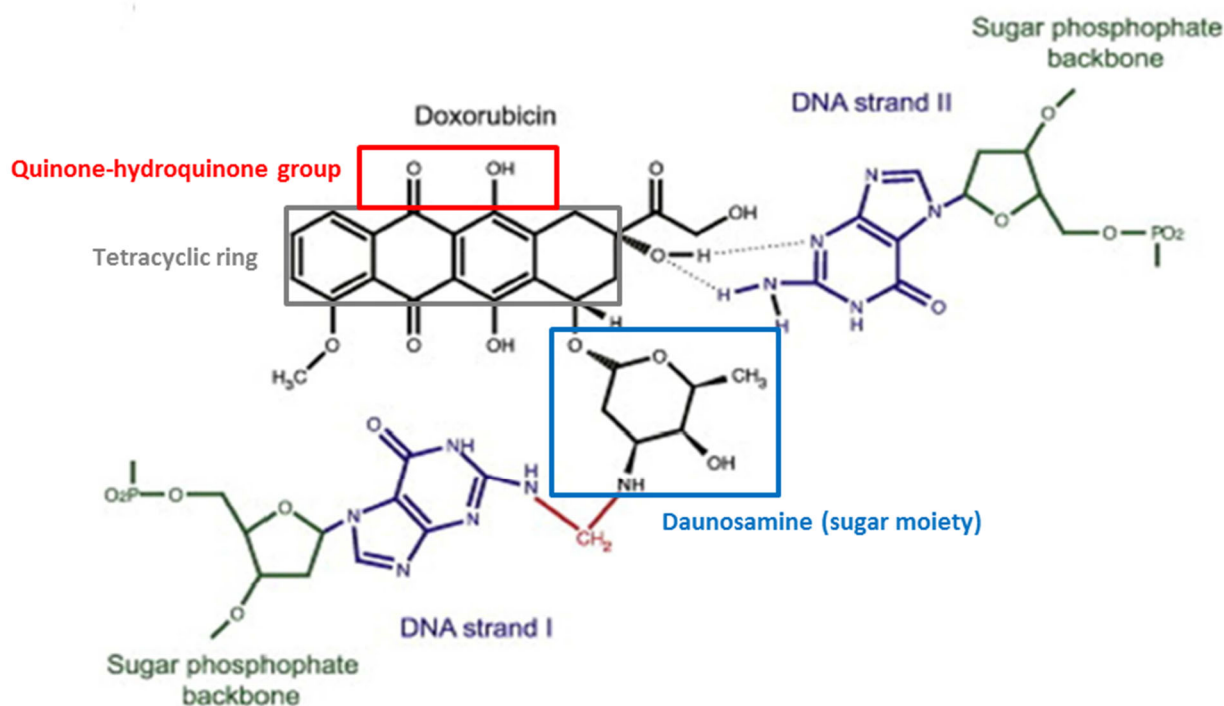
Figure 2.6: Chemical structure of doxorubicin in complex with DNA. Image modified from Di Marco et al. (1969).

Due to its systemic administration and thus non-specific mode of action, doxorubicin elicits significant side effects by targeting healthy cells. Doxorubicin induces its cytotoxic action through: (i) Topoisomerase II inhibition, (ii) Interference with cell respiration and electron transport resulting in oxidative stress, (iii) disruption of cellular membrane phospholipids.

i) Topoisomerase II inhibition

Topoisomerases are ubiquitous enzymes that exercise regulation over DNA replication, transcription and other nuclear processes (Bakshi et al. 2001, Cuvier and Hirano 2003). These enzymes act to manipulate the topology of DNA function at various steps of replication in both pro and eukaryotes by cutting and re-joining single and double stranded DNA, thereby either catalysing addition or removal of supercoils to rapidly make DNA accessible for interaction with proteins, which regulate different

functions of chromatin (Cuvier and Hirano 2003). Previous studies have demonstrated that doxorubicin functions as a topoisomerase II inhibitor (Heart et al. 2016). The inhibition of topoisomerase II by doxorubicin prevents cell division, which results in programmed cell death via apoptosis or necrosis (Minotti et al. 2004).

ii) Interference with cell respiration and electron transport resulting in oxidative stress

Oxidative stress is considered one of the major mechanisms of doxorubicin's antitumour action. Doxorubicin is prone to the generation of oxygen free radicals, since it possesses a quinone-hydroquinone group within its structure. Once this quinone structure is oxidized, an unstable semiquinone radical is formed which rapidly reacts with oxygen to generate free radicals such as superoxide and hydrogen peroxide which damages DNA (Yang et al. 2014). Semiquinone radical formation can increase aglycone lipid solubility within its structure by further oxidizing the glycosidic bond between daunosamine and the aglyconic subunit, allowing for its intercalation into biological membranes as well as the production of ROS. Furthermore, iron overload may develop in doxorubicin treated cancer patients (Lipshultz et al. 2013) due to blood transfusions, blood loss or iron supplementation resulting in doxorubicin-iron complex formation which catalyses the conversion of hydrogen peroxide to highly reactive hydroxyl radicals (Yang et al. 2014). These free radicals increase oxidative stress within tumours, leading to DNA damage and cell death (Yang et al. 2014).

iii) Disruption of cellular membrane phospholipids

Doxorubicin has been shown to bind to phospholipids such as cardiolipin within the heart mitochondrial membrane (Goormaghtigh et al. 1990, Kavazis et al. 2017); this binding has been suggested as a possible explanation of selectivity of action within this class of drugs (Lown et al. 1983). Further evidence for the alteration of cell surface

architecture by doxorubicin is described in other studies (Arancia et al. 1995, Suwalsky et al. 1999) as well, where it was shown that doxorubicin perturbed the polar head and acyl chain regions of lipids and was able to fluidize their hydrophobic moieties. The aforementioned studies used a variety of methods to prove doxorubicin's interaction with erythrocyte membranes. It was found to be incorporated into either the inner or outer leaflets of the erythrocyte membrane (Suwalsky et al. 1999) causing modifications of the cell morphology and membrane ultrastructure (Arancia et al. 1995), which might induce anaemia, in cancer patients. Furthermore, it was also demonstrated that doxorubicin is incorporated into the membranes of skeletal muscle (Peters et al. 1981, Hayward et al. 2013), where current research indicates that long term effects may persist in skeletal muscle for many years following chemotherapy treatment (Fabris and MacLean 2015), independent of cancer cachexia.

2.5 Doxorubicin-induced mechanisms of muscle protein degradation

It is well documented that doxorubicin treatment causes skeletal muscle atrophy as well as loss of function, However, mechanisms of doxorubicin-induced skeletal muscle atrophy are not well characterised, although it has been proposed that the induction of oxidative stress constitutes a common denominator. Since doxorubicin strongly binds the membranes of mitochondria which are specifically dense in skeletal muscle, it seems plausible that myotoxicity is mediated via doxorubicin-induced mitochondrial ROS mediated dysfunction, especially since symptoms of myotoxicity include fatigue, weakness and exercise intolerance. As previously described, doxorubicin's unique chemical structure contains an unsubstituted quinone that is able to form a redox cycle with electron donors thereby promoting superoxide radical production at complex I of the electron transport chain (Minotti et al. 2004), thus removing electrons needed for ATP production. Since one of doxorubicin's primary functions is to fragment DNA,

poly-ADP-ribose polymerases (PARPs) will be activated in order to repair damaged DNA at a metabolic cost. PARPs require energy cofactors such as NAD^+ for repair to DNA, thus consuming ATP, adding to energy imbalances. Interestingly, this leads to reduced SIRT-1 activity - a protein that provides protection against chronic inflammation, induces mitochondrial biogenesis and increases skeletal muscle oxidative capacity (Bai et al. 2011). Gilliam et al. (2012) demonstrated that ROS levels were increased following exposure of C2C12 cells in culture to doxorubicin. The elevation in ROS was followed by an upregulation of E3 ubiquitin ligases and caspase-3. Furthermore, when an antioxidant was added, mitochondrial ROS formation was inhibited. Moreover, it has been shown that ROS can contribute to the activation of calpain by acting as a signalling molecule (Storr et al. 2011). ROS is also largely implicated in the activation of caspase-3 and subsequent apoptosis, thereby promoting atrophy (Storr et al. 2011, Tsai et al. 2010, Smuder et al. 2011). These muscle degradative systems may then catalyse the release of myofilament proteins, allowing activation of the ubiquitin-protease system (Powers et al. 2005, Du et al, 2004, Smuder et al. 2011).

In a recent study by Gilliam et al. (2016) it was demonstrated that the overexpression of catalase in mitochondria of muscle diminished mitochondrial H_2O_2 emission and protein oxidation, preserving mitochondrial and whole muscle function despite doxorubicin treatment in an *in vivo* cancer model. Gouspillou et al. (2015) studied doxorubicin & dexamethasone-induced long-term mitochondrial respiration impairment in skeletal muscle in non-tumour bearing mice. The authors showed that treated animals had lower muscle mass and impaired growth as well as an attenuation in mitochondrial respiration and increased ROS production. They further observed reduced protein levels of Parkin which suggested a novel pattern of chemotherapy-

induced mitochondrial dysfunction in skeletal muscle that persists due to an acquired defect in mitophagy signalling.

It is postulated that the generation of mitochondrial ROS by doxorubicin could lead to activation of proteolytic and catabolic pathways. This was demonstrated by Kavazis et al. (2014), who showed that doxorubicin administration increased FoxO1 and FoxO3 mRNA expression in skeletal muscle. The transcription of FoxO targeted genes namely, MaFbx, MuRF-1, and BNIP3 were also found to be elevated in both heart and *soleus* muscles. Exercise training prevented these doxorubicin-induced elevations by increasing PGC-1α, which is a known suppressor of FoxO activity. Smuder et al. (2011) first showed that markers of autophagy (mRNA and protein) were increased following doxorubicin administration in rat *soleus* muscle and also found that prophylactic endurance exercise protected skeletal muscle against DOX-induced activation of autophagy. Doxorubicin may also indirectly promote muscle degradation via systemic mechanisms.

2.5.1 Doxorubicin-induced anaemia and leukopenia and iron-binding in muscle tissue

Doxorubicin targets and destroys rapidly dividing cells, which are characteristic of cancer. However, many other rapidly dividing normal cell types are destroyed in the process which include bone marrow and by extension, blood cells, a condition known as myelosuppression (Bhinge et al. 2012). Doxorubicin interacts with free iron in red blood cells forming a doxorubicin-iron complex which self reduces, resulting in the generation of potent free radicals causing lipid peroxidation and DNA damage (Vaidyanathan and Boroujerdi 2000). Shinohara and Tanaka (1980) reported that doxorubicin peroxidises human red blood cell membrane lipids as well as thiols which result in glutathione instability and the oxidation of haemoglobin and membrane

protein components. Doxorubicin treatment is also strongly associated with aplastic anaemia, leukopenia and thrombocytopenia (Nurgalieva et al. 2011). Furthermore, doxorubicin-induced leukopenia (Henry et al. 1993) considerably weakens the immune system and is associated with poor prognosis in cancer patients (Bhinge et al. 2012).

The iron binding properties of doxorubicin extend beyond blood cells to other tissue including cardiac and skeletal muscle (Octavia et al. 2012). Miranda et al. (2003) showed that *HFE* (an iron regulatory gene) deficient mice were more susceptible to doxorubicin-induced cardiotoxicity. It is however argued that not enough free iron is present within most cells for doxorubicin to couple with under physiological conditions, therefore doxorubicin-iron coupling may not always result in myopathy (Minotti et al. 2004). However, Minotti et al. (1998) provided evidence that the major metabolite of doxorubicin, doxorubicinol forms complexes with iron regulatory proteins, thus stabilizing transferrin mRNA resulting in the prevention of iron sequestration and protein translation. This leads to increased free iron, which could propagate the cycle of free radical generation. Panjrath et al. (2007) found that cardiotoxicity was enhanced in an iron loading rat model, further supporting the role of iron in the pathogenesis of doxorubicin-induced cardiotoxicity.

It is thought that iron-containing protein complexes which are plentiful in muscle tissues are one of doxorubicin's preferred accumulation sites in skeletal muscle (Octavia et al. 2012). Indeed, muscle iron dyshomeostasis has been linked to pathogenesis of both sarcopenia of aging and disuse-induced muscle atrophy (Xu et al. 2010). Furthermore, in a model of disuse atrophy in aged rodents significantly increased iron levels in skeletal muscle have been associated with widespread oxidative damage (Hofer et al. 2008). Gilliam et al. (2013) speculated that potential

sources of doxorubicin-induced oxidants within skeletal muscle are from redox cycling within mitochondria, and the inactivation of the electron transport system. The authors further speculated that hydroxyl radicals are formed from H_2O_2 leading to lipid peroxidation in the presence of redox active iron, thus altering mitochondrial and myofilament proteins (Gilliam et al. 2013).

2.5.2 Doxorubicin and satellite cells

Little is known about the effects of doxorubicin on satellite cell function. In an *in vitro* study to determine if the overexpression of antioxidants protects against doxorubicin-induced cell death in primary satellite cell cultures derived from the hindlimb muscles of mice, the investigators found that overexpression of the cytosolic antioxidant enzyme, CuZnSOD, protects against DNA fragmentation, and enhanced cell survival. The overexpression of the mitochondrial enzyme, MnSOD, also enhanced cell survival in response to an apoptosis-inducing dose of doxorubicin (Soltow et al. 2007).

As mentioned earlier in the introduction, doxorubicin is used clinically to permanently remove muscle from the eyelids of patients to treat blepharospasm (Wirtschafter and Mcloon 1998). Nguyen et al. (1998) further demonstrated that doxorubicin chemomyectomy was more effective when introduced two days post muscle injury since this coincided with peak satellite cell division, demonstrating doxorubicin's effect is most potent during mitosis.

Survivors of childhood cancers, such as acute lymphoblastic leukaemia, have increased rates of long-term skeletal muscle dysfunction due to treatment with doxorubicin and other anthracyclines (Gouspillou et al. 2015). During cytotoxic conditions such as oxidative stress, activated satellite cells seem to be more susceptible to oxidative stress damage than quiescent satellite cells (Pallafacchina et

al. 2010). The average age of these cancer patients when seeking treatment is 4 years which corresponds with the period in life when satellite cells are still highly active and proliferating due to skeletal muscle growth. Thus, exposure of activated muscle satellite cells to chemotherapy-induced oxidative stress during this time, may impair satellite cell function and the ability to adequately replenish the quiescent cell population (Chen et al. 2007). This lead Scheede-Bergdal and Jagoe (2013) to infer that chemotherapy-induced early depletion of muscle satellite cells may be one mechanism for chemotherapy-related muscle dysfunction.

2.6 Managing cancer and cachexia

2.6.1 Current treatment strategies for cancer cachexia

Current treatments targeting cancer cachexia appear to be limited or have not shown the desired results. This is proposedly due to the complex nature of cancer cachexia, which involves an array of factors such as metabolic abnormalities, pro-inflammatory cytokines, tumour-derived catabolic factors and decreased food intake (Macciò et al. 2012, Mantovani et al. 2001). Various treatment strategies have been suggested to target muscle wasting, from multimodal pharmacological to nutritional and antioxidant approaches, with some treatments showing significant increases in skeletal muscle strength and size in clinical trials (Greig et al. 2014), and improvements in appetite, body weight, and caloric intake (Gagnon and Bruera 1998, Mantovani et al. 2001, Mantovani and Madeddu 2008). Corticosteroids have also shown some promise, with results from clinical studies reporting increased appetite, improved pain control, and improved perceived quality of life in patients suffering from cancer cachexia (Gagnon and Bruera 1998; Mantovani et al. 2001). Other strategies under investigation are either to enhance chemotherapeutic drug delivery via PEGylated liposomes (Cohen et al. 2018, Ma and Mumper 2013) or to administer agents which target the

proteasome system. Some proteasome inhibitors successfully attenuate atrophy in various animal models (Supinski et al. 2009, Caron et al. 2011, Jamart et al. 2011, Bonaldo and Sandri 2013); however this was ineffective in human clinical trials (Jatoi et al. 2005).

Another category of drug treatments are those which target ROS and cytokine release, such as melatonin (Leja-Szpak et al. 2018, Mantovani et al. 2001), eicosapentaenoic acid (EPA) (Jatoi et al. 2004), nonsteroidal anti-inflammatory drugs, or those antagonizing muscle wasting, such as clenbuterol and anabolic agents, growth hormone and insulin-like growth factor (Kim et al. 2011, Mantovani et al. 2001).

Due to the aberrant nature of cancer cachexia, it is proving quite difficult to completely stunt excessive muscle wasting. Below the potential for targeting reactive oxygen species in attenuating muscle wasting associated with cancer, with particular emphasis on the contribution of antioxidants, will be discussed.

2.6.2 Antioxidants

Positive results for antioxidant treatment in muscle disease models have been reported in a number of studies. Alpha linoleic acid (ALA) an n−3 fatty acid with antioxidant activities, has been shown to induce protein synthesis in C2C12 myotubes via the PI3K/Akt signalling pathway thereby modulating skeletal muscle turnover (Jing et al. 2016). Furthermore, antioxidant-supplementation (resveratrol) improved weight gain in tumour-bearing mice (Shadfar et al. 2011). However, in two separate *in vivo* C26 adenocarcinoma-induced mouse cachexia models, it was found that the selected antioxidants failed to attenuate muscle loss (Tian et al. 2011, Assi et al. 2016). In the first study by Tian et al. (2011) it was demonstrated that conjugated linoleic acid, which is thought to have anti-cancer and antioxidant properties, failed to attenuate muscle

wasting and furthermore it was found that the supplement increased expression of inflammatory markers in muscle. In the more recent study by Assi et al. (2016), an antioxidant cocktail, comprised of catechins (polyphenol), quercetin and vitamin C, failed to attenuate muscle loss in the C26 tumour-bearing mouse model, but instead the cocktail combination resulted in cachexia enhancement and reduced survival. The successful and the unsuccessful studies used the same C26 adenocarcinoma tumour bearing mouse model and tumours were allowed to grow for 17 days. Tumour weight in the study by Tian et al. (2011) weighed approximately double in mass compared to the successful study by Shadfar et al. (2011), as 250 000 more C26 adenocarcinoma cells were inoculated on study day 0.

In humans, Persson et al. (2005) investigated the effects of fish oil, melatonin or a combination of the supplements in cachectic patients with advanced gastrointestinal cancer. They found that the supplements did not induce major biochemical changes in the muscle of patients, but they did observe a weight-stabilizing effect. In a different clinical trial, treatment with antioxidants (alpha-lipoic acid + carbocysteine lysine salt + vitamin E + vitamin A + vitamin C), drugs (medroxyprogesterone acetate and selective cyclooxygenase-2 inhibitor celecoxib) and pharmaco-nutritional support was beneficial in a population of advanced cancer patients with anorexia/cachexia. In this study increased body weight and appetite, as well as decreased pro-inflammatory cytokines and improved fatigue symptom scores were observed (Mantovani et al. 2006). It is clear from literature that the roles of antioxidants in cancer cachexia are not fully elucidated yet. In a recent review by Assi and Rebillard (2016), it was concluded that antioxidant therapy may: 1) benefit cancer cachectic patients' deficient in antioxidants; 2) be of harm to cancer patients with sufficient antioxidant status, and 3) not be recommended whilst undergoing radiotherapy.

2.7 Melatonin

2.7.1 Structure, function, synthesis and secretion

First isolated in the late 1950's (Lerner et al. 1959), melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring light sensitive multi-tasking hormone produced in the brain and primarily secreted by the pineal gland (Tasdemir et al. 2012). Numerous other non-pineal tissue also have the ability to synthesize melatonin such as the retina, ciliary body, lens, Harderian gland, brain, thymus, airway epithelium, bone marrow, gut, ovary, testicles, placenta, lymphocytes and skin (Tan et al. 2007). Both its release and synthesis are sensitive to light-dark conditions. Photosensitive retinal ganglion cells relay information regarding light-dark exposure to the suprachiasmatic nucleus of the hypothalamus, the region of the brain which coordinates biological clock signals, thus regulating melatonin's secretion (Brennan et al. 2007). The synthesis of melatonin gradually rises at night and levels are extremely low during the day, occurring in a diurnal fashion. This compound's main function is to regulate the central circadian clock and oscillators in peripheral tissue and organs, thereby influencing almost all cell processes and other body functions which include antioxidant (Tasdemir et al. 2012), anti-inflammatory (Laste et al. 2012), analgesic (Laste et al. 2012), immunomodulatory (Srinivasan et al. 2008), thermoregulatory (Krauchi et al. 2006) and neuroprotective (Alonso-Alconada et al. 2013) properties. Furthermore, melatonin's potential applications are being explored in areas as diverse as cancer (Cutando et al. 2012), ophthalmology (Rosenstein et al. 2010), haematology (Li et al. 2017), toxicology (Malhotra et al. 2004), sleep (Bramley et al. 2017), cardiology (Zhang et al. 2017), ageing (Bubenik and Konturek 2011), immunity (Srinivasan et al. 2008), and osteoporosis (Liu et al. 2013).
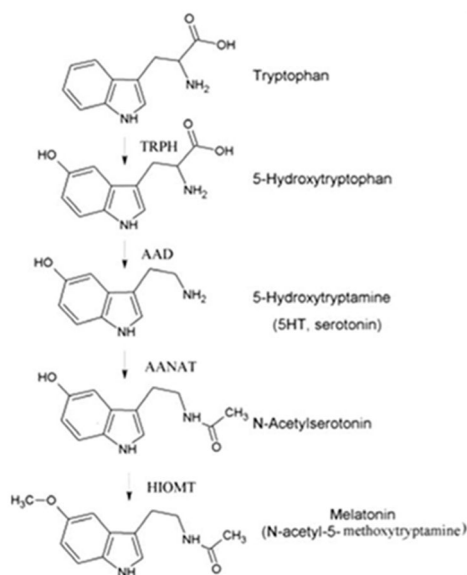
Figure 2.7: Biochemical pathway for the synthesis of melatonin from tryptophan (Hickman et al. 1999).

Serotonin is a precursor of melatonin and is derived from tryptophan. This neurotransmitter first undergoes acetylation via a serotonin N-acetyltransferase (NAT) (which is considered the rate limiting enzyme in the process) into N-acetylserotonin. N-acetylserotonin next undergoes methylation via an enzyme methyltransferase in the pineal gland to yield melatonin (see Figure 2.7) (Hickman et al. 1999). Evidence contrary to the NAT as rate limiting enzyme paradigm suggests that melatonin synthesis in fact may be limited by the speed of conversion from N-acetylserotonin to melatonin, which is mediated by methyltransferase (Chattoraj et al. 2009). Once synthesised, melatonin is immediately released into circulation where (Chattoraj et al. 2009) peak levels would range from 54 – 75 pg/mL and 18 – 40 pg/mL for young and old individuals respectively. The serum half-life of endogenous melatonin typically ranges from 30-60 mins whereas exogenous melatonin levels have a shorter half-life of between 12-48 mins.

Melatonin exerts its effects directly via two main membrane bound receptors namely melatonin receptor 1 and melatonin receptor 2 (MT1 and MT2 respectively), both of

which are G protein-coupled transmembrane receptors. These receptors are ubiquitously expressed throughout the body, including the brain, immune system, testes, skin, retina, kidneys and in the breasts. Melatonin has been shown to activate several signalling pathways; most notably the Ca^{2+}-dependent pathways, which include the cAMP and Phospholipase C pathways. Dubocovich et al. (2003 & 2010) argue that melatonin's biological activity may also be mediated via non-receptor mediated antioxidant activity. Moreover, melatonin is highly lipophilic and water soluble which means it can readily diffuse across cell membranes and into circulation for effective delivery to the CNS and other organs (Nosjean et al. 2001). Melatonin further may mediate its actions indirectly via nuclear orphan receptors from the RORα/RZR family; this however remains controversial and researchers studying nuclear receptors question whether melatonin may be a ligand of the receptor tyrosine kinase-like orphan receptors (ROR) (Slominski et al. 2016). Researchers have also described a third melatonin receptor (MT3), however this turned out to be a cytosolic enzyme quinone reductase 2 (Ferry et al. 2010).

2.7.2 MT receptors in skeletal muscle

Omnipresent MT receptors also operate within skeletal muscle tissue. Recently, Owino, et al. (2016) analysed relative mRNA levels in skeletal muscle of wild type, $MT_1^{-/-}$ and $MT_2^{-/-}$ mice. They found that the daily rhythms in blood glucose levels were lost in $MT_1^{-/-}$ and $MT_2^{-/-}$ mice and interestingly that signalling via the MT receptor produced negligible effects on rhythmic expression patterns of clock genes in insulin sensitive tissues such as the liver, adipose tissue, and skeletal muscle. More recently MT1a mRNA expression was found to be up-regulated in contusion injured skeletal muscle of male Wistar rats treated with MLT (Mehanna et al. 2017). Treatment with MLT further significantly increased twitch force and decreased Bax levels of injured

soleus muscle compared to the injured non-treated group. Histological analysis of MLT treated injured *soleus* muscle revealed less inflammatory cellular infiltration and vascular congestion at 1 and 4 days after injury compared to the injured non-treated group. The authors concluded that the presence of MT receptors in injured muscle may have direct effects in contused muscle, whilst co-ordinating possible systemic effects which further indirectly support the restoration of injured muscle (Mehanna et al. 2017). This data provides evidence that MLT supports muscle restorative capacity in contusion injured muscle.

2.7.3 Melatonin and Breast Cancer

The International Agency for Research on Cancer has classified "shift-work that involves circadian disruption" as "probably carcinogenic to humans" (Rondanelli et al. 2013). Melatonin secretion disruption by night-time light exposure has been hypothesised to play a role in breast cancer (Jasser et al. 2006, Ravindra et al. 2006, Shadan 2007), since melatonin secretion is impaired in breast cancer patients (Lissoni 2002). Indeed many *in vitro* (Sainz et al. 2003, Garcia-Santos et al. 2006, Garcia-Navarro et al. 2007) and *in vivo* (Srinivasan et al. 2008) studies have confirmed melatonin's oncostatic and antiproliferative effects in different cancer cell types as well as in a variety of solid tumours.

In a study by Bartsch et al. (1981), it was shown that urine melatonin levels in postmenopausal women with advanced stage breast cancer was 31% lower compared to controls. Furthermore, melatonin excretion patterns in these patients were not synchronised as compared to synchronised controls, suggesting both melatonin secretion and rhythm are modified in breast cancer patients (Bartsch et al. 1981). In a clinical trial by Lissoni et al. (1995), ER-negative metastatic breast cancer patients who were unable to continue with chemotherapy were treated with tamoxifen and

melatonin. Patients treated with melatonin as adjuvant together with tamoxifen had a significantly higher one year survival rate compared to tamoxifen treatment alone. No melatonin related toxicity was observed; furthermore, melatonin reduced anxiety and depression in these patients. In a recent study by Innominato et al. (2016), where sleep quantity and quality of life in patients with advanced breast cancer were evaluated, it was found that melatonin treatment improved fatigue and sleep problems in these patients. The results of this study were supported by another study which demonstrated that melatonin significantly changed sleep efficiency after breast cancer surgery (Madsen et al. 2016). Additionally, melatonin has been found to be beneficial as adjuvant therapy when used in conjunction with chemotherapeutic agents in patients suffering from melanoma and breast cancer (Kilic et al. 2013). Although mounting evidence points toward melatonin's potential as adjuvant therapy for breast cancer patients, clinical evidence is still limited.

2.7.4 Melatonin the unique antioxidant

The indole moiety of the melatonin molecule is characterised as having high resonance stability and a very low activation energy barrier toward oxidative reactions, while, the molecule's methoxy and amide side chains add to melatonin's antioxidant capacity, the methoxy group prevents pro-oxidative activity of the molecule (Tan et al. 2002). Since the early nineties when it became evident that melatonin possesses potent radical-scavenging properties, numerous *in vitro* and *in vivo* studies have further confirmed this (Tesoriere et al. 1999, Tan et al. 2002). For a summary, see Table 1. These studies have demonstrated that melatonin scavenges a wide variety of radicals, including reactive oxygen (Zang et al. 1998) and nitrogen species (Reiter et al. 2001) hydroxyl radicals (Kaneko et al. 2000), hydrogen peroxide (Tan et al.

2000), singlet oxygen, nitric oxide and peroxynitrite anions (Cagnoli et al. 1995, Tan et al. 2002, Aydogan et al. 2006, Schaefer and Hardeland 2009).

Table 1: Studies where the anti-oxidant effect of melatonin is demonstrated *in vitro* and *in vivo*.

Study	[Melatonin]	Effect	References
In vivo	Up to 1mM	↓ iNOS	Leon *et al*., 1998
In vivo	10-60mg/kg BW	↓ NO by ↓iNOS	Crespo *et al*., 1999
In vivo	10 mg X 2	↓ Lipid peroxidation products (MDA & 4-HDA) in septic newborns	Gitto *et al*., 2001
In vivo	10-20 mg/kg	↓ MDA & NO	Taysi *et al*., 2003
In vivo	5 or 10 mg	↓ MDA and ischemia-reperfusion induced markers of tissue damage	Şener *et al*., 2006
in vivo	3 μg/mL water	↓ cadmium-induced lipid peroxidation	Poliandri *et al*., 2006
In vivo	4 mg/kg	↓ eNOS in alcoholic rats	Sönmez *et al*., 2009
In vitro	5 mg/kg	↓ paraquat-induced MDA, 4-HAD & mortality	Melchiorri *et a*l., 1996
In vitro	N/A	↓ lipid peroxidation induced by linoleic acid	Longoni *et a*l., 1998
In vitro	N/A	↓ OH-induced DNA damage	Romero *et al*., 1999
In vitro	100-2000 μM	↓ hydroperoxide induced lipid peroxidation	Zavodnik *et al*., 2006
In vitro	100 μg	↓ *t*-BHP induced toxicity, ROS, and apoptosis	Hibaoui *et al*., 2009

Abbreviations: MDA, malondialdahyde; 4-HAD, 4-hydroxyalkenals; NO, nitric oxide; iNOS, nitric oxide synthase; OH, hydroxide; *t*-BHP, Tert-butylhydroperoxide.

Melatonin is capable of functionally supporting several antioxidant enzymes, as well as inducing the activity of enzymes responsible for stimulating the production of the antioxidant, glutathione (Korkmaz et al. 2008). Moreover, melatonin has been shown to be more effective and potent than vitamin E, beta-carotene or vitamin C as antioxidants (Gultekin et al. 2001, Montilla et al. 2001, and Lamont et al. 2015). What further sets melatonin apart from other antioxidants is that a single melatonin molecule is capable of scavenging up to 4x or more reactive species than other antioxidants such as vitamin C and E (Tan et al. 2002). Furthermore, all intermediates stemming

from its interaction with reactive species are also free radical scavengers absent of pro-oxidative activity, a property which other antioxidants do not possess (Poeggeler et al. 2002, Tan et al. 2002). The downstream benefits of these antioxidant properties are illustrated below (see Figure 2.8).
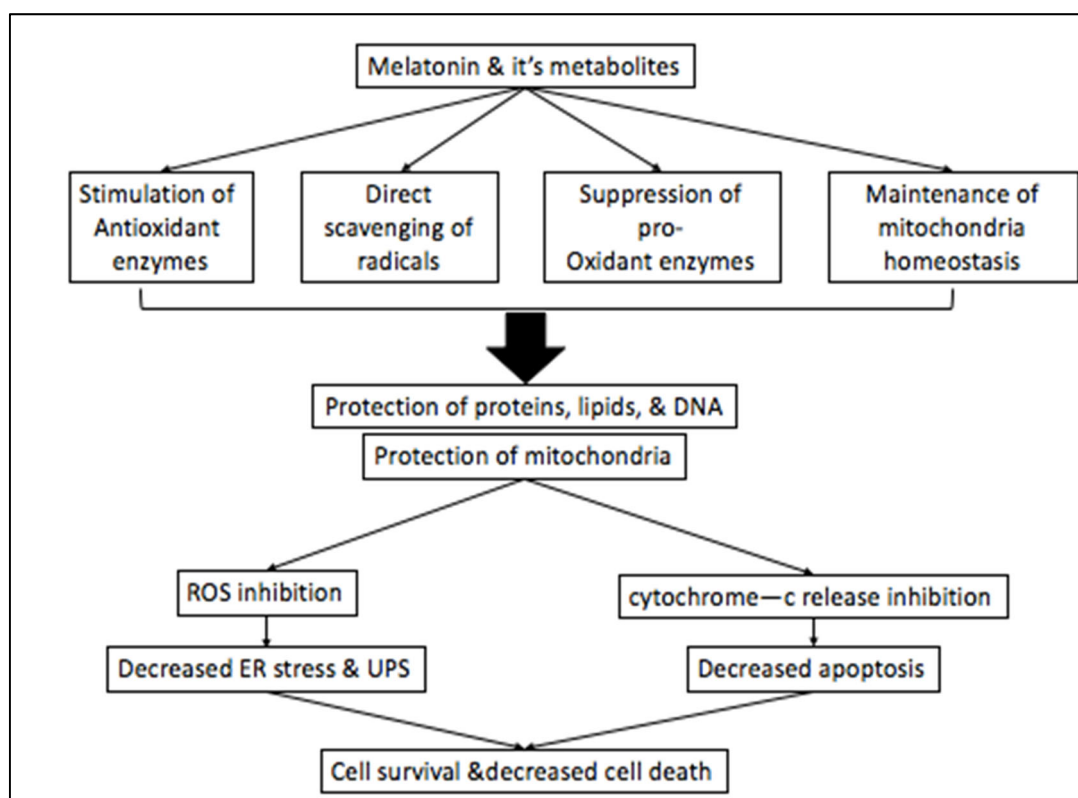
Figure 2.8: The antioxidant cascade of melatonin and mechanisms of protection against oxidative damage. In this review, melatonin is considered to independently protect the cell as well as via three further mechanisms which may be involved in maintaining mitochondrial homeostasis. Adapted from Zhang and Zhang (2014).

2.7.5 Circulatory effects of melatonin (Anaemia and Leukocytosis)

It has previously been demonstrated that the pineal gland through melatonin controls haematopoiesis of both red and white blood cells (Maestroni 1998, Lissoni et al. 2003), confirming the hormone's immunoregulatory role (Maestroni 1999). This was clearly demonstrated in a clinical trial where twenty metastatic lung cancer patients were treated with the chemotherapeutic drugs, cisplatin (a treatment notorious for its anaemia inducing properties) and etoposide (Lissoni et al. 2003). Patients received

either chemotherapy alone or a combination of chemotherapy and 5-methoxytryptamine (5-MTT is a metabolite of melatonin). It was demonstrated in this study that 5-MTT administration reduced cisplatin-induced anaemia and significantly increased haemoglobin levels when compared to the group which only received chemotherapy (Lissoni et al. 2003). With the aid of an electrochemical technique, adsorptive stripping voltammetry, it was found that similar to doxorubicin, melatonin also has a binding affinity for iron (Limson et al. 1998, Maharaj et al. 2003). This evidence suggests a further role for melatonin in reducing free radical generation. It was also discovered that melatonin was actively taken up into oxidatively stressed human red blood cells which protected them against haemolysis and attenuated modifications to haemoglobin and membrane proteins (Tesoriere et al. 1999). Lissoni et al. (1996) investigated the effects of melatonin treatment in patients who suffered from thrombocytopenia due to a variety of causes. An oral dosage of 20 mg/day of melatonin normalised platelet number in 57% of patients and significantly increased average platelet number. The same group later observed that melatonin significantly improved thrombocytopenia and leukopenia in patients with untreatable advanced solid tumours receiving supportive care (Lissoni 2002).

Espino et al. (2013) treated human leukocytes with TNF-α and cycloheximide (caspase-8 promoter) which induced an increase in apoptotic cell death of leukocytes; pre-treatment with melatonin reduced leukocyte apoptotic cell death. The authors showed that melatonin binding to its receptors induced an ERK-dependent signalling cascade which increased levels of the anti-apoptotic protein, cFLIP (Espino et al. 2013).
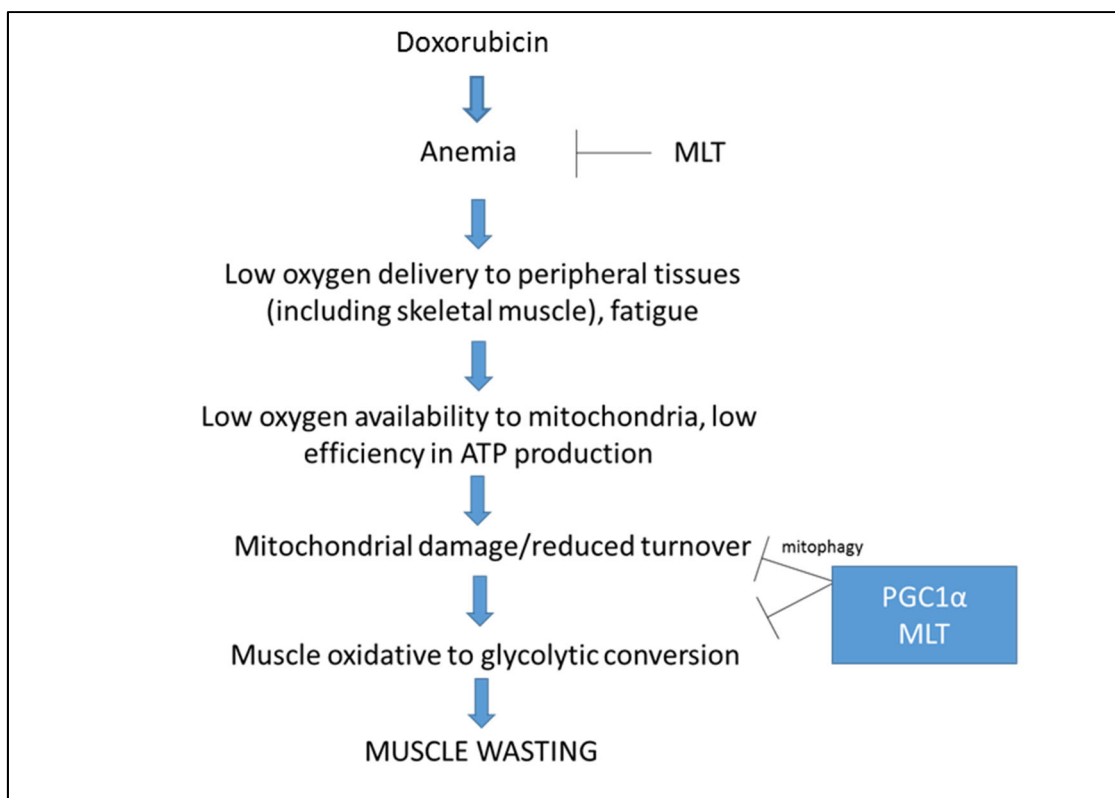
Figure 2.9: Proposed mechanism of action of melatonin in counteracting tumour/doxorubicin-induced muscle alterations. Diagramme modified from Pin et al. (2015).

2.7.6 Melatonin and skeletal muscle atrophy

It is suggested from the evidence in several *in vitro* studies that melatonin should be able to attenuate skeletal muscle atrophy (Park et al. 2013, Favero et al. 2015). A recent study by Salucci et al. (2017) provided evidence that melatonin was able to modulate cell death in C2C12 myotubes where pre-treatment of myotubes with melatonin, followed by known apoptotic and oxidative stress inducing compounds such as, H_2O_2, etoposide and staurosporine, reduced membrane blebbing, chromatin condensation, myonuclei loss and in situ DNA cleavage. Furthermore, melatonin also prevented mitochondrial dysfunction.

A literature search revealed only three *in vivo* studies where the effects of melatonin treatment in the prevention of skeletal muscle atrophy were investigated. In the first study, the effect of melatonin treatment (6 mg/kg) on *soleus* muscle fibre diameter and

ultrastructure in a rat castration model was investigated (Oner et al. 2008); melatonin treatment was found to increase skeletal muscle fibre diameter and also to preserve the muscle ultrastructure of castrated rats. It was concluded that melatonin treatment was as effective as testosterone treatment in the prevention of skeletal muscle atrophy through the IGF-1 axis. A more recent study conducted on rats treated with melatonin (10 mg/kg) using a contusion spinal cord injury model, demonstrated a decrease in m-RNA expression of atrophy markers, MAFbx and MuRF-1, as well as the down regulation of expression of autophagy proteins LC3-II and Beclin-1 with melatonin treatment (Park et al. 2012). Furthermore, Lee et al. (2012) demonstrated that long term prophylactic treatment of melatonin (8 weeks) was able to attenuate *gastrocnemius* and *soleus* muscle loss in stroke-induced muscle atrophy in rats. Atrophy related genes, MuRF1 and MAFbx, were significantly down-regulated in the *gastrocnemius* muscle whereas only MAFbx mRNA levels were attenuated in the *soleus* muscle, while insulin-like growth factor-1 receptor (IGF-1R) was found to be significantly over-expressed in both *gastrocnemius* and *soleus* muscle of rats treated with melatonin.

IGF-1 is a mitogenic factor which regulates muscle satellite cell proliferation and differentiation as well as myofibril growth and regeneration. It has also previously been proposed that IGF-1 can regulate the central clock in the suprachiasmatic nucleus (Zheng and Sehgal 2010). It was observed that daily rhythmic control of IGF-1 levels in breast cancer patients differed to that of other growth factors, further supporting this proposition (Haus et al. 2001). An *in vivo* experimental cancer rat model (Costelli et al. 2006) and a doxorubicin-induced nephrotic syndrome model (Feld and Hirschberg 1996) also associated disease with lower circulating levels of IGF-1 than controls. However, treatment with exogenous IGF-1 did not prevent cancer cachexia in an *in*

vivo experimental model (Costelli et al. 2006). It has also previously been shown *in vitro* that melatonin stimulates glucose transport (Ha et al. 2006) and glucose synthesis (Nduhirabandi et al. 2012) by increasing the phosphorylation level of insulin receptor substrate-1 (IRS-1) and hence the phosphoinositide 3-kinase (PI-3-kinase) pathway. The way in which melatonin stimulates the phosphorylation of IRS-1 remains unknown.

2.7.7 Melatonin and its effects on skeletal muscle protein degradation in response to cancer cachexia

Several pro-inflammatory cytokines derived from tumour cells and activated leukocytes including IL-1, IL-6 and TNF-α are cachectic mediators (Reid and Li 2001, Tisdale 2008, Fearon et al. 2012). TNF-α in particular has direct catabolic effects on skeletal muscle as it increases the ubiquitin proteasome pathway (Reid and Li 2001). Results from clinical trials where patients received supportive care or supportive care together with 20 mg/kg melatonin treatment daily for 3 months suggest that melatonin may decrease circulating TNF-α levels in cancer patients with untreatable metastatic solid tumours (Lissoni et al. 1996).
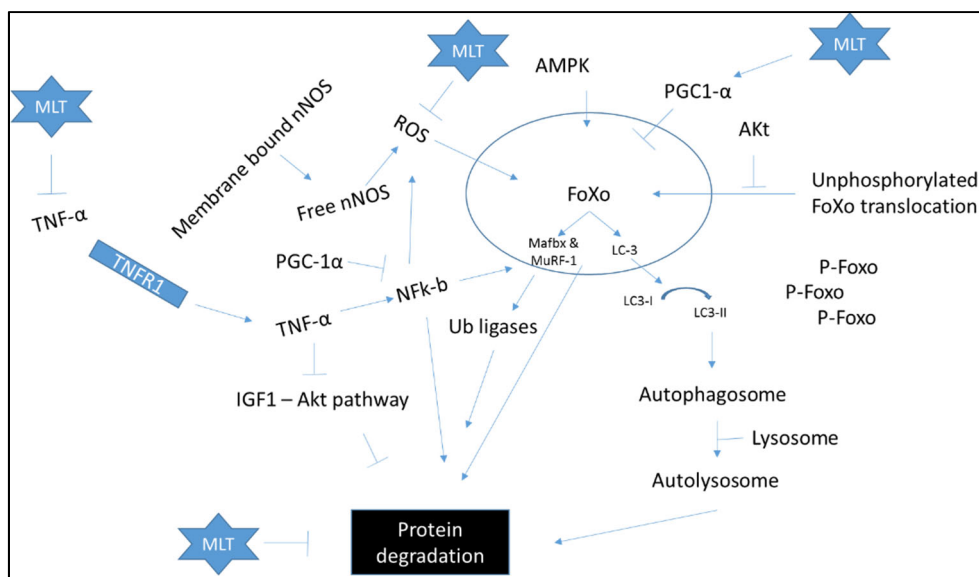
Figure 2.10: The effects of cancer-cachexia induced TNF-α expression on protein degradation as well as possible beneficial actions of melatonin. Adapted from VanderVeen et al. (2017).

In a randomized, non-placebo controlled study where 24 patients with advanced gastrointestinal cancer with a body weight reduction of at least 10% in a period over 6 months were either treated with melatonin (18 mg/day), fish oil (30 mL/day), or a combination of both (Persson et al. 2005), no significant reduction in plasma inflammatory cytokines were observed; however a weight stabilising effect was observed in all 3 groups. A major limitation of this study was the exclusion of a control group, however, these findings nonetheless implied a potential anticachectic role for melatonin in cancer cachexia. In an *in vitro* study conducted by Park et al. (2013) where L6 myotubes were exposed to TNF-α (100 ng/mL), ROS generation was induced and a decreased cell viability was observed. The authors demonstrated that the treatment with TNF-α compromised L6 myotubes, but if administered with melatonin, the addition of melatonin significantly attenuated the TNF-α induced reduction in myotube width and decreased proteolysis, apoptosis and ROS generation (Park et al. 2013). Moreover, in an elegant study by Sacco et al. (1998), melatonin's effect on TNF- production both *in vitro* and *in vivo* was investigated (Sacco et al. 1998).

In vitro results showed that melatonin directly inhibits TNF-α production in human peripheral blood mononuclear cells treated with lipopolysaccharide. Furthermore, *in vivo* results showed that TNF-α production was inhibited in adrenalectomised or hypophysectomised mice in an endotoxic shock model. The authors concluded that the mechanism by which melatonin inhibited the effects of TNF-α is mainly due to its anti-oxidant activity and that it does not act via the hypothalamic–pituitary–adrenal axis (Sacco et al. 1998).

2.7.8 Melatonin and its effects on the mitochondria in cancer cachexia

Cancer cachectic patients often complain of fatigue. It is well known that cytokine regulation and oxidative metabolism are disrupted in these patients, which may well contribute to reduced metabolic function and muscle mass loss (Suzuki et al. 2013, Carson et al. 2016). It has previously been shown that ATP infusion significantly inhibited loss of body weight, fat mass and fat-free mass in patients with advanced lung cancer (Agteresch et al. 2002). The inability to efficiently produce ATP further highlights the dysfunctional muscle oxidative metabolism cancer cachectic patients experience (Agteresch et al. 2000, McLean et al. 2014), which may involve mitochondrial dynamics, mitophagy, and biogenesis (see Figure 2.11) (VanderVeen et al. 2017). Melatonin has previously been shown to regulate ATP production through complexes I and IV of the electron transport chain by increasing oxidative phosphorylation enzymes in different tissue types (Martin et al. 2002, Paradies et al. 2015). A recent publication by Ozkok et al. (2016) demonstrated that melatonin treatment prevents muscle damage by increasing ATP levels in a rodent model of endotoxemic shock.

Chen et al. (2010) observed fragmented in skeletal muscle mitochondria and accumulated mitochondrial DNA mutations in a mitofusin (Mfn1&2) double knockout

mouse model. Mitofusin is present in both mitochondria and sarcoplasmic reticulum and is required for normal endoplasmic reticulum (Zorzano 2009) and mitochondrial morphology (Zorzano 2009, Ainbinder et al. 2015). Ainbinder et al. (2015) investigated the role Mfn2 plays in sarcoplasmic reticulum-mitochondrial crosstalk in a Mfn2 mouse knockdown model during repetitive high frequency tetanus stimulation. The investigators observed that mitochondrial calcium ions were significantly reduced in *flexor digitorum brevis* muscles. An increase in calcium ion transients in the contractile portion of muscle tissue, as well as reduced mitochondrial membrane potential were further noticed. In a recent study, Xi et al. (2016) demonstrated that Mfn2 is downregulated in *rectus abdominus* biopsies of cancer patients with cachexia, whereafter these authors then further explored Mfn2's role both *in vitro* and *in vivo*. The *in vitro* experiments revealed that Mfn2 is downregulated with TNF-α induced atrophy in myotubes. *In vivo* experiments demonstrated that Mfn2 expression was decreased in *gastrocnemius* of mice in a cancer cachexia-induced atrophy model and that the overexpression of Mfn2 partially attenuated this muscle loss.

Melatonin preserved mitochondrial morphology and enhanced Mfn2 expression in renal proximal convoluted tubules of mice in an obesity model (Stacchiotti et al. 2014). Furthermore, melatonin was also able to enhance Mfn2, DRP-1 and PGC-1α expression in rat liver exposed to carbon tetrachloride poisoning (Kang et al. 2016). These mitochondrial effects may have been mediated via AMPK, since AMPK expression levels were increased with melatonin treatment and reduced in animals exposed to carbon tetrachloride alone.

Accelerated mitochondrial fission is associated with mitochondrial dysfunction in cancer cachexia (Youle and van der Bliek 2012). However, it was found that accelerated fission also occurs in healthy muscle during exercise which stimulates

mitochondrial biogenesis, possibly via AMPK activation (Romanello et al. 2010). These findings may indicate that upregulation of fission is necessary and a positive process for mitochondrial adaptation and remodelling in exercised muscle whereas fission in cachexia may be even more accelerated and hence has detrimental effects. Indeed, the overexpression of the fission protein, hFIS1, in muscle has been demonstrated to induce apoptosis, to increase ROS and atrophy *in vivo* (Romanello et al. 2010). A recent *in vitro* study by Parameyong et al. (2015), demonstrated that melatonin inhibited increases in mitochondrial hFis1 levels in methamphetamine-induced toxicity of neuronal cells.

The role of mitophagy in cancer cachexia is still unclear, and it is not known if this process is crucial for the maintenance of an efficient mitochondrial network through the removal of damaged and redundant mitochondria. Evidence suggests that mitophagy is impaired (accumulation of damaged mitochondria) in cancer cachectic patients, since a reduction in PTEN-induced putative kinase 1 (PINK1) (marker of mitophagy) expression was observed in these individuals together with a trend toward Bnip3 (mitophagy regulator) upregulation (Aversa et al. 2016). Marzetti et al. (2017), recently showed that mitophagy regulators, PINK1 and Parkin, were both decreased in muscle samples from aged gastric cancer patients presenting with cachexia.
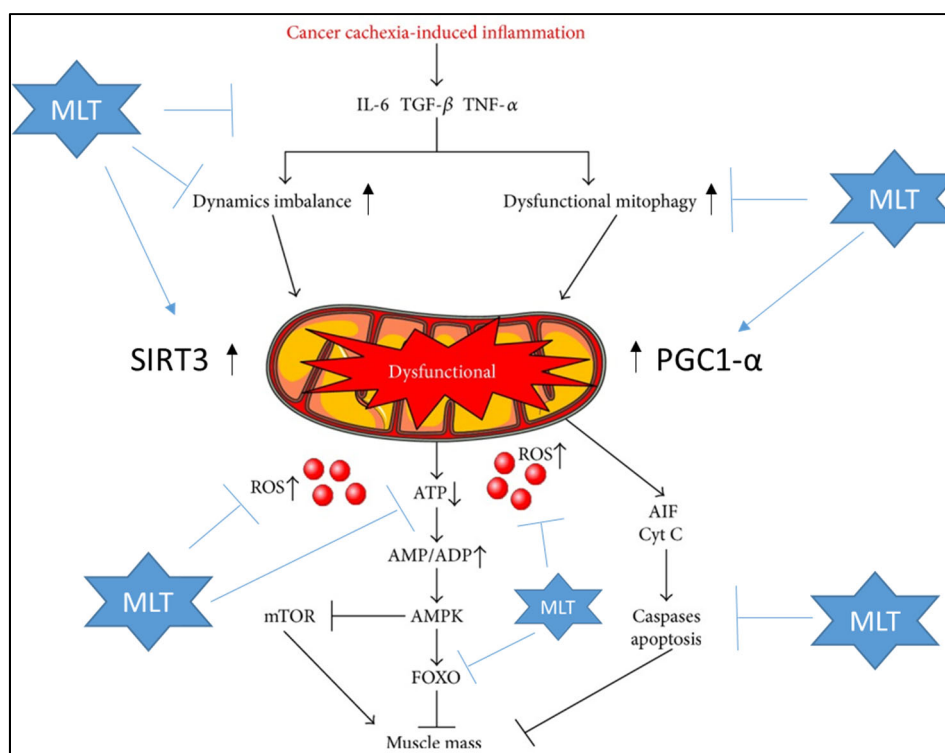
Figure 2.11: The effects of cancer-cachexia induced inflammation on mitochondrial dysfunction in context of protein degradation and possible beneficial actions of melatonin. Adapted from VanderVeen et al. (2017).

In addition to the effects on mitophagy and mitofission, it has been demonstrated in a variety of models that melatonin improves mitochondrial function (Vassilopoulos and Papazafiri 2005, Petrosillo et al. 2009, Agil et al. 2015), for example in diabetic obese rats (Agil et al) in cardiac ischemia-reperfusion injury (Petrosillo et al 2009). In a recent *in vivo* study melatonin was found to enhance mitochondrial biogenesis by increasing expression of PGC-1α (Kang et al. 2015). Moreover, melatonin also increased mitochondrial biogenesis in cadmium-induced hepatotoxicity *in vitro* (Guo et al. 2014). This effect was found to be mediated via the MT1 receptor through the SIRT1-dependent PGC-1α pathway. SIRT1 activates PGC-1α by deacetylation of lysine residues resulting in increased mitochondrial biogenesis (Nemoto et al. 2005, Rodgers et al. 2005 and Rasbach and Schnellmann 2008).

2.7.9 Melatonin and its effects on skeletal muscle protein degradation: Doxorubicin-induced toxicity

Doxorubicin is a very effective chemotherapeutic agent, but myotoxicity limits the clinical use of this drug (Sin et al. 2016). The mechanisms of doxorubicin-induced myotoxicity is not yet fully understood, however, reactive oxygen species generation and long term impairment of mitochondrial respiration appear to be the major contributors (Sin et al. 2016). Lissoni, et al. (1999), demonstrated that melatonin improved one year survival rates in advanced cancer patients receiving chemotherapy. Studies where the effects of melatonin on reducing the toxic effects of doxorubicin on striated muscle have been investigated are, however, limited to cardiac muscle. Treatment of mice with doxorubicin induced cardiotoxicity using melatonin, improved survival rates and significantly improved cardiac function, ultrastructural alterations, and inhibited apoptosis (Liu et al. 2002). Ahmed et al. (2005) showed that melatonin was cardioprotective and reduced ROS markers and improved the antioxidative capacity in rat heart tissue. An excellent recently published review by Govender et al. (2017) describes potential mechanisms by which melatonin may confer mitochondrial protection against doxorubicin-induced cardiotoxicity.

The majority of evidence points to the argument that melatonin could potentially attenuate skeletal muscle degradation during cancer cachexia and doxorubicin treatment; primarily through its antioxidant capacity, thereby providing a possible therapeutic mechanism for improving clinical outcome in cancer patients (Lissoni et al., 1996, 1999; Srinivasan et al., 2008). However, the role of melatonin on mitophagy in the context of doxorubicin-induced skeletal muscle atrophy remains largely unclear.
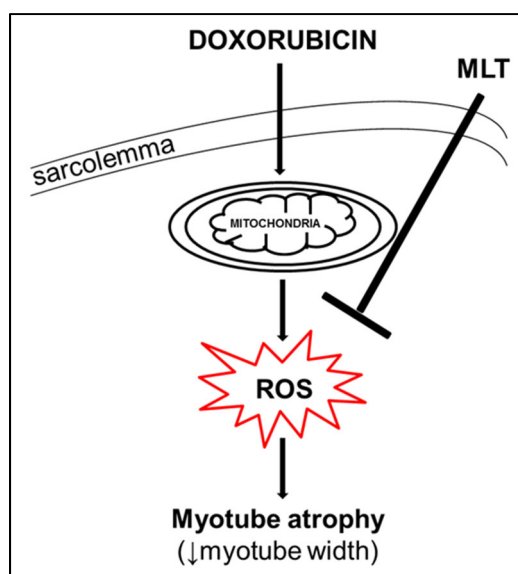
Figure 2.12: Summarized mechanism of DOX-induced myotube atrophy and MLT intervention. ROS, reactive oxygen species and MLT, melatonin. Image modified from Gilliam et al. (2015).

2.7.10 Melatonin and Satellite Cells

Evidence in literature suggests that melatonin may enhance muscle function by enhancing muscle regeneration, directly and indirectly. Melatonin treatment was found to increase skeletal muscle fibre diameter and preserve the muscle ultrastructure of castrated rats (Oner et al. 2008). In another study, insulin-like growth factor-1 receptor (IGF-1R) was significantly over-expressed in melatonin-administered rats in a model of stroke induced muscle atrophy, complementing the previous findings (Lee et al. 2012).

As mentioned above, melatonin affects mitochondrial biogenesis via PGC-1α. Interestingly, in a recent study, it was shown that PGC-1α modulates satellite cell number and proliferation by remodelling the stem cell niche (Dinulovic et al. 2016). PGC-1α modified the extracellular matrix composition, including the levels of fibronectin (Dinulovic et al. 2016). Furthermore, SIRT-1 was shown to act as an epigenetic regulator connecting changes in satellite cell metabolism towards myogenic

commitment (Ryall et al. 2015). Melatonin could thus potentially regulate satellite cell number indirectly via SIRT1 and PGC-1α.

2.8 Hypothesis revisited

We hypothesise, that melatonin treatment will prevent doxorubicin-induced skeletal muscle atrophy in a rodent tumour-bearing model.

This hypothesis will be tested using the following models: *in vivo* rat tumour-bearing model of DOX-induced skeletal muscle atrophy and an *in vitro* C2C12 differentiated myotube model of DOX-induced myotoxicity.

The muscle protective effect of melatonin is likely to occur via multiple mechanisms simultaneously, including the attenuation of muscle degradative pathways, the stabilization of mitochondrial function and subsequent reduction of ROS levels, enhancement of oxygen and nutrient delivery to muscle and by improving muscle regeneration during DXR-induced myotoxicity.

Objective:

- To assess MLT as a low risk adjuvant therapeutic intervention which can be used in conjunction with doxorubicin to treat cancer patients.

Chapter 3: Materials & Methods

3.1 *In vivo* Study design – Part 1

3.1.1 Culture of LA7 rat mammary tumour cell line

Experiments were performed using the rat mammary adenocarcinoma cell line LA7 (ATCC CRL-2283, Renato Dulbecco, the Salke Institute, San Diego, CA). This cell line was a kind gift by Dr A. Krygsman (Stellenbosch University, Stellenbosch, Western Cape, RSA). During routine maintenance, cells were grown as monolayers in Dulbecco's Modified Eagles Medium (DMEM, Gibco. Ltd) supplemented with 10% foetal bovine serum (FBS, Sigma Chemical Co., St Louis, MO, USA) and 1% penicillin/streptomycin (P/S) (Sigma-Aldrich, Johannesburg, South Africa) at 37°C in a humidified atmosphere of 95% air plus 5% CO_2 in T75 flasks (75 cm^2 flasks, Nest Scientific, USA) until they reached 80% confluency. LA7 cells were then subcultured. Subculturing was accomplished by washing the cell monolayer with warm phosphate buffered saline (PBS) followed by incubation with 4 ml trypsin/EDTA (Sigma Chemical Co., St Louis, MO, USA) at 37°C, until cells detached completely or for a maximum of four minutes. Experiments were performed using exponentially growing cells.

3.1.2 Animals and interventions

3.1.2.1 Tumour-bearing rat model

Sixty, sixteen week old adult female Sprague-Dawley rats (housed in the laboratory animal facility at the Department of Physiological Sciences, Stellenbosch University) weighing approximately 270 g were used for this study. All animals had access to food (standard rat chow, supplied by the Medical Research Council animal unit, Parow) and tap water *ad libitum*. Rats were exposed to a 12 hr light/dark cycle (lights on at 06h00). Ambient temperature was controlled at 22°C, and rooms ventilated at a rate of 10

changes/hour. Prior to the initiation of intervention protocols, rats were accustomed to the researcher by frequent handling and weighing before the start of experimental procedures. All experimental protocols were approved by the Animal Research Ethics Committee of Sub-Committee B of Stellenbosch University (Ethical clearance: SU-ACUM13-00015).
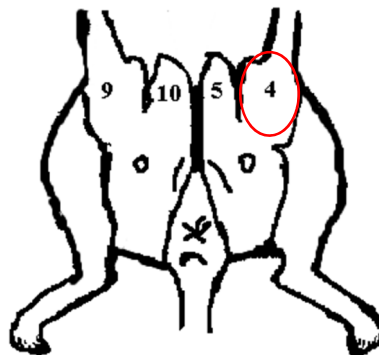
Figure 3.1: Illustration depicting the position of mammary fat pad no. 4. A 300 ul cell suspension (containing LA7 $16x10^6$ cells) was injected in the lower abdomen of rats, in the no. 4 mammary fat pad.

Rats were inoculated subcutaneously on the left pad of the fourth mammary gland with 300 µl of $16x10^6$ LA7 cells suspended in Hanks Balanced Salt Solution (Sigma Chemical Co., St Louis, MO, USA), using a 23-gauge needle. This protocol was modified and developed from a mouse breast cancer tumour model previously established by our research group at Stellenbosch University (Thomas M., PhD thesis, University of Stellenbosch and Govender Y., PhD thesis, Stellenbosch University).
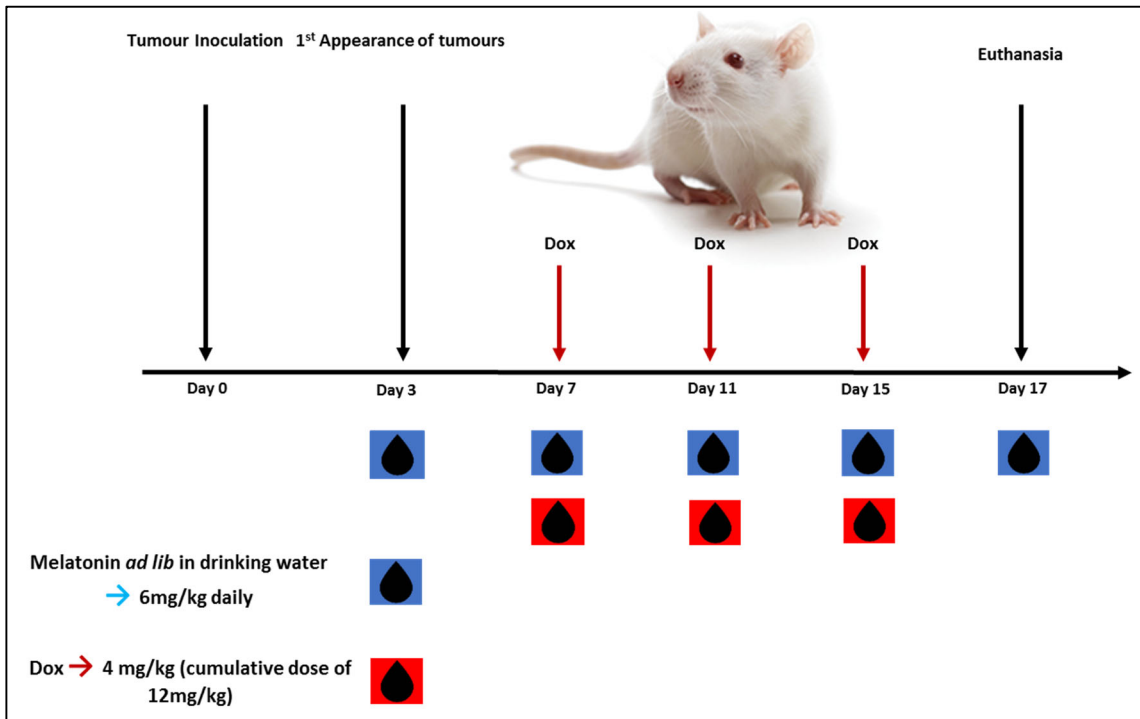
Figure 3.2: Diagrammatic depiction of experimental procedure.

Figure 3.3: Injection site of Sprague-Dawley rat and typical tumour growth two weeks after injection with LA7 cell suspension.

3.1.2.2 Experimental treatment groups

Rats were allocated to 6 groups, namely control group; vehicle control group (Hanks balanced salt solution); tumour control group; melatonin group; doxorubicin group and doxorubicin+melatonin combination group (see Fig. 3.5).
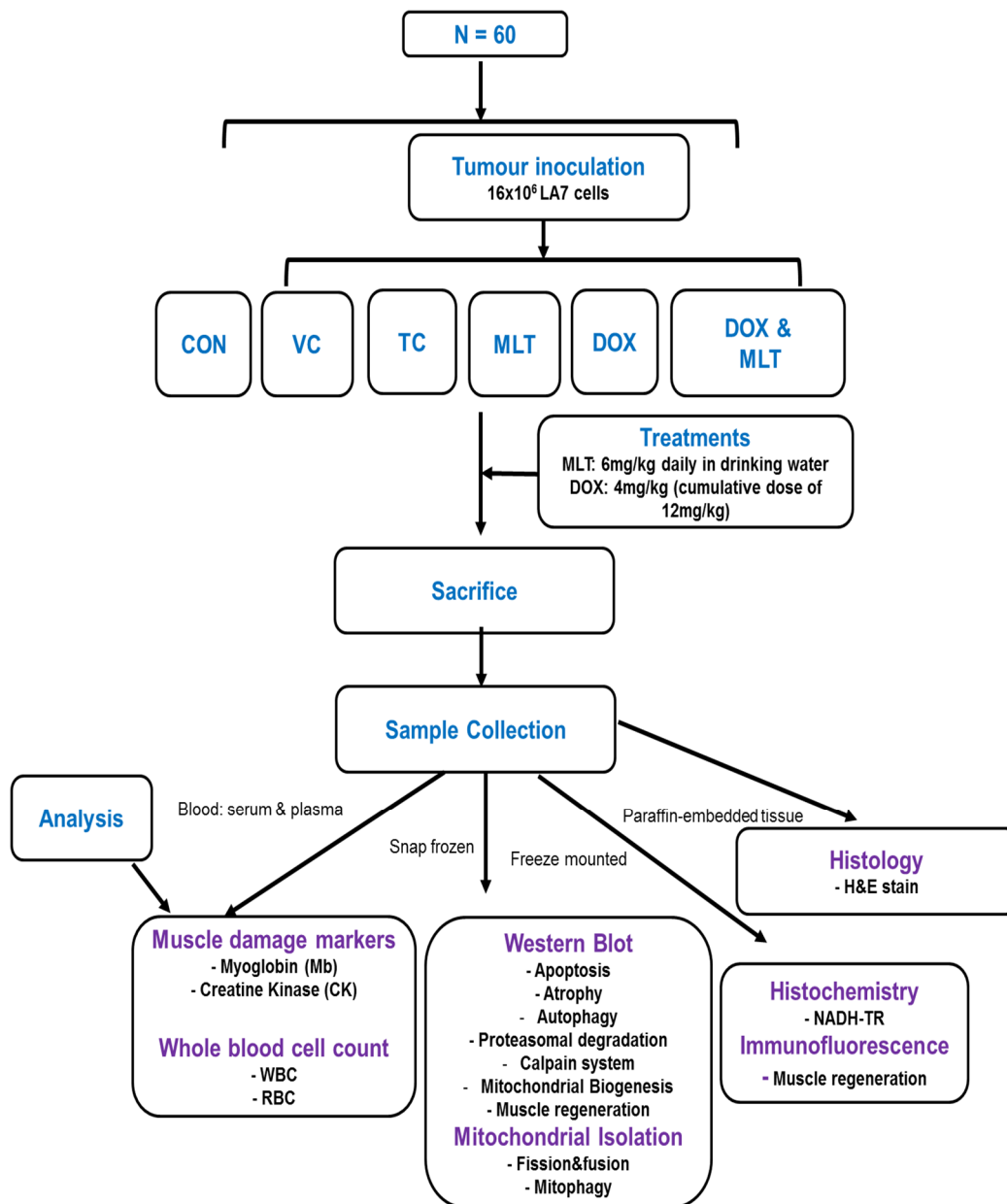
Figure 3.4: Experimental design (Abbr. CON - control, VC - vehicle control, TC - tumour control, MLT - melatonin, DOX - doxorubicin and DOX+MLT – doxorubicin+melatonin)

All groups except the control animals (Con) were inoculated with LA7 cells. The vehicle control (VC) group received three intraperitoneal injections of Hanks balanced salt solution (Sigma Chemical Co., St Louis, MO, USA); the melatonin (MLT) group received a daily 6 mg/kg dosage in drinking water after the initial appearance of tumours; the doxorubicin (DXR) group received 4 mg/kg dose of doxorubicin

intraperitoneally three times over the period of 9 days adding up to a cumulative dosage of 12 mg/kg.

Table 2. Experimental groups and treatments.

Group	Tumour Inoculation (LA7 cells)	Treatment
Control (Con)	X	X
Vehicle Control (VC)	✓	Hanks balanced salt solution
Tumour Control (TCON)	✓	X
Melatonin (MLT)	✓	6 mg/kg MLT daily
Doxorubicin (DXR)	✓	12 mg/kg DXR total
Doxorubicin+Melatonin (DM)	✓	6 mg/kg MLT daily + 12 mg/kg total DXR

3.1.2.2.1 Vehicle administration

Hanks Balanced Salt Solution (Sigma Chemical Co., St Louis, MO, USA) was used as vehicle control and administered three times intraperitoneally over 9 days. Hanks Balanced Salt Solution was first administered after two weeks of tumour growth and twice more with three days separating subsequent injections.

3.1.2.2.2 Melatonin administration & solution preparation

Rats were housed 5 per cage with one bottle of water per cage. Melatonin (Sigma Chemical Co., St Louis, MO, USA) was administered in the drinking water (Lamont et al. 2015) daily (a dosage of 6 mg/kg melatonin) from the day of tumour inoculation and allowed *ad libitum* intake.

Water consumption by the group of animals per cage was monitored and recorded one week prior to the start of the study and the average volume of drinking water

consumed per animal could be calculated. Water consumption was determined at 50 ml/rat/day, MLT was dissolved in 700 ml of drinking water at a final concentration of 30 µg/ml. Water bottles containing MLT were covered with aluminum foil since melatonin is a light sensitive and fresh solutions were prepared daily. The dosage of 6 mg/kg/day was adjusted according to the body weight gain of the animals. Since a small volume of MLT was used in this study, it was completely dissolved in the large volume of drinking water provided.

3.1.2.2.3 Doxorubicin administration

Doxorubicin (Sigma Chemical Co., St Louis, MO, USA) was prepared in Hanks Balanced Salt Solution (Sigma Chemical Co., St Louis, MO, USA). It was administered intraperitoneal three times, three days apart at 4mg/kg for a cumulative dose of 12 mg/kg. Doxorubicin was first administered after two weeks of tumour growth and twice more with three days separating subsequent injections.

3.1.2.3 Euthanasia and sample collection

3.1.2.3.1 Sacrifice

During the sacrifice procedures, all animals received an overdose of pentobarbitone sodium (200 mg/kg i.p.) injection and were exsanguinated.

3.1.2.3.2 Sample collection

3.1.2.3.2.1 Blood collection

Following euthanasia by pentobarbitone overdose, the thorax cavity was opened and the heart exposed. Whole blood samples were obtained from blood in the chest cavity after the heart was excised. Blood was collected using a 5 ml syringe and immediately transferred to EDTA-K$^+$ and SST tubes (Vacutainer, Beckton Dickinson). Blood

samples were immediately inverted and placed on ice and analysed within 4 hrs following collection.

3.1.2.3.2.2 Muscle collection

Following blood collection, the *gastrocnemius* muscle of the right (non-tumour side) hind leg was exposed by cutting and removing the skin and connective tissue surrounding the muscle. The *gastrocnemius* muscle was harvested and cut in cross section along the muscle belly; one half was processed for immunofluorescence, and the other snap-frozen for biochemical analysis. Both the *plantaris* and *soleus* muscles was fixed and processed for histological analysis.

3.1.3 Sample Analysis

The analytical profile included the following parameters and procedures: a) serum myoglobin (Mb) concentration, b) protein concentration, c) plasma and muscle oxygen radical absorbance capacity (ORAC), d) qualitative histological analysis of skeletal muscle, e) immunohistochemical staining, f) immunofluorescent staining for visualisation of muscle sections for SCs measuring identified by staining for Pax7 along with appropriate co-stains (see below for detail), g) qualitative histological analysis of muscle, h) mitochondria isolation and i) Western blot analysis.

3.1.3.1 Myoglobin (Mb) concentration

Serum myoglobin a marker for the presence of muscle damage, was independently determined by PathCare pathology laboratory (Medi Clinic, Stellenbosch, Western Cape, RSA) using an automated enzymatic method. The normal range for rat Mb concentration is 10 – 95 ng/ml at 37°C.

3.1.3.2 Whole blood cell differential counts

Whole blood cell counts were performed using a CELL-DYN 3700 CS Haematology Analyser (Abbott Diagnostics, Fullerton, CA) for blood collected in EDTA-K$^+$ tubes (Vacutainer, Beckton Dickinson).

3.1.3.3 Haemotoxylin and Eosin (H&E)

H&E stained sections were used to qualitatively assess the muscle structure. Using a rotary microtome (Leica Microsystems CM1850, Nussloch, Germany) 5 μm muscle cross-sections were cut and then stained with H&E. Haematoxylin stains the basophilic structures blue-purple and alcohol-based eosin stains the eosinophilic structures bright pink. The basophilic structures include nucleic acids, such as in ribosomes and cell nuclei, and cytoplasmic regions rich in RNA.

3.1.3.4 Histochemistry and immunofluorescence

All histopathological and immunohistochemical evaluations were conducted on at least four rats per time point per treatment group.

3.1.3.4.1 Histochemistry - Nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR)

Tissue frozen in isopentane was sectioned at -23°C, 8 μm muscle cross-sections were prepared for NADH-TR staining to distinguish between muscle fibre types I and II. Via enzymatic activity NADH is reduced to tetrazolium thus forming a formazan precipitate which is deposited at sites of mitochondria in the sarcoplasmic network. Type I fibres stain darker than type II fibres.

3.1.3.4.2 Immunofluorescence

Cross-sections were labelled with: (a) Pax7 for labelling SCs, (b) laminin for labelling muscle membranes and (c) Hoechst for labelling nuclei. PBS controls were also used in this part of the study and the antibodies used were specific for rat samples. For appropriate dilutions and suppliers of antibodies used, see Table 2.

Cross sections of the muscle tissue were cut at 8 μm using a cryostat microtome (Leica CM1850 UV, Leica Microsystem Nussloch GmbH, Germany) at -23ºC and mounted on slides. The following day, the sections were brought to room temperature, and rinsed in 0.01 M phosphate buffered saline containing 0.25% Triton X-100 (15 min) and washed with PBS (3 X 5 min). The sections were incubated with Pax7 (1:50; mouse monoclonal antibody, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for 1 hour, after which the sections were washed with PBS (3 X 5 min), incubated for 1 h at RT with Alexa fluor 488 conjugated secondary antibody (1:250, goat anti-mouse, Invitrogen, Eugene, Oregan, USA), washed again with PBS (3 X 5 min), incubated for 1 h at RT with laminin (1:200, rabbit polyclonal, Z 0097, Dako Diagnostech), washed again with PBS (3 X 5 min) and incubated for 1 h at RT with Alexa fluor 594 conjugated secondary antibody (1:250, goat anti-rabbit, Invitrogen, Eugene). The sections were then washed in PBS (3 X 5 min), and mounted with the fluorescent mounting medium (Dako, Denmark). Samples were observed with a direct fluorescence microscope (Leica DM 5000 CTR) using x40 objective. Pax7 positive cells were counted in the midbelly region of the whole *gastrocnemius* muscle.

Table 3. List of primary antibodies and secondary fluorescent antibodies used for immunofluorescence methods.

Primary Antibodies (Markers)	Dilution	Catalogue number and supplier
Pax7	1/50	Pax7, Developmental Studies Hybridoma Bank
anti- Laminin	1/200	Z 0097, Dako Diagnostech
Fluorescent labelled Secondary antibodies:		
Alexa Fluor 488 goat anti-mouse	2 mg/ml 1/250	A11029, Invitrogen
Alexa Fluor 594 goat anti rabbit	2 mg/ml 1/250	A11012, Invitrogen

3.1.3.5 Protein concentration determination

To determine muscle total protein concentrations, either for preparing samples for the measurement of muscle oxygen radical absorbance capacity (ORAC), western blot analysis or to isolate mitochondria, the Direct Detect Infrared Spectrometer (Merck Millipore, headquarters Darmstadt, Germany) was used (see section 3.1.7.2).

3.1.3.6 Antioxidant Capacity - muscle oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed on homogenised muscle tissue as described by Huang et al. (2002). This assay is based on the measurement of oxygen radical-induced quenching of a fluorescent probe, through the change in the fluorescence intensity of the probe in solution over time. Thus, the oxygen radical quenching

capacity of the probe in the sample solution can be determined and named ORAC (Meeusen and Lievens 1986; Safran et al., 1989; Noonan et al., 1993; Olson and Ley 2002). The fluorescence probe, fluorescein (F6377, Sigma-Aldrich) and the peroxyl radical, AAPH (2,2'azobis(2-amidino-propane) dihydrochloride) (440914, Sigma-Aldrich) were used to determine the ORAC value. Fluorescence decay is represented as the area under the curve (AUC), which was used to quantify total antioxidant capacity. The results were then compared to the antioxidant Trolox^{TM} (238813, Sigma-Aldrich) standard curve and expressed as µmol Trolox^{TM} equivalents (TE) per L of sample.

3.1.3.7 Mitochondrial Isolation

Mitochondria were isolated from muscle *gastrocnemius* tissue using a Mitochondria isolation kit (ab110169, Abcam). Tissue homogenates were prepared with a Dounce homogenizer as indicated in the Abcam kit protocol (ab110169) and protein concentration determined as described in section 3.2.2.6. Briefly, 0.2 - 0.4 g of muscle tissue was washed, minced and suspended in isolation buffer. Cells were disrupted by homogenizing the tissue with 40 dounce strokes after which the homogenate was centrifuged at 1000 g for 10 min at 4°C. The supernatant was collected and further spun down at 12000 g for 15 min and the pellet was collected, this step was then repeated. The pellet was re-suspended in RIPA buffer, aliquoted and frozen at -80°C for further analyses.

3.1.3.8 Western Bot Analysis

a) Western blot analysis for markers of mitophagy, mitochondrial fission and fusion proteins from isolated mitochondria was performed.

b) Western blot analysis for markers of Apoptosis, Atrophy, UPP, Autophagy and Ca^{2+}-dependent cysteine proteases, mitochondrial biogenesis and markers of muscle regeneration was performed.

Protein levels were determined by standard western blot techniques. Briefly, muscle homogenates of each sample was prepared, either snap frozen or cryo-sectioned tissue in RIPA buffer. Samples were then separated on 4 - 20% precast gradient gels and were immunoblotted for apoptotic markers - PARP and Caspase-3, atrophy markers – Murf-1 and FoxO3a, autophagy markers – p62, LC3-II and mTOR, Ca^{2+}-dependent cysteine proteases – Calpain-3. Total protein was used to determine equal loading. For a full-detailed description of all the reagents used, as well as the protocol and method see appendix I.

3.1.3.8.1 Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Electrotransfer and Immuno-detection

The methodology is described in sections 3.1.7.3. Protein expression was normalized to the corresponding total protein per sample. Proteins of interest were detected using the manufacturers' recommended dilutions and the list of primary and secondary antibodies are listed in the table below (Table 5.).

Table 4. Primary and enzyme-linked secondary antibodies used for western blot analysis.

Mitochondrial Isolation		
Primary Antibodies	**Dilution**	**Catalogue number & supplier**
Mitochondrial fission & fusion		
Mitofusin-1	1:1000	Ab57602, Abcam
Mitofusin-2	1:1000	Ab57889, Abcam
OPA-1	1:1000	Ab157457, Abcam
DRP-1	1:1000	Ab56788, Abcam
hFis1	1:1000	Ab71498, Abcam
Mitophagy		
PINK1	1:1000	6946 , Cell Signalling
PARKIN	1:1000	2132, Cell Signalling
Crude Extract		
Primary Antibodies	**Dilution**	**Catalogue number and supplier**
Markers of muscle regeneration		

Pax7	1/1000	Ab110111, Abcam
MyoD	1:1000	SAB1410813, Sigma-Aldrich
Myogenin	1:1000	SAB1305721, Sigma-Aldrich
Apoptosis Markers		
c-Caspase3	1:1000	9664, Cell Signalling
cPARP	1:1000	9541, Cell Signalling
Atrophy Markers		
FoxO3a	1:1000	9467, Cell Signalling
MURF-1	1:1000	IMX-3924, IMGENEX
MAFbx	1:1000	Sc-33782, Santa Cruz Biotechnology
Autophagy Markers		
LC3-IIB	1:1000	3868, Cell Signalling
p62	1:1000	8025, Cell Signalling
pmTOR (Ser2448)	1:1000	ab84400, Abcam
UPP		

K48	1:1000	8081, Cell Signalling
K63	1:1000	sc-8017, Santa-Cruz Biotechnology
Calpains		
Calpain-3	1:1000	NCL-CALP-12A2, Novocastra
Mitochondrial Biogenesis		
PGC-1α	1:1000	Ab54481, Abcam
Sirtuins		
SIRT-1	1:500	9475S, Cell Signalling
SIRT-3	1.500	sc-99146, Santa-Cruz Biotechnology
Enzyme-linked Secondary antibodies:		
Anti-mouse	1:10 000	7076P2, Cell Signalling
Anti-rabbit	1:15 000	7074S, Cell Signalling

3.1.4 Image Acquisition and Analysis

Brightfield images were acquired with a Nikon ECLIPSE E400, equipped with a colour digital camera (Nikon DXM1200) at 20X magnification. Muscle cross sectional area analysis was performed using the ImageJ Java 1.8.0_77 software (National Institute of Health, USA) imaging software.

3.1.5 Statistical Analyses

Results are presented as means ± standard error of the mean (SEM), unless otherwise specified. Differences between groups were analysed using one-way analysis of variance (ANOVA). When significant interactions were found, Bonferroni post hoc tests were performed. All statistical analyses were performed using Graph pad Prism 7 (Graph pad Software, Inc., CA, USA). The accepted level of significance was $p < 0.05$.

3.2 *In vitro* Study design – Part 2

The mouse myoblast cell line, C2C12, was used to perform the experiments. These cells were cultured and differentiated, with 7 treatment groups established i.e. (i) control; (ii) vehicle control; (iii) doxorubicin; (iv) melatonin; (v) melatonin pre-treatment followed by doxorubicin exposure; (vi) luzindole (a selective melatonin receptor antagonist was added together with MLT at a concentration of 50 nM over 48 h (50 nM 24 h pre-treatment + 50 nM 24 h); and (vii) luzindole & melatonin pre-treatment followed by doxorubicin exposure.
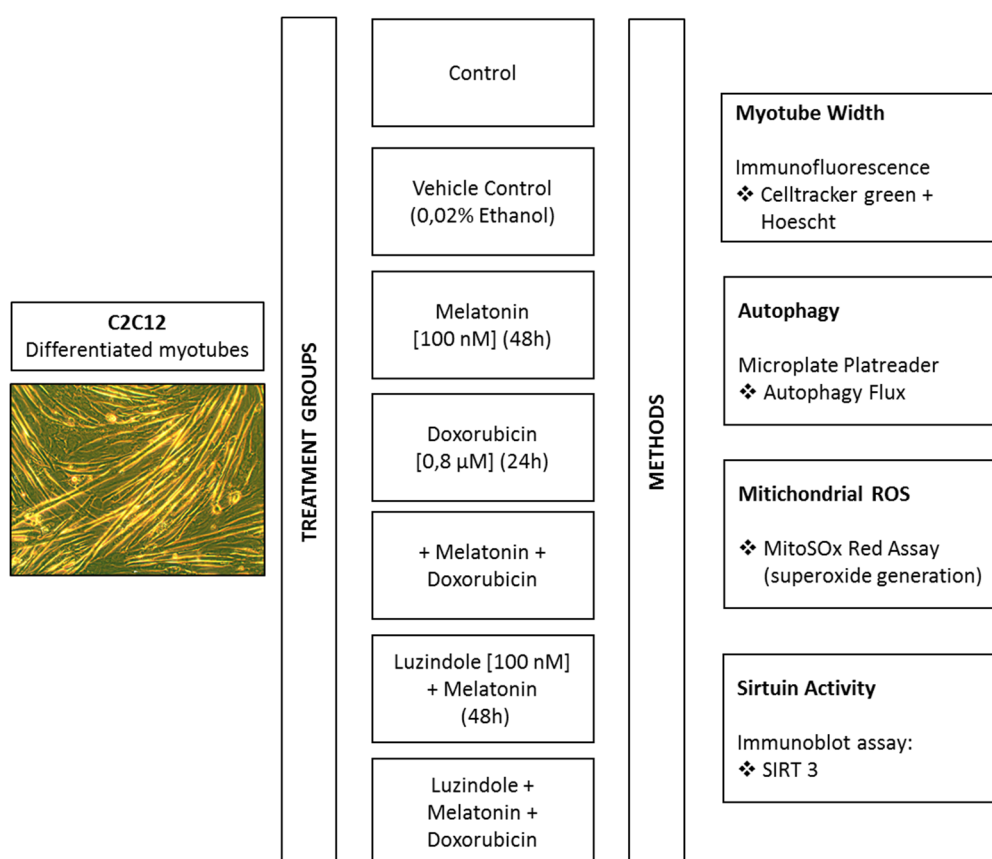
Figure 3.5: Schematic representation of the *in vitro* study design. Differentiated C2C12 myotubes were treated as indicated above, and were used to assess various parameters. A Luzindole only group was omitted as seen in previous studies (Ha et al. 2006, Quan et al. 2015).

3.2.1 Culture of C2C12 mouse cell Line

The mouse myoblast cell line, C2C12 (ATCC CRL-2283, Renato Dulbecco, The Salke Institute, San Diego, CA) was used to perform the experiments. C2C12 myoblasts were grown as monolayers in Dulbecco's Modified Eagles Medium (DMEM, Gibco. Ltd) supplemented with 10% foetal bovine serum (FBS, Sigma Chemical Co., St Louis, MO, USA) and 1 % penicillin/streptomycin (P/S) (Sigma-Aldrich, Johannesburg, South Africa) at 37°C in a humidified atmosphere of 95% air plus 5% CO_2 in T75 flasks (75 cm^2 flasks, Nest Scientific, USA) for 4 days (media change every two days). Upon reaching 80% confluence, cells were serum restricted (2% heat-inactivated horse serum, DMEM) and grown for an additional 3 days. On the fourth day following serum restriction intervention experiments commenced and myotubes were treated with MLT (Sigma Chemical Co., St Louis, MO, USA) and luzindole (Sigma Chemical Co., St Louis, MO, USA). On the fifth day myotubes received doxorubicin (Sigma Chemical Co., St Louis, MO, USA) treatment and on day six myotubes were harvested for biochemical analysis or prepared for staining.

3.2.2 Differentiated myotube cell culture treatments

During treatment of myotubes, differentiation medium was replaced with fresh differentiation medium containing respective treatments after myotubes were washed with a 37°C phosphate buffered saline (PBS) solution. Based on studies in literature (Gilliam et al. 2012), cells were treated with DOX at a concentration of 0.8 µM for 24h which was used in all *in vitro* experiments. A cumulative concentration of 100 nM of MLT for 48h (50 nM for 24 h + 50 nM for 24 h) was used in all *in vitro* experiments (Maldonado et al. 2016).

Myotubes were thus pre-treated with MLT (50 nM) for 24h followed by MLT (50nM) and DOX (0.8 µM) the next 24 hours. Luzindole (50 nM, Sigma Chemical Co., St Louis, MO, USA) a MLT 1 (MT1) and 2 (MT2) receptor antagonist was added to myotubes 1h before MLT treatment. A total of seven treatment groups were used for the *in vitro* study as shown in Fig. 3.1 (study design).

3.2.2.1 Melatonin (MLT) treatment

A 21.5 mM stock solution of MLT (Sigma Chemical Co., St Louis, MO, US) was prepared by dissolving the compound in 50% ethanol. The freshly prepared stock solution was dissolved in warm differentiation media to a working solution of 50 nM, added to myotubes in appropriate flasks which were covered in foil as MLT is light sensitive. Flasks were then incubated for 24h and refreshed with MLT working solution for an additional 24h. Since the MLT was dissolved in ethanol, a vehicle control group was included in all experiments. The vehicle control group was treated with 0.02% ethanol.

3.2.2.2 Doxorubicin (DOX) treatment

A stock solution of DOX (Sigma Chemical Co., St Louis, MO, US) at a concentration of 3.4 mM was prepared by dissolving the compound in differentiation medium. During treatment, DOX was added to fresh warm growth medium to a final concentration of 0.8 µM. Flasks containing DOX treated myotubes were covered in foil since DOX is light sensitive whereafter it was incubated for 24 hours.

3.2.2.3 Luzindole (LUZ) treatment

A stock solution of Luzindole (Sigma Chemical Co., St Louis, MO, US) at a concentration of 21.5 mM was prepared by dissolving the compound in differentiation medium. The freshly prepared stock solution was dissolved in warm differentiation

media to a working solution of 50 nM, added to myotubes in appropriate flasks and covered in foil. Flasks were then incubated for 24h and refreshed with Luzindole working solution for an additional 24h.

3.2.3 Myotube width

To measure myotube width, myotubes were tagged with a fluorescent label CellTracker™ Green CMFDA CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes, C7025) freely traverses cell membranes into cells and is excited at 492 nm and emits fluorescence at 517 nm, where it is transformed into cell membrane-impermeant reaction products. C2C12 cells were cultured, differentiated and treated as previously described (Figure 3.5) in 24 well plates (1.9 cm^2 plates, Nest Scientific, USA). The myotube width measurements is described in section 3.2.8.

3.2.4 Mitochondrial ROS generation

ROS generation was assessed using the MitoSOX Red probe (Invitrogen, Molecular Probes, M7514). MitoSOX Red is a fluorescent probe used to measure superoxide production in the mitochondrial matrix. Due to its cationic properties, MitoSOX Red is able to rapidly target the mitochondria, where it is oxidized by superoxide. Oxidation of MitoSOX Red yields a fluorescent product known as 2- hydroxymitoethidium, which is excited at 510 nm and emits fluorescence at 580 nm.

Following myotube differentiation and treatment, a 50 µg vial of MitoSOX™ Red reagent (Invitrogen, Molecular Probes, M36008) was thawed and allowed to reach room temperature before opening. A 50 µg vial of MitoSOX™ mitochondrial superoxide indicator was dissolved in 13 µL of dimethylsulfoxide (DMSO) to prepare a 5 mM MitoSOX™ reagent stock solution. The stock solution was further dissolved in

differentiation media up to a final working concentration of 5 µM; added to cultured cells and incubated for 10 mins at 37˚C, while protected from light. Next, myotubes were gently washed with warm differentiation medium and fixed in 4% paraformaldehyde for 5 mins. The myotubes were then washed with warm PBS and wells were sealed fluorescent mounting medium (Dako, Denmark). The culture plates were then briefly stored at 4ºC until being imaged.

3.2.5 Autophagy Kit Assays

The CYTO-ID® Autophagy Detection kit (ENZ-51031, Enzo Life Sciences) selectively labels accumulated autophagic vacuoles and monitors autophagic flux in lysosomal inhibited live cells. The probe works as a cationic amphiphilic tracer dye that rapidly partitions into cells enabling labelling of vacuoles associated with the autophagy pathway without accumulating in lysosomes, possible due to careful selection of titratable functional moieties on the dye itself.

C2C12 cells were differentiated in 96 well plates and appropriately treated (Fig. 3.1). Autophagy induction was detected using the Cyto-ID Autophagy detection kit (ENZ-51031, Enzo Life Sciences) as per the manufacturer's instruction. Briefly, treated differentiated myotubes were plated in biological triplicate in 96 well plates (0.32 cm^2 plates, Nest Scientific, USA) and incubated overnight. Next, myotubes were treated with 500 nM rapamycin (positive control) or an equivalent volume of DMSO (negative control) for 18 h, after which 60 mM chloroquine was added to each well to enhance fluorescence signal. Cells were then washed with 1 × assay buffer and incubated with Cyto-ID Green detection reagent and Hoechst 33432. Following incubation, cells were washed with 1 × Assay buffer and mean fluorescence signal acquired on a FLx800™ Microplate Fluorescence Reader (BioTek™, USA).

3.2.6 Immunofluorescence

Differentiated myoblasts were labelled with: (a) CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes, C7025) for myotube width measurement (see section 3.2.3) (b) MitoSOX Red for labelling mitochondrial ROS (see section 3.2.4) and (c) Hoechst 33432 for labelling nuclei.

3.2.7 Western Blot analysis

3.2.7.1 Protein extraction and quantification

Following treatment, myotube supernatant was discarded and placed on ice. Myotube monolayers were then washed 3X in 5 mL of pre-lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM sodium orthovanadate). Next, myotubes were incubated on ice for 10 mins in 1 mL of modified RIPA buffer (pH 7.4, containing: Tris–HCl 2.5 mM, EDTA 1 mM, NaF 50 mM, NaPPi 50 mM, dithiothreitol 1 mM, phenylmethylsulfonyl fluoride 0.1 mM, benzamidine 1 mM, 4 mg/mL SBTI, 10 mg/mL leupeptin, 1% NP40, 0.1% SDS and 0.5% Na deoxycholate) to extract total cell protein. Adherent cells were then harvested from culture dishes by scraping. The resulting lysates were then sonicated to disrupt the cell membranes to release protein contents before being centrifuged at 4°C and 8000 rpm for 10 minutes. Lysates were next stored at -80°C or had their protein content determined immediately.

3.2.7.2 Protein Determination and Sample Preparation

To estimate muscle total protein concentrations, the Direct Detect Infrared Spectrometer (Merck Millipore, headquarters Darmstadt, Germany) was used. Direct Detect™ Assay-free Cards each contain four slots for loading 2 µl of sample. The first slot on all cards was loaded with RIPA buffer which served as a blank reading. Cards

were next inserted into the spectrometer and protein content analysed in µg/mL following an automated drying cycle of the samples.

Following protein quantification, aliquots diluted in Laemmli sample buffer were prepared for all samples, each containing 20 µg of protein and analysed by western blotting.

3.2.7.3 Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Electrotransfer and Immuno-detection

After protein concentrations were determined, 20 µg/mL was loaded into each gel and separated on 4 - 20% midi-precast gradient gels (Bio-rad). Proteins were separated by SDS-PAGE (Midi-PROTEAN®, Biorad) at a constant voltage of 100 V (Biorad Power Pac 1000) for 10 minutes (stacking gel), and thereafter at constant voltage of 200 V for 50 minutes (resolving gel). The separated proteins were transferred to PVDF (polyvinylidine fluoride) membranes (Trans-Turbo® midi PVDF transfer pack, 1704157, Bio-Rad) using the Trans-Blot Turbo System (170-4155, Bio-Rad) at a voltage of 12V and constant current of 12A for 10 minutes. Following transfer, membranes were washed 3X5 min in TBS-T (TRIS-buffered saline-Tween Solution) and non-specific binding sites blocked for one hour in 5% fat-free powdered milk in TBS-T (TRIS-buffered saline-Tween Solution), after which membranes were first washed and then incubated overnight at 4°C in specific primary antibody solutions. Proteins of interest were detected using the manufacturers recommended dilutions (see table 3.1). Following overnight incubation, membranes were washed 3X5 min in TBS-T and then incubated with their corresponding secondary antibodies at room temperature for 1 hour (see table 3.1). The secondary antibodies; HRP-linked anti-Rabbit secondary antibody (7074, Cell Signalling), Goat anti-Rabbit IgG H&L (HRP) secondary antibody (ab97069, Abcam) and Goat anti-Mouse IgG H&L (HRP)

secondary antibody (ab97040, Abcam) were utilized. After washing the membranes with TBS-T (3X5 min), they were incubated with Clarity^{TM} Western ECL Substrate (170-5061, Bio-rad) and exposed with the ChemiDoc^{TM} XRS+ System. Densitometric analysis was performed with the Image Lab^{TM} 5.2.1 Software (Bio-Rad) and protein expression was normalized to the corresponding total protein per sample. Antibodies were then stripped from membranes in low glycine stripping buffer, pH 2.2 for 2x 5 min and membranes re-probed.

Table 5: Primary and enzyme-linked secondary antibodies used for Western blot analysis.

Crude Extract		
Primary Antibodies	**Dilution/Molecular Weight**	**Catalogue number & supplier**
Sirtuins		
SIRT-3	1:500	sc-99146, Santa-Cruz Biotechnology
Enzyme-linked Secondary antibodies:		
Anti-rabbit	1:15 000	7074S, Cell Signaling

3.2.8 Image Acquisition and Analysis

To determine myotube width, five fluorescent images of randomly selected areas were captured per well. The mitochondrial ROS assay was similarly conducted and cells analysed and analysed for mean fluorescent intensity. Fluorescent signal was detected with a Nikon ECLIPSE E400, equipped with a colour digital camera (Nikon DXM1200) at the appropriate wavelengths and at 20X magnification.

All images were analysed using ImageJ Java 1.8.0_77 imaging software (National Institute of Health, USA). All values obtained from treated groups were expressed as

a percentage of the control values. Three experiments were done in series with triplicates for each group.

3.2.9 Statistical Analyses

Results are presented as means ± standard error of the mean (SEM). Differences between groups were analysed using one-way factorial analysis of variance (ANOVA) or two-way factorial analysis of variance (TWO WAY ANOVA), as appropriate. When significant interactions were found, Bonferroni post hoc tests were performed. All statistical analyses were done using Graph pad Prism 7 (Graph pad Software, Inc., CA, USA). The accepted level of significance was $p < 0.05$.

Chapter 4: Results

4.1 *In vivo* study

4.1.1 The effect of MLT and DOX treatment on body mass over time (A) and post-sacrifice (B) in a tumour-bearing rat model

Rats in the different groups were matched for body mass at the start of the protocol. A: A significant reduction in body mass was observed in DOX treated rats on days 10 [228 ± 6.7 g ($p<0.01$)], 12 [218 ± 7.3 g ($p<0.001$)] and 14 [222g ± 6.5 g ($p<0.01$)] in comparison to the MLT group on days 10 [268 ± 8.3 g ($p<0.01$)], 12 [268 ± 8.0 g ($p<0.001$)] and 14 [271g ± 9.0 g ($p<0.01$)] and DM days 10 [282 ± 10.1 g ($p<0.01$)], 12 [285 ± 7.2 g ($p<0.001$)] and 14 [284g ± 6.5 g ($p<0.01$)] groups. B: Post-sacrifice mean body mass was significantly (*** $p < 0.001$) higher in the DM group compared to the DOX group (Fig. 4.1.1B).
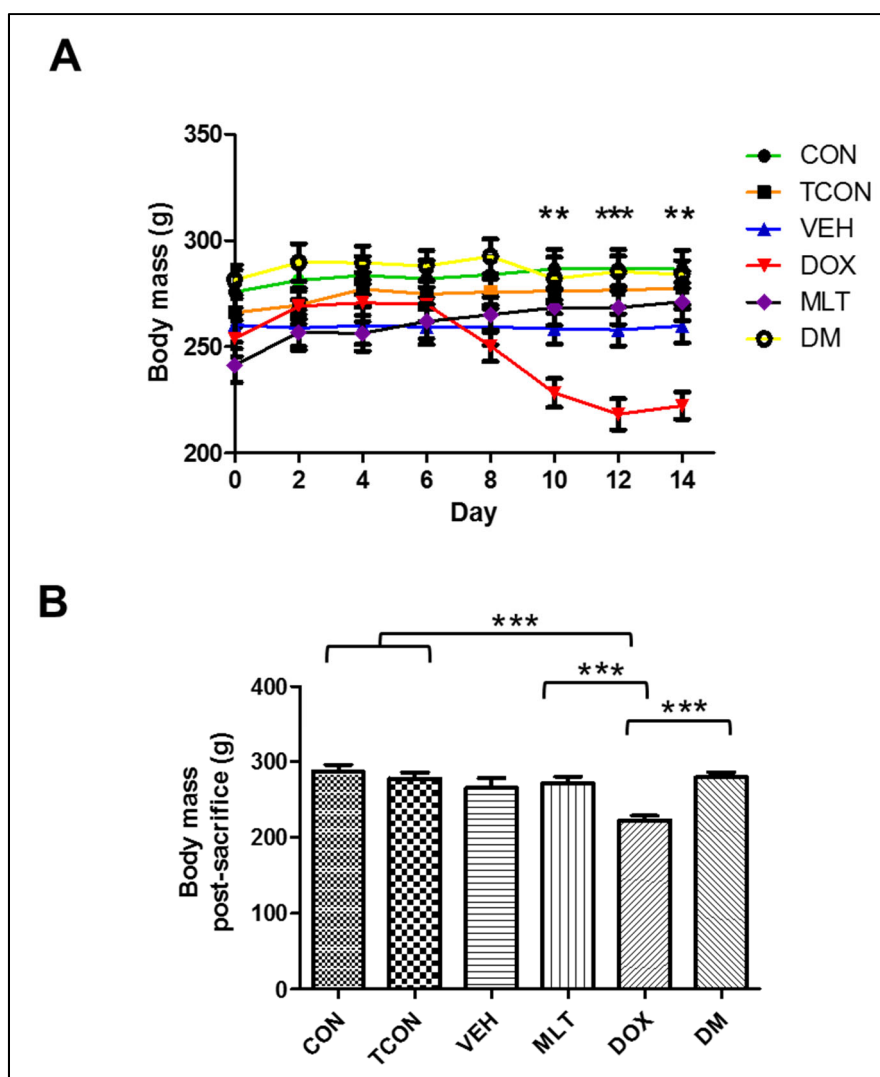
Figure 4.1.1: Comparison of mean body mass (g) in treated groups (MLT 6 mg/kg or DOX 4 mg/kg or both MLT and DOX). (A) Body mass over time and (B) body mass post-sacrifice. Abbreviations- CON: control n = 8; TCON: tumour control n = 10 control; VEH: vehicle control n = 8; MLT: melatonin n = 10; DOX: doxorubicin n = 10 and DM: doxorubicin+melatonin. n = 10. ** $p < 0.01$; *** $p < 0.001$. Data are expressed as mean ± SEM.

4.1.2 Qualitative comparison of muscle cross sectional area following MLT and DOX treatment in a tumour-bearing rat model

Skeletal muscles were harvested from rats after sacrifice. Tissue histologic features were assessed with the use of conventional haematoxylin and eosin (H&E) staining. Qualitative assessment showed no major ultrastructural changes between the groups, however the DOX group (E) appeared to have smaller muscle cross-sectional at the

mid-belly and more compact fibre bundles, when compared to all other groups (Fig. 4.1.2).
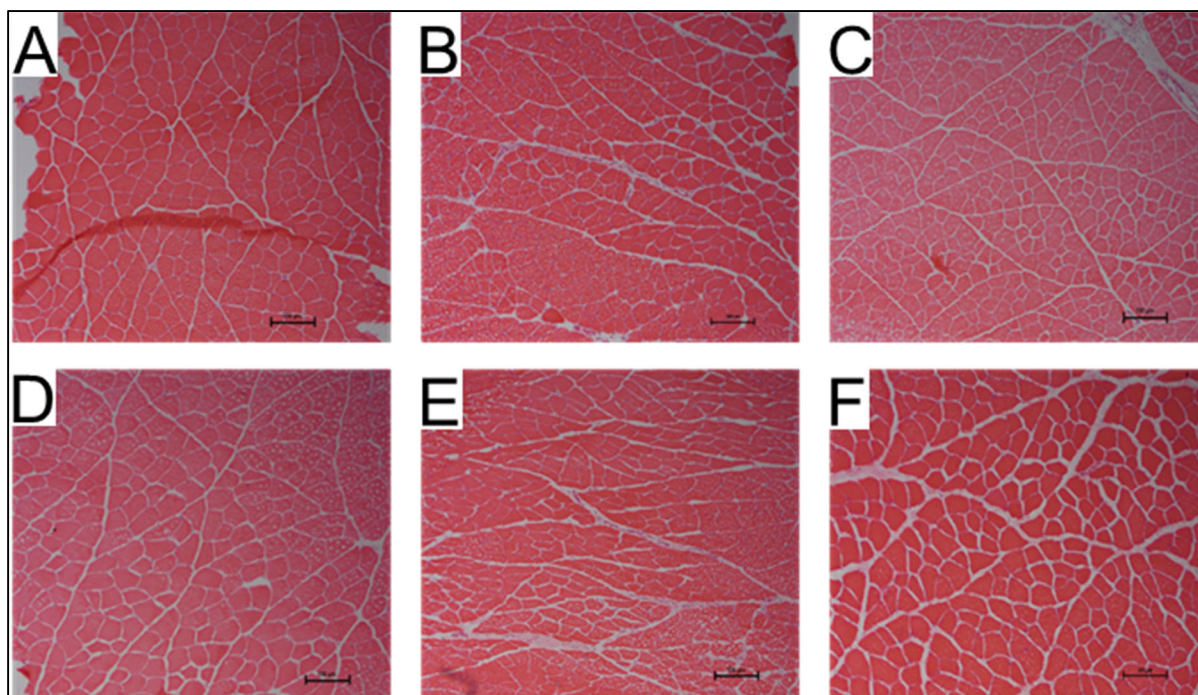
Figure 4.1.2 Representative H&E images of *gastrocnemius* muscle harvested from rats. Images displayed are from (A) Control (CON); (B) Tumour control (TCON); (C) Vehicle (VEH); (D) Melatonin (MLT); (E) Doxorubicin (DOX) & (F) Doxorubicin+melatonin (DM). Scale bar represents 100 μm.

4.1.3 The effect of melatonin and doxorubicin treatment on muscle mass and cross-sectional area in a tumour-bearing rat model

At the end of the experimental protocol, rats were sacrificed and rat muscle wet weights (*soleus*, *plantaris* & *gastrocnemius*) and CSA's were recorded.

4.1.3.1 *Soleus* muscle weight to body weight ratio and total cross-sectional area

Rat mean *soleus* muscle weight & body weight ratios indicated no significant changes between groups. Total *soleus* CSA *was* determined by analysing H&E stained sections, using ImageJ Java 1.8.0_77 software (National Institute of Health, USA). *Soleus* CSA data indicated that the DOX group had a significantly (** $p < 0.01$) reduced

soleus muscle CSA (n=4) compared to the TCON, VEH, MLT and DM groups (Fig. 4.1.3).
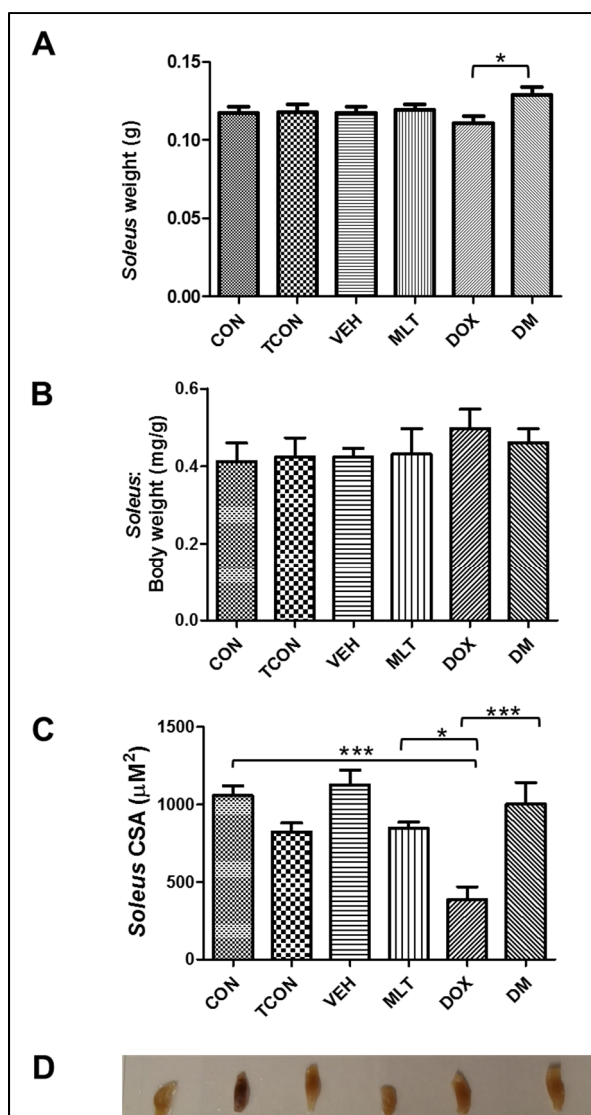
Figure 4.1.3: Comparison of average A) *soleus* muscle weight (g), B) *soleus*/body weight ratio (mg/g) and C) total *soleus* cross-sectional area (CSA) between groups. D) Illustration of the *soleus* muscle for the different groups. *** p < 0.01, *** p < 0.001. Data is expressed as mean ± SEM. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin. n = 3-10.

4.1.3.2 *Plantaris* muscle weight to body weight ratio and total cross-sectional area

Rat mean *plantaris* muscle weight & body weight ratios indicated that the DOX treatment led to a significant (* p < 0.05) reduction in *plantaris* muscle weight & body weight compared to the MLT and DM groups. Total *plantaris* muscle CSA was

determined by analysing H&E stained sections, using ImageJ Java 1.8.0_77 software (National Institute of Health, USA). *Plantaris* muscle CSA was significantly (** $p < 0.05$) reduced in the DOX group compared to the CON and MLT groups (Fig. 4.1.4).
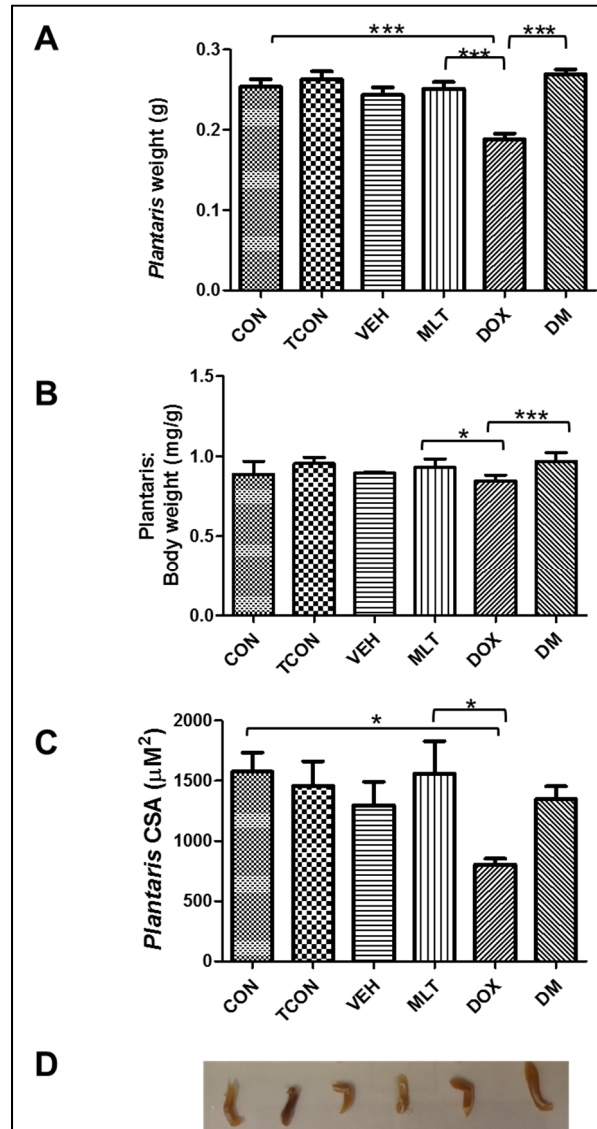
Figure 4.1.4: Comparison of average A) *plantaris* muscle weight (g), B) *plantaris*/body weight ratio (mg/g) and C) total *soleus* cross-sectional area (CSA) between groups (μm). D) Illustration of the *plantaris* muscle for the different groups. Data is expressed as mean ± SEM; * $p < 0,05$; ** $p < 0.01$; *** $p < 0.001$. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin. n = 3-10.

4.1.3.3A *Gastrocnemius* muscle weight and body weight ratio

Analyses of the rat mean *gastrocnemius* muscle weight & body weight ratios indicated that the DOX group had significantly (*** $p < 0.001$) reduced *gastrocnemius* muscle weight compared to the MLT and DM groups (Fig. 4.1.5).
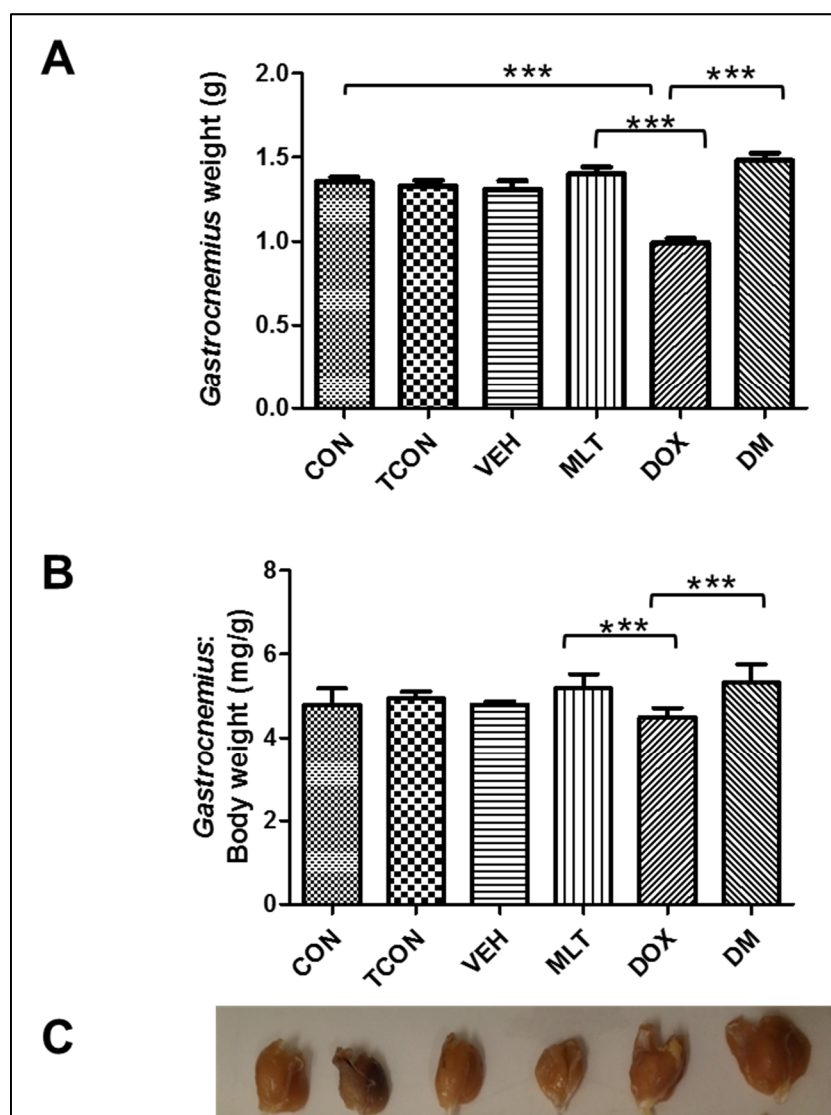
Fig. 4.1.5: Comparison of average A) *gastrocnemius* muscle weight (g) and B) body weight ratio (mg/g) between groups. C) Illustration of the *gastrocnemius* muscle for the different groups. *** $p < 0.01$, *** $p < 0.001$. Data is expressed as mean ± SEM. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin. n = 3-10.

4.1.3.3B *Gastrocnemius* muscle cross-sectional area

Gastrocnemius muscle CSA was quantitatively assessed with the use of NADH-tetrazolium reductase (NADH-TR) histochemical stain. The DOX treated group had significantly decreased type I muscle fibre CSA compared to all other groups. Similarly the DOX treated group displayed significantly decreased type II muscle fibre CSA compared to all other groups, excluding the TCON group (Fig. 4.1.6).
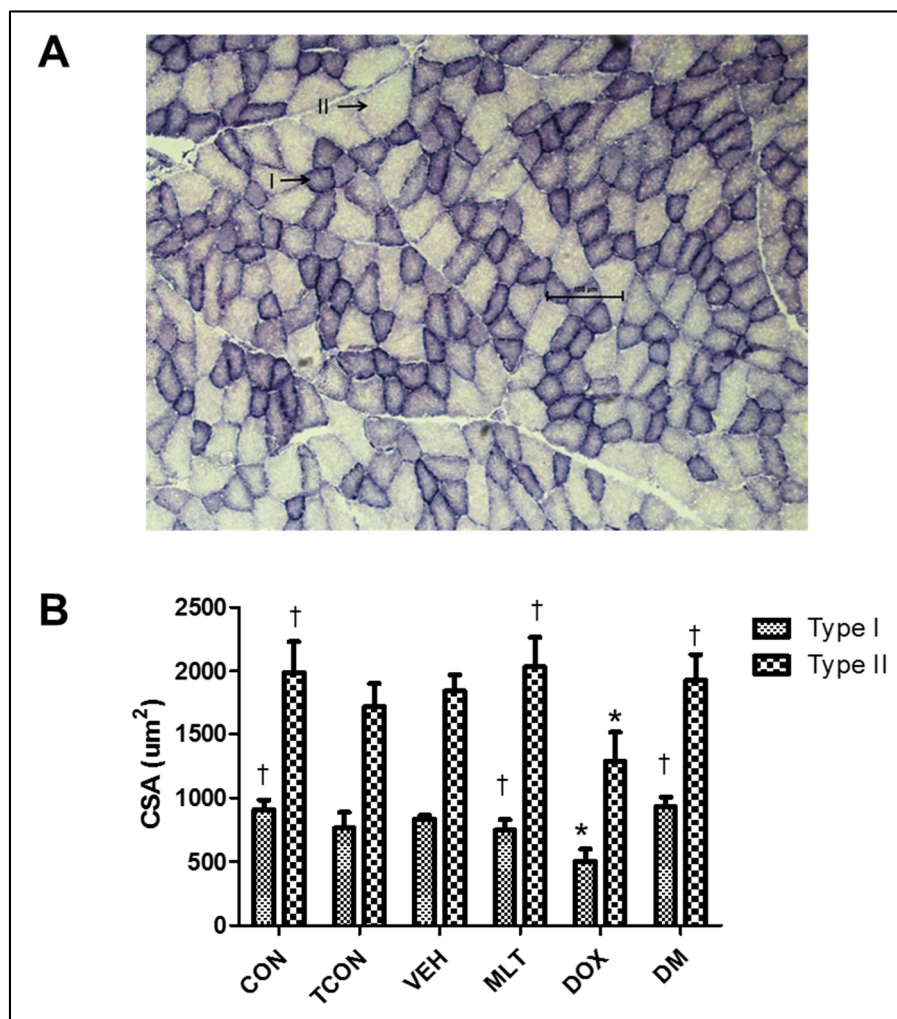
Figure 4.1.6: A) NADH-TR stained *gastrocnemius* muscle: I = dark type I fibres and II = light type II fibres. B) Differences in fibre-specific cross-sectional area (CSA). The dotted and blocked bars indicate changes in CSA (um^2) for type I and type II fibres respectively. *significantly different from CON, (** $p < 0.05$) and † significantly different from DOX, (* $p < 0.05$). Data are expressed as mean ± SEM. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin. n = 3-10. Scale bar represents 100 μm.

4.1.4 The effect of melatonin and doxorubicin treatment on blood parameters in a tumour-bearing rat model

4.1.4.1 Serum Myoglobin (Mb)

Serum Myoglobin (Mb) levels were significantly increased when treating with DOX, compared to MLT and DM groups (* $p < 0.05$) groups (Fig. 4.1.7).
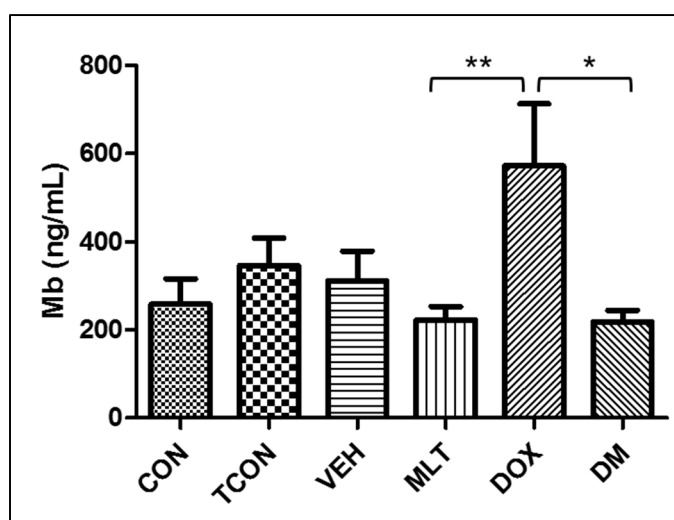
Figure 4.1.7: Difference in serum myoglobin (Mb) levels between groups. Data are expressed as mean ± SEM; * $p < 0.05$; ** $p < 0.01$. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.4.2 Differential whole blood counts

WBCs ($1.49 \pm 0.4 \times 10^9 L^{-1}$, * $p < 0.05$), neutrophils ($1.6 \pm 0.5 \times 10^9 L^{-1}$, * $p < 0.05$), RBCs ($4.0 \pm 1.52 \times 10^{12} L^{-1}$, * $p < 0.05$), haematocrit ($39 \pm 10.43 \times 10^{12} L^{-1}$, * $p < 0.05$) and haemoglobin ($8.3 \pm 2.22 \times 10^{12} L^{-1}$, * $p < 0.05$) levels were significantly decreased in the DOX group compared to all other groups (Table 3). Eosinophil levels were significantly increased in the TCON [$0.4 \pm 0.20 \times 10^9 L^{-1}$ (* $p < 0.01$)] and VEH [$0.3 \pm 0.20 \times 10^9 L^{-1}$ (* $p < 0.01$)] group compared to the DOX group [$0.04 \pm 0.33 \times 10^9 L^{-1}$ (* $p < 0.01$)]. Lymphocytes [$2.2 \pm 2.3 \times 10^9 L^{-1}$ (* $p < 0.05$)] in the DM group were significantly increased when compared to CON [$0.04 \pm 0.03 \times 10^9 L^{-1}$ (* $p < 0.01$)] and MLT [$0.1 \pm$

0.1 x 10^9L^{-1} (* $p < 0.01$)]. Basophil levels were significantly increased in the MLT group [0.1 ± 1.40 x 10^9L^{-1} (* $p < 0.01$)] compared to the TCON group [0.003 ± 0.003 x 10^9L^{-1} (* $p < 0.01$)], while monocyte levels remained unchanged.

Table 6: Leukocyte (WBC) and red blood cell (RBC) indices in peripheral blood following MLT and DOX treatment in a tumour-bearing rat model.

	CON	TCON	VEH	MLT	DOX	DM
WBC (10^9L^{-1})	$5.61 \pm 1.5^*$	$5.31 \pm 2.0^*$	$5.22 \pm 1.6^*$	$4.80 \pm 1.3^*$	1.49 ± 0.4	$5.34 \pm 3.3^*$
Neutrophils (10^9L^{-1})	$5.4 \pm 1.3^*$	$4.6 \pm 1.6^*$	$5.5 \pm 1.3^*$	$5.0 \pm 1.1^*$	1.6 ± 0.5	$4.6 \pm 1.2^*$
Lymphocytes (10^9L^{-1})	$0.04 \pm 0.03^\#$	0.1 ± 1.8	0.5 ± 1.0	$0.1 \pm 0.1^\#$	0.4 ± 0.6	2.2 ± 2.3
Monocytes (10^9L^{-1})	0.01 ± 0.01	0.01 ± 0.01	0.004 ± 0.003	0.02 ± 0.03	0.004 ± 0.004	0.01 ± 0.01
Eosinophils (10^9L^{-1})	0.2 ± 0.10	$0.4 \pm 0.20^*$	$0.3 \pm 0.20^*$	0.2 ± 0.16	0.04 ± 0.33	0.2 ± 0.04
Basophils (10^9L^{-1})	0.01 ± 0.02	$0.003 \pm 0.003^\Psi$	0.03 ± 0.10	0.1 ± 1.40	0.01 ± 0.02	0.04 ± 0.09
RBC $(10^{12}L^{-1})$	$6.8 \pm 0.21^*$	$6.4 \pm 0.74^*$	$5.9 \pm 1.11^*$	$5.1 \pm 1.06^*$	4.0 ± 1.52	$6.0 \pm 1.09^*$
Haemoglobin (g.dL)	$13 \pm 1.71^*$	$13.1 \pm 0.74^*$	$12 \pm 2.50^*$	$12 (2.24)^*$	8.3 ± 2.22	10.9 ± 1.82
Hematocrit (%)	$58 \pm 7.90^*$	$58 \pm 6.44^*$	$53 \pm 10.23^*$	$55 \pm 6.15^*$	39 ± 10.43	51 ± 8.84

Abbreviations- WBC: white blood cell; RBC: red blood cell; CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin. * $p < 0.05$ compared to DOX; Ψ $p < 0.05$ compared to DM; # $p < 0.05$ compared to DOX. Data is expressed as mean ± SEM.

4.1.5 The effect of melatonin and doxorubicin treatment on muscle injury and degradation processes in a tumour-bearing rat model

4.1.5.1 Apoptosis

A significant increase in cCaspase3 expression was evident in the DOX group compared to CON (* $p < 0.05$). The TCON group was significantly decreased from the MLT (* $p < 0.05$), DOX (* $p < 0.001$) and DM (* $p < 0.05$) groups. No significant differences between groups were observed in cPARP protein levels (Fig. 4.1.8).
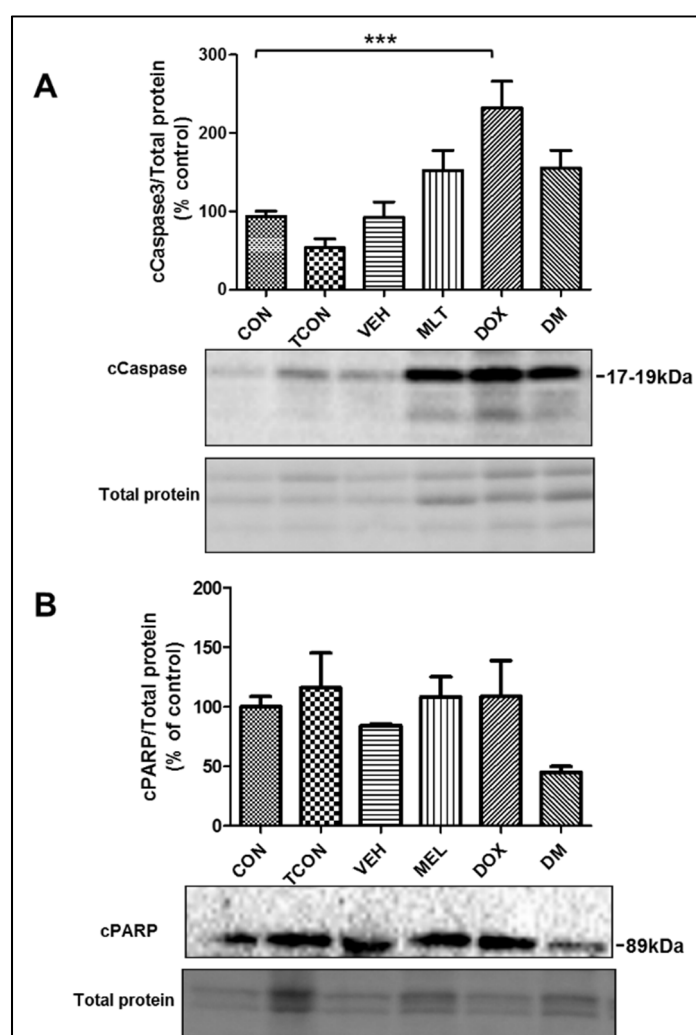
Figure 4.1.8: Apoptosis marker protein levels A) cCaspase3 and B) cPARP. Below bar graphs A and B are illustrations of protein bands. Values are expressed relative to the control values (mean ± SEM). *** $p < 0.001$, n = 3-10, average n = 8. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.5.2 Atrophy

MAFbx protein levels were significantly reduced in the DM group compared to all other groups, while a significant increase in MuRF1 protein expression was evident in the DOX treated groups compared to CON (* $p < 0.05$). FoxO3a protein levels were significantly increased in the DOX group compared to CON and DM groups (Fig. 4.1.9).
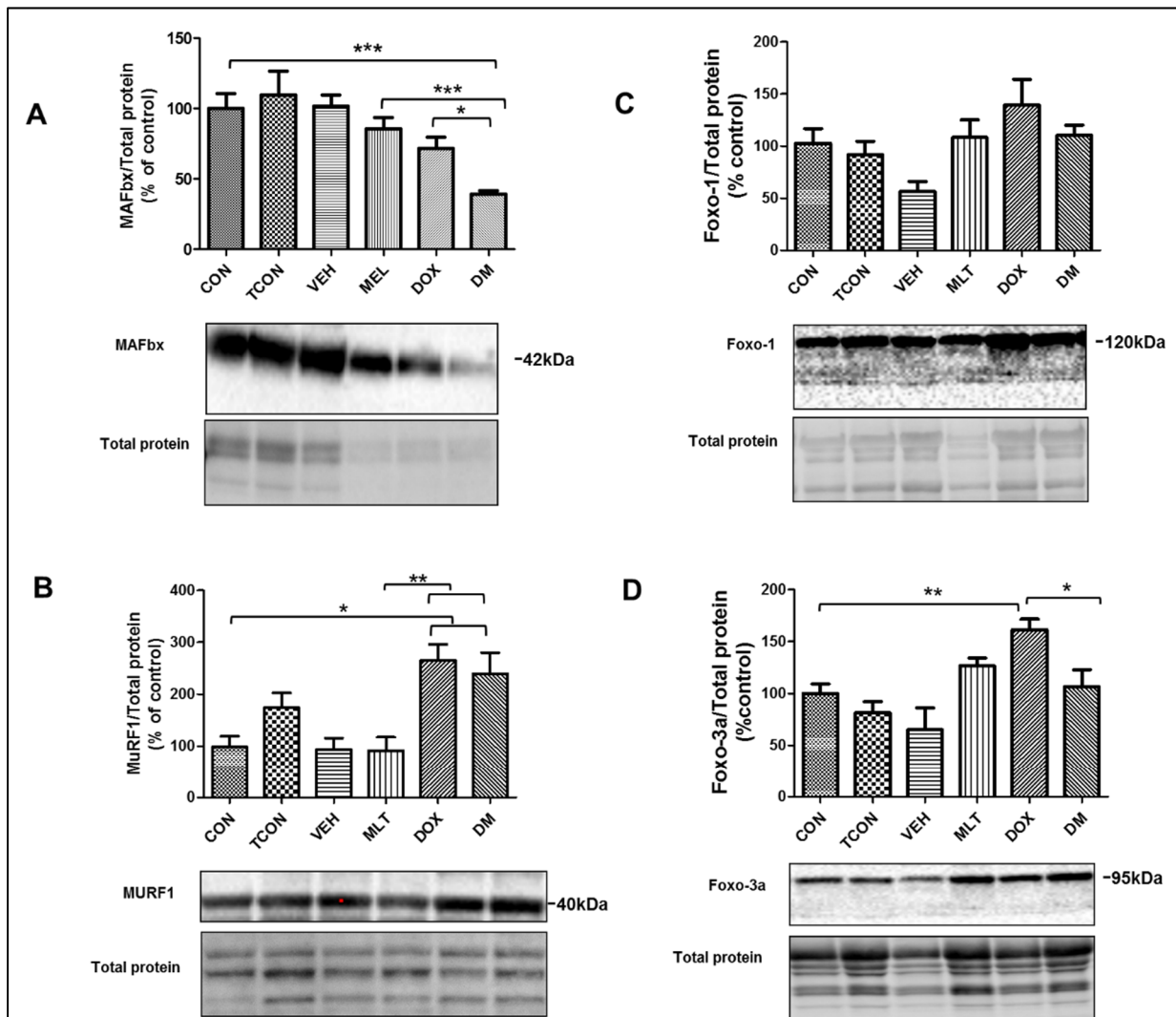
Figure 4.1.9: Atrophy marker: MAFbx, MuRF-1, FoxO-1 & FoxO3a protein levels. Values are expressed relative to the control values (mean ± SEM).* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ n = 3-10. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.5.3 Ubiquitin proteasome pathway (UPP)

No significant changes were observed for UPP markers; K48 and K63 protein levels (Fig. 4.1.10).
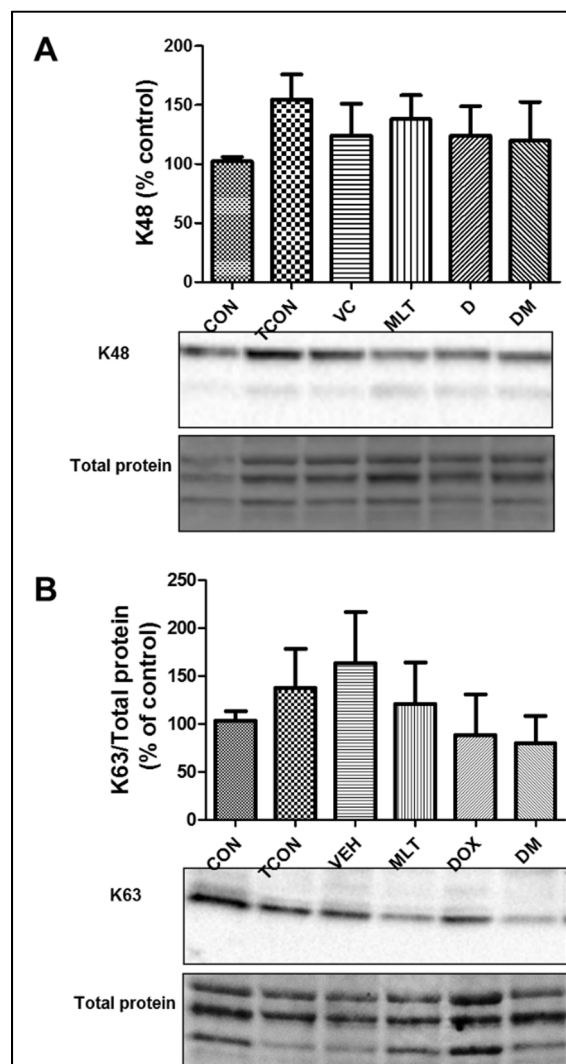
Figure 4.1.10: Markers of the ubiquitin proteasome pathway. Values are expressed relative to the control values (mean ± SEM), n = 5-7. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.5.4 Autophagy

Key proteins involved in autophagy were assessed, namely A) Phospho-mTOR; B) p62 and LC3-I to LC3-II conversion were assessed by western blot analysis. Phospho-mTOR protein levels were significantly increased in the DOX group compared to the

CON (*p < 0.05). p62 protein levels were significantly increased (*p < 0.05) in the DOX group as compared to the DM group; whereas LC3-I to LC3-II conversion was significantly (*** p < 0.001) decreased in the DOX group compared to all the other groups (Fig. 4.1.11).
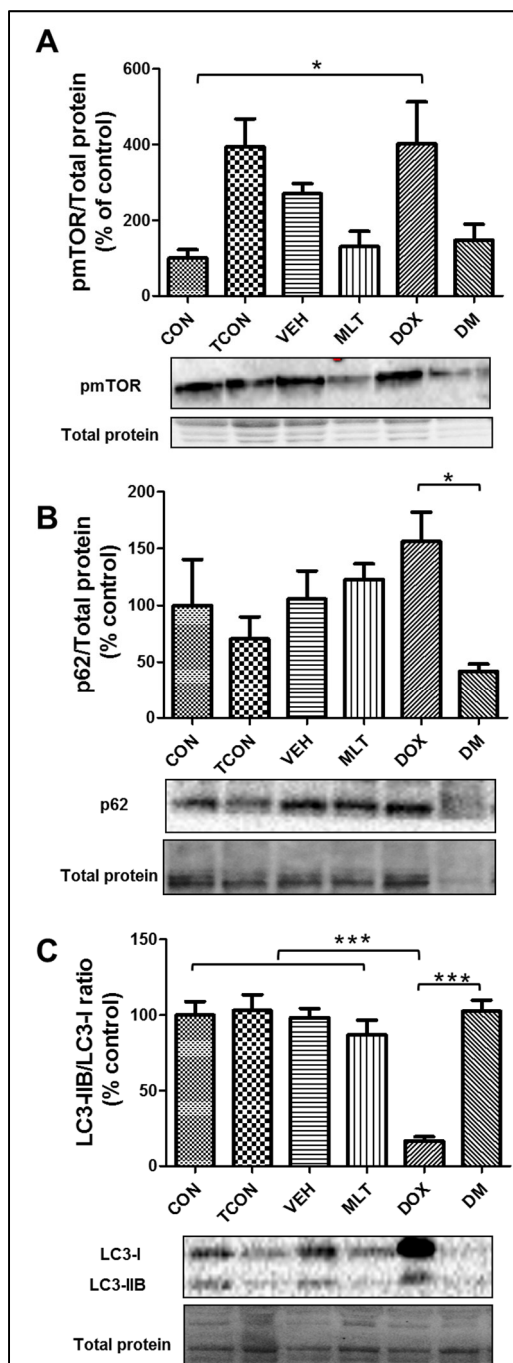
Figure 4.1.11: Markers of the autophagy pathway. Values are expressed relative to the control values (mean ± SEM). *p < 0.05; ** p < 0.01; *** p < 0.001, n = 3-10. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.5.5 Calpain System

Ubiquitously expressed and skeletal muscle specific Calpain:

Western blot analysis revealed no significant differences between groups in protein levels of ubiquitous non-lysosomal, Ca^{2+}-dependent cysteine proteases, Calpain-1 or 2. While Calpain-3 protein level was significantly higher (* $p < 0.05$) in the DOX group compared to the MLT and DM groups (Fig. 4.1.12).
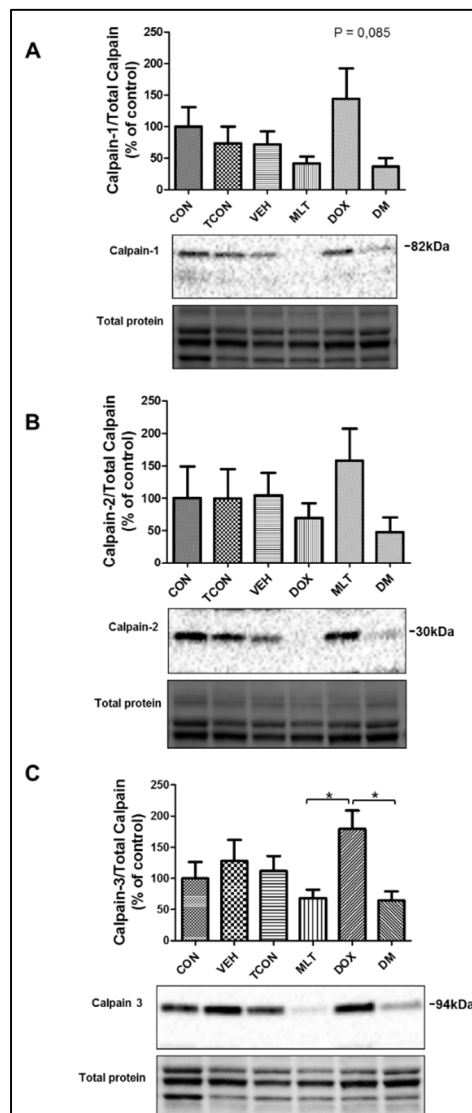
Figure 4.1.12: Ubiquitously expressed calpain-1 (A), calpain-2 (B) and skeletal muscle specific calpain-3 (C) protein levels protein levels. Values are expressed relative to the control values (mean ± SEM), * $p < 0.05$, n = 3-10. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.6 The effect of melatonin and doxorubicin treatment on muscle antioxidant capacity in a tumour-bearing rat model

Intramuscular Oxygen Radical Absorbance Capacity indicated that both MLT and DM treatment groups had significantly (* $p < 0.05$) increased muscle ORAC levels compared to all the other groups. No significant difference was observed between the CON and DOX group (Fig. 4.1.13).
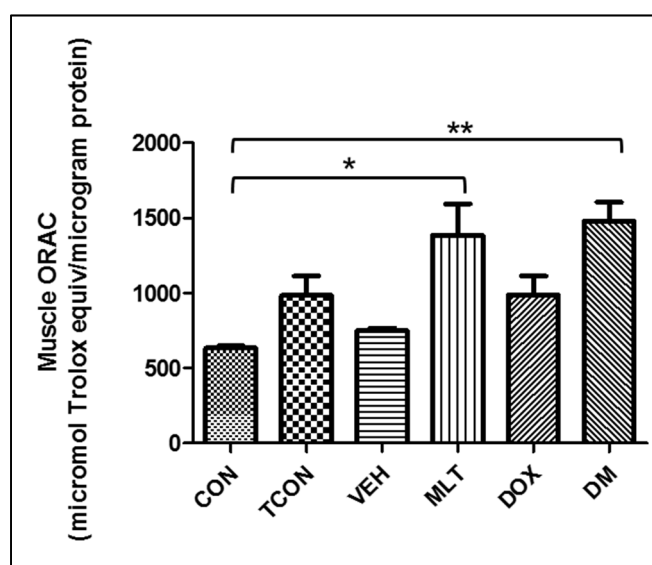
Figure 4.1.13: Intramuscular *gastrocnemius* antioxidant capacity of treatment groups. * $p < 0.05$; ** $p < 0.01$. Data are expressed as mean ± SEM. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin. n = 3-10.

4.1.7 The effect of melatonin and doxorubicin treatment on mitochondrial biogenesis in a tumour-bearing rat model

Mitochondrial biogenesis marker PGC-1α protein levels were significantly decreased in the DOX group compared to the Control (*$p < 0.05$), MLT (** $p < 0.01$) and DM (*** $p < 0.001$) groups (Fig. 4.1.14).
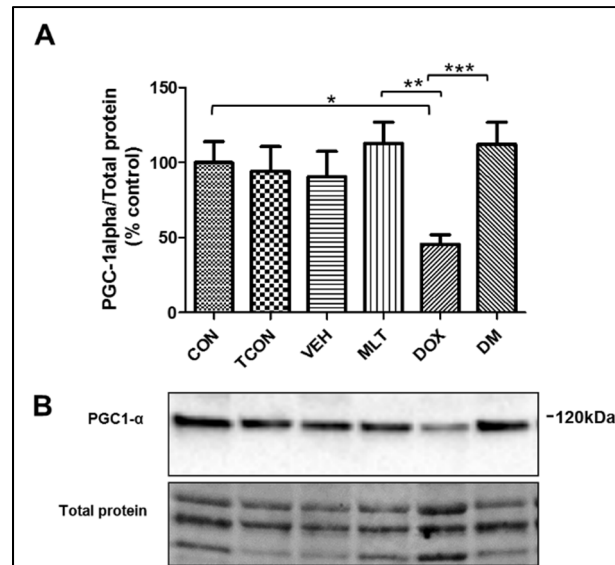
Figure 4.1.14: PGC-1α protein levels in skeletal muscle. Values are expressed relative to the control values (mean ± SEM). * p < 0.05; ** p < 0.01; ** p < 0.001, n = 3-10. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.8 The effect of melatonin and doxorubicin treatment on mitochondrial dynamics in a tumour-bearing rat model

4.1.8.1 Fission protein

Mitochondrial fission protein marker, Hfis1, expression showed no significant changes between groups (Fig. 4.1.15).
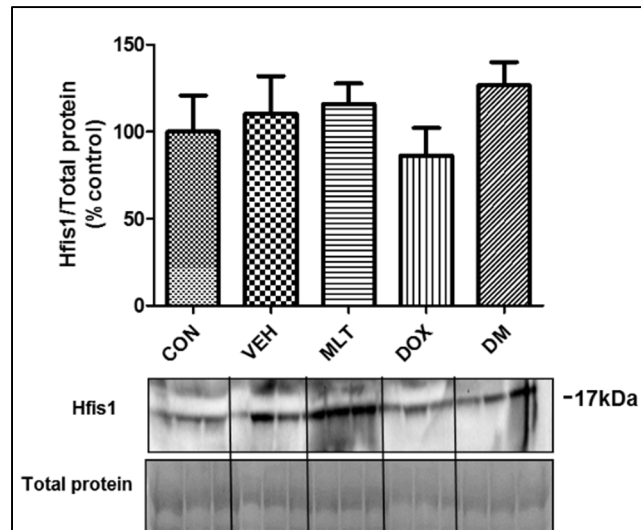
Figure 4.1.15: Hfis1 protein levels in skeletal muscle. Values are expressed relative to the control values (mean ± SEM). * $p < 0.05$; ** $p < 0.01$, n = 3. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.8.2 Fusion proteins

Mitochondrial fusion markers A) mitofusin1 (Mfn1) and B) mitofusin (Mfn2) protein levels. No significant difference was observed in Mfn1 protein levels, whereas Mfn2 protein levels in the DM group was significantly (* $p < 0.05$) decreased compared to the control group (Fig. 4.1.16).
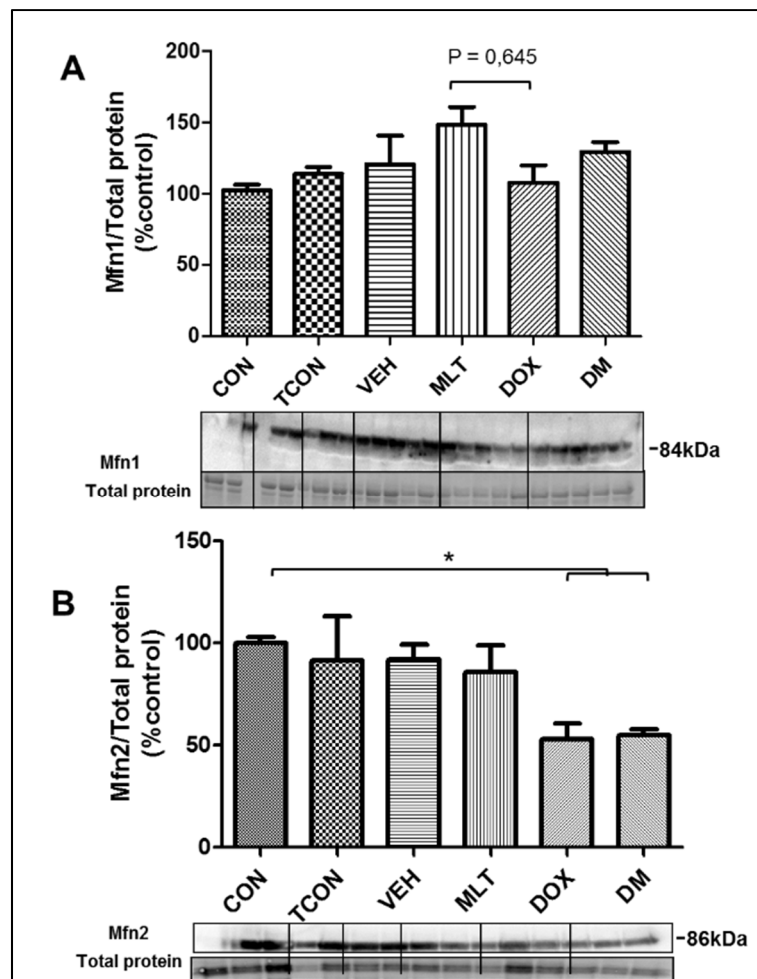
Figure 4.1.16: Mitofusion protein levels in skeletal muscle. Values are expressed relative to the control values (mean ± SEM). * p < 0.05, n = 3-5. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.9 The effect of melatonin and doxorubicin treatment on marker PTEN-induced putative kinase 1 in a tumour-bearing rat model

PINK1 is a mitochondrial serine/threonine kinase which protects cells from stress induced mitochondrial dysfunction. A significant (* p < 0.05) increase was observed in PINK1 protein levels in the MLT group compared to all other groups (Fig. 4.1.17).
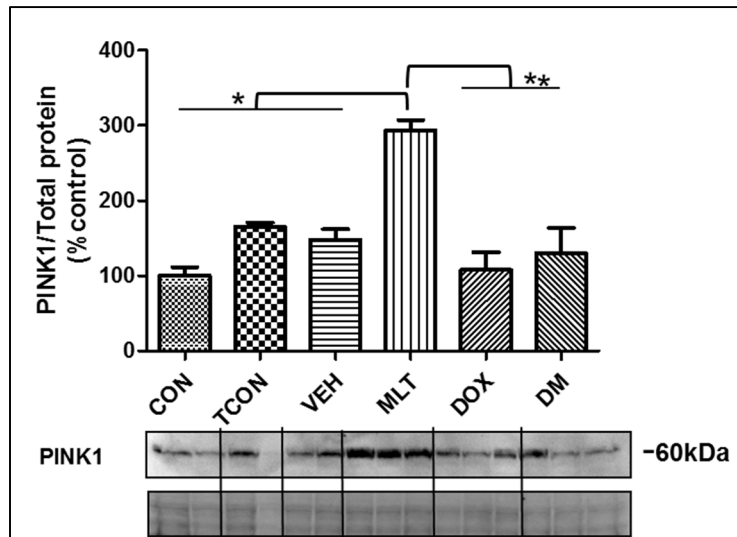
Figure 4.1.17: PINK1 protein levels in skeletal muscle. Values are expressed relative to the control values (mean ± SEM). * $p < 0.05$, n = 3. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.10 The effect of melatonin and doxorubicin treatment on muscle satellite cell number in a tumour-bearing rat model

The satellite cell marker, Pax7, was used to determine satellite cell number per myofibre (section 4.1.12.1) and Pax7 protein levels in skeletal muscle (section 4.1.12.2).

4.1.10.1 Pax-7 positive cell count

Pax-7 is a marker expressed on quiescent satellite cells (Cornelison and Wold 1997, Scime and Rudnicki 2006). MLT caused a significant (* $p < 0.05$) increase in satellite cells per myofibre. No significant difference was detected when DOX was compared with the control group. Pax7 protein levels significantly (* $p < 0.05$) increased in both the MLT and DM group compared to the control group, thus mimicking the Pax7 positive cell count result (Fig. 4.1.18).
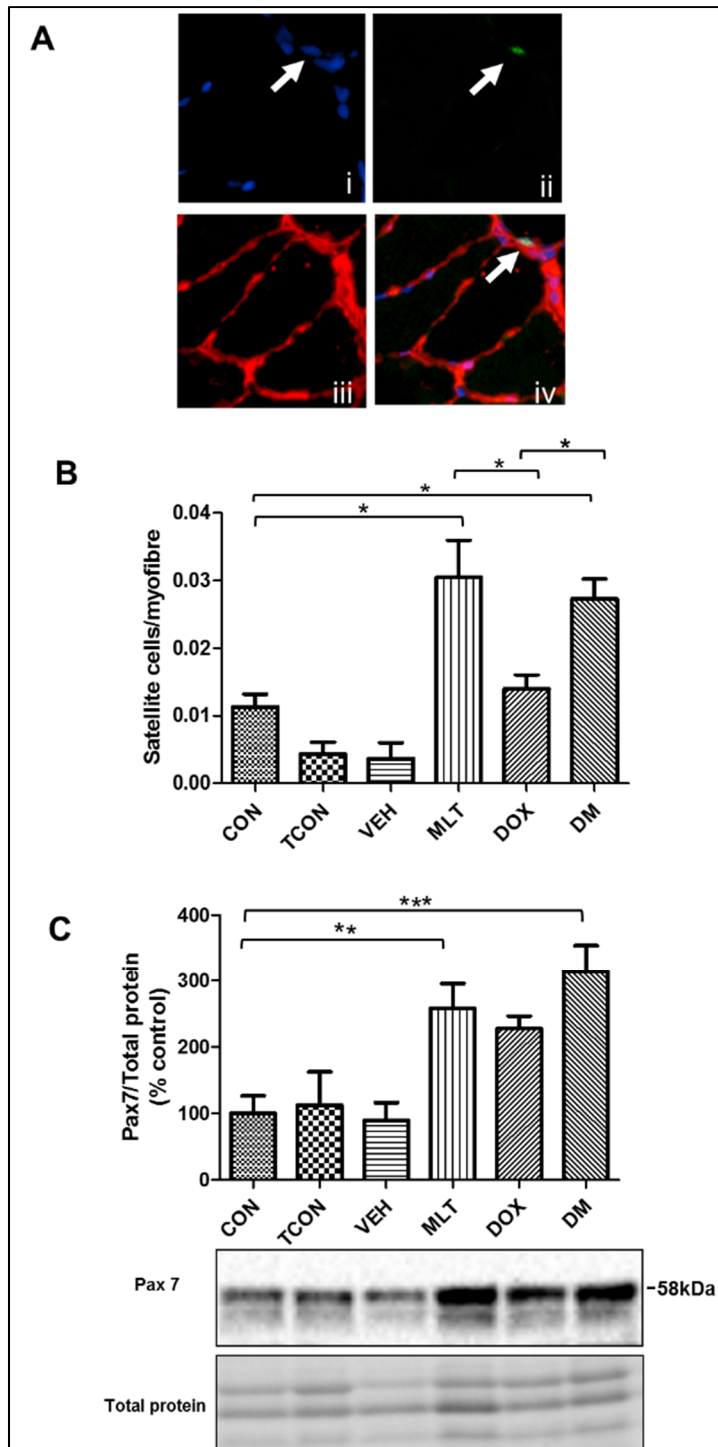
Figure 4.1.18: Pax7 positive cell count. A) Example of satellite cell – i) Hoechst 33342 stained nuclei; ii) FITC anti-Pax7; iii) Texas Red anti-Laminin and iv) image overlay. B) Differences in average satellite cell/myofibre ratio between groups. C) Pax7 protein levels in skeletal muscle. * $p < 0.05$; ** $p < 0.01$, n = 3-9. Data are expressed as mean ± SEM. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.10.2 Markers of myogenic regulation

Western blot analysis of Myogenin and MyoD protein levels. MyoD protein levels were significantly (*p < 0.05) higher in the DOX group compared to the MLT group (Fig. 4.1.19).
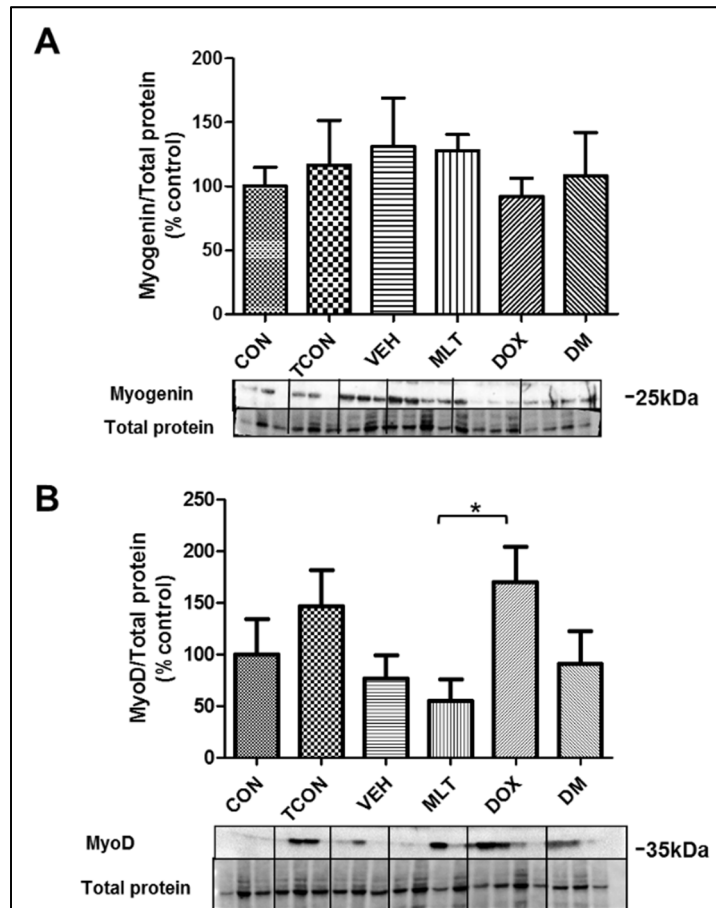
Figure 4.1.19: A) Myogenin and B) MyoD protein levels in skeletal muscle. Values are expressed as arbitrary units (mean ± SEM). Significance (** p < 0.01), n = 3-5. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.1.11 The effect of melatonin and doxorubicin treatment on muscle sirtuin 1 and 3 protein levels during in a tumour-bearing rat model

SIRT-3 protein levels in the DOX treated groups were significantly increased compared to the CON group (Fig. 4.1.20).
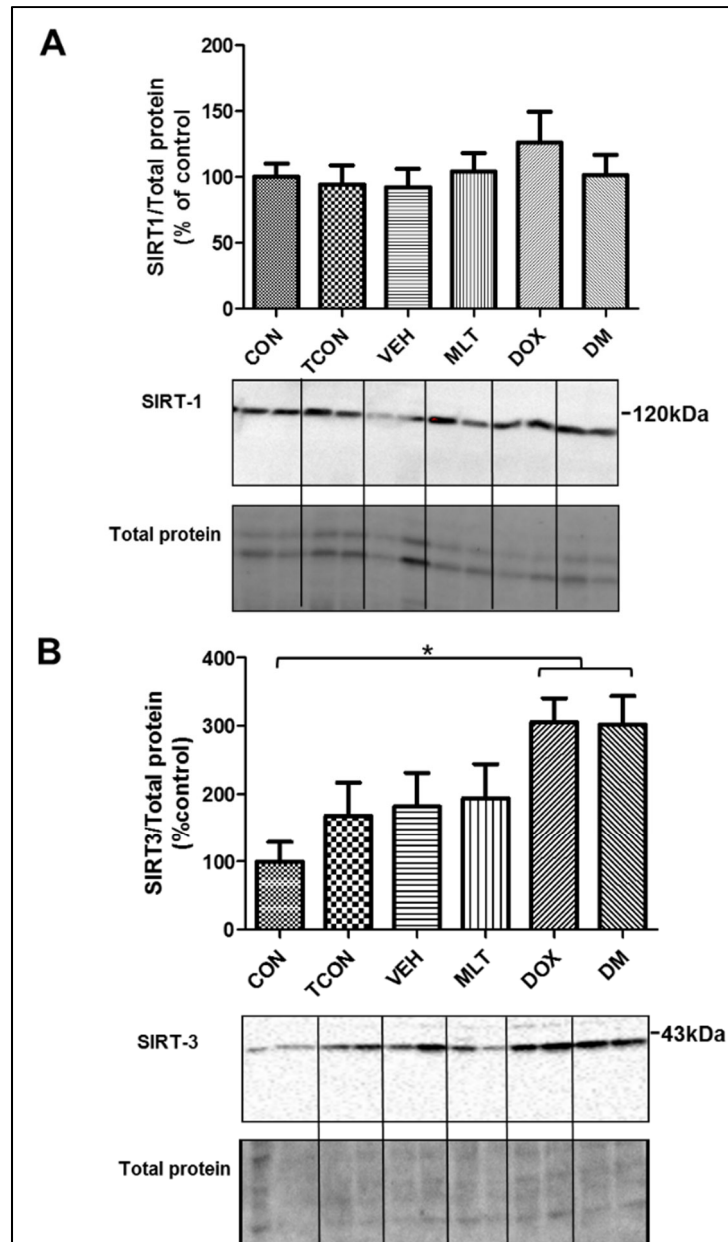
Figure 4.1.20: A) SIRT-1 and B) SIRT-3 protein levels in skeletal muscle mitochondrial extract. Values are expressed relative to the control values (mean ± SEM). Significance (* $p < 0.05$; ** $p < 0.01$), n = 3-5. Abbreviations- CON: control; TCON: tumour control; VEH: vehicle control; MLT: melatonin; DOX: doxorubicin and DM: doxorubicin+melatonin.

4.2 *In vitro* Study

4.2.1 Inhibition of Melatonin Receptor

To establish whether melatonin acts through its receptor two additional treatment groups were added, i.e. MLT+luzindole and MLT+DOX+ luzindole. Luzindole inhibits the MT1 receptor and partially inhibits the MT2 receptor. Myotube diameter was assessed by analysing 2D images with ImageJ using the FITC Celltracker green to label myotubes (Fig. 4.2.1&2).

4.2.1.1 Qualitative observations of myotubes

Upon visual inspection, myotubes in the MLT and DM groups had increased tube diameter compared to all other groups. The DOX group possessed less differentiated myotubes as more single nuclei were observed in this group, whereas the DMI group had more differentiated tubes which were decreased in diameter but more pronounced in length (Fig. 4.2.1).
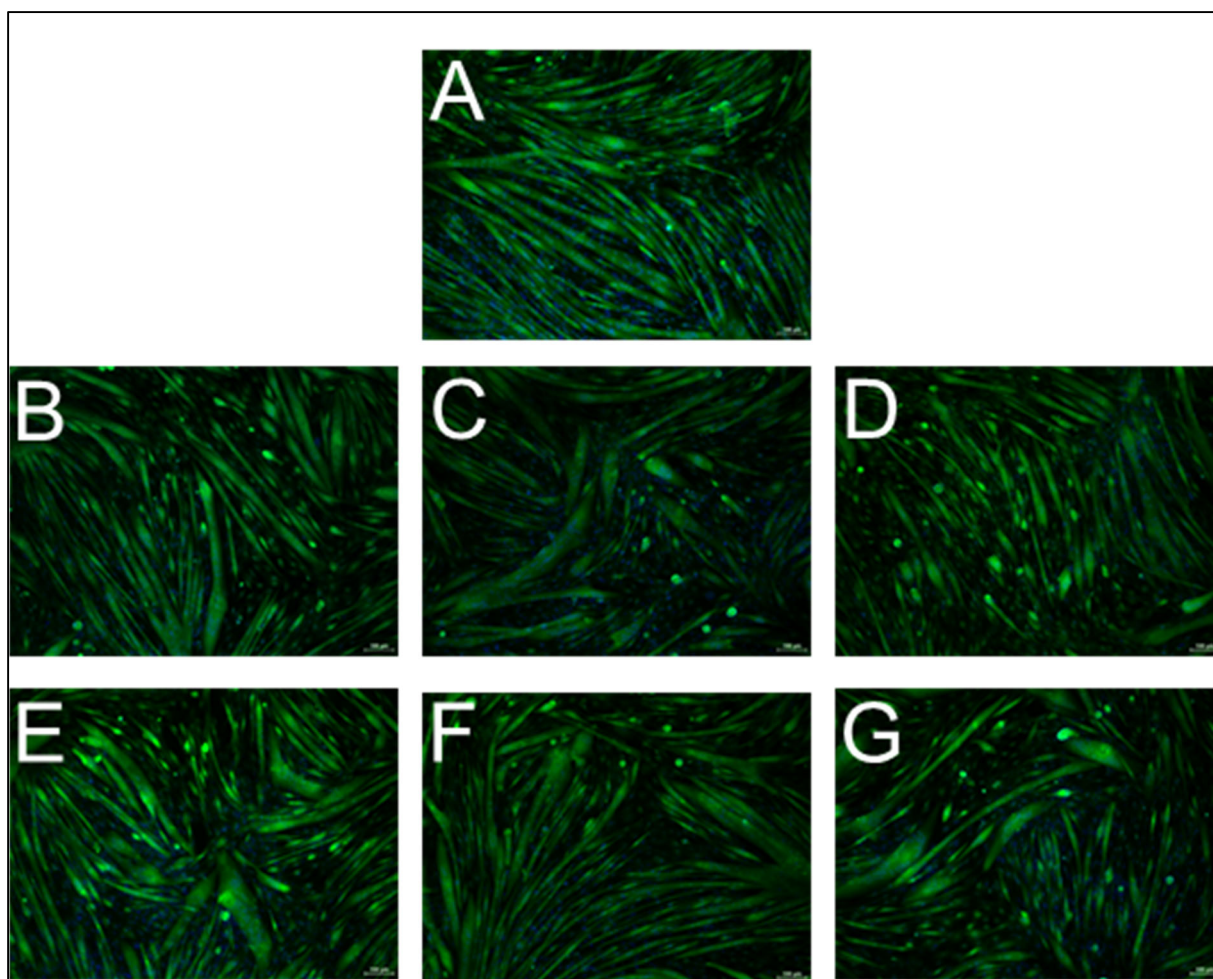
Figure 4.2.1: A comparison of myotubes in all treatment groups: A) CON; B) VEH; C) MEL; D) DOX; E) DM; F) MI and G) DMI. Abbreviations- CON: control; VEH: vehicle; MLT: melatonin; DOX: doxorubicin; DM: DOX+MLT; MI: MLT+luzindole; DMI: DOX+MLT+luzindole. Myotubes were labelled with FITC Celltracker green and Hoechst (nuclei). Scale bar represents 100 µm.

4.2.1.2 Quantitative analysis of myotube diameter

Myotube width in the control group was significantly increased compared to the DOX group and significantly reduced compared to the DM group. The MLT group myotube widths were significantly increased compared to all groups except the control and DM groups. Moreover, the DM group displayed thicker myotube formation compared to all other groups except the MLT group (Fig. 4.2.2).
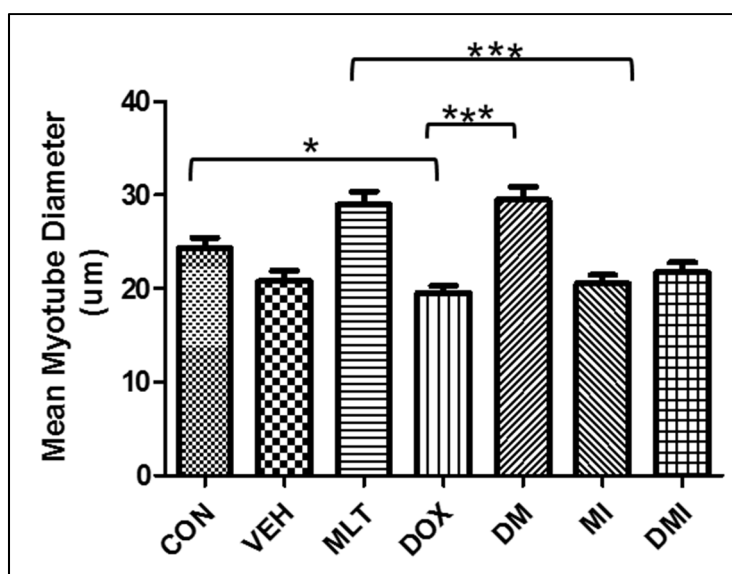
Figure 4.2.2: A comparison of mean myotube diameter (um) in treatment groups: A) CON; B) VEH; C) MEL; D) DOX; E) DM; F) MI and G) DMI. Abbreviations- CON: control; VEH: vehicle; MLT: melatonin; DOX: doxorubicin; DM: DOX+MLT; MI: MLT+luzindole; DMI: DOX+MLT+luzindole. Mean myotube tube diameter analysed.

4.2.2 The effect of melatonin and doxorubicin treatment on mitochondrial reactive oxygen species generation

Below are representative images showing MitoSOX red fluorescent signal in myotubes in the treatment groups (Fig. 4.2.3). MitoSOX red fluorescent signal intensity was brightest in the DOX group (Fig. 4.2.3 - D) and significantly (** $p < 0.01$) elevated compared to all other groups (Fig. 4.2.3).
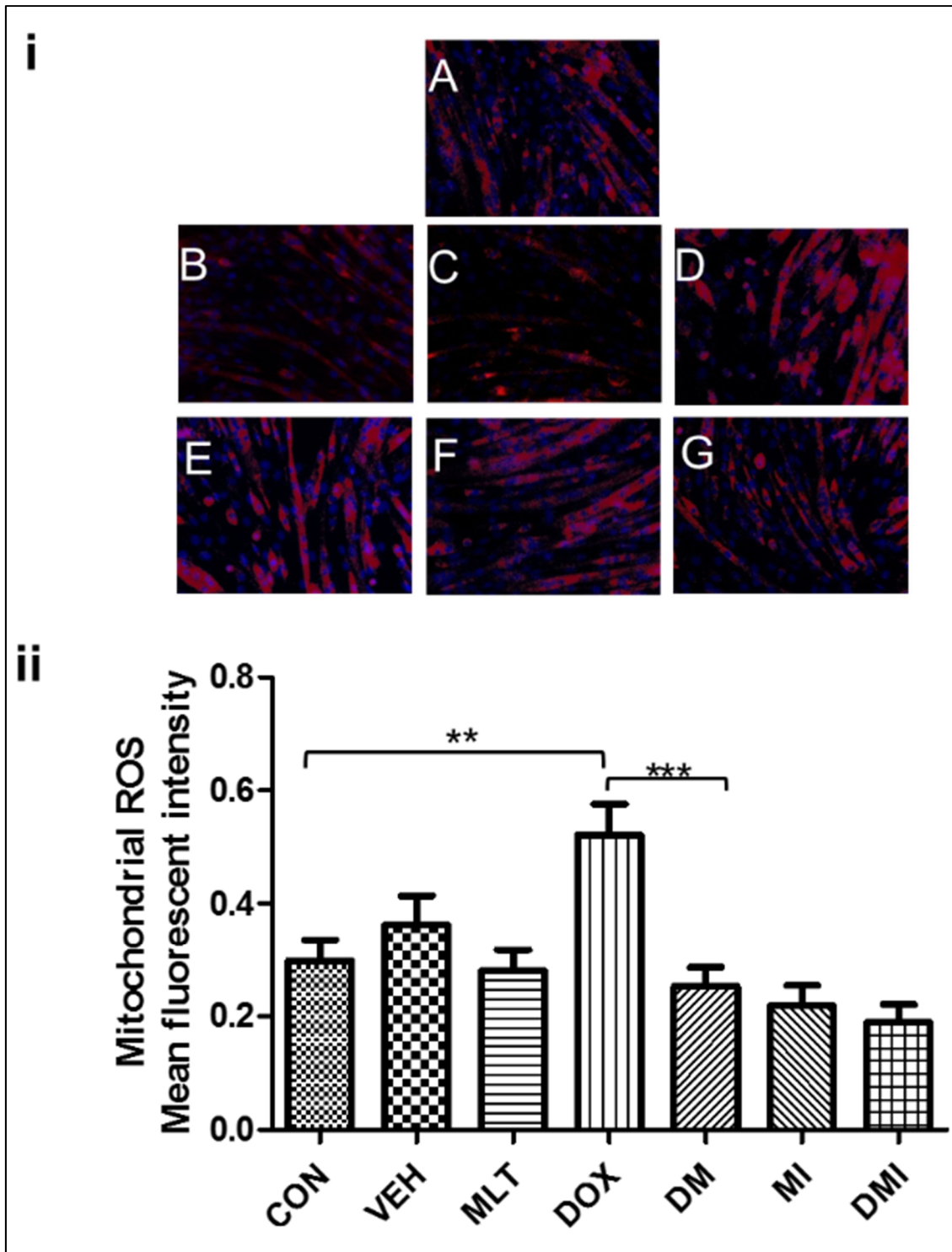
Figure 4.2.3: A comparison of mitochondrial ROS production in treatment groups. i) Myotubes were labelled with MitoSOX red and Hoechst (nuclei), and were overlaid with phase contrast images. Representative Images (A) CON; (B) VEH; (C) MLT; (D) DOX, (E) DM, (F) MI & DMI. ii) The data indicate the relative red fluorescence intensity. Data represent the mean from three wells, 100 µm scale. Abbreviations- CON: control; VEH: vehicle; MLT: melatonin; DOX: doxorubicin; DM: doxorubicin+melatonin; MI: melatonin+luzindole; DMI: dox+melatonin+luzindole.

4.2.3 The effect of melatonin and doxorubicin treatment on mitochondrial sirtuin levels

A significant (* $p < 0.05$) increase in SIRT3 expression was evident in the DOX and MI group compared to the control (Fig. 4.2.4).
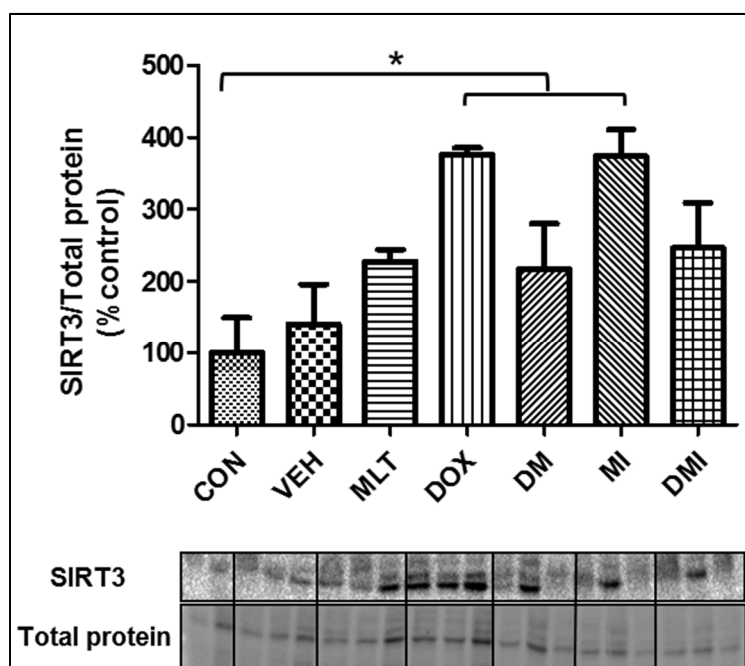
Figure 4.2.4: Mitochondrial SIRT3 expression. Values are expressed relative to the control values (mean ± SEM). * $p < 0.05$, n = 3. Abbreviations- CON: control; VEH: vehicle; MLT: melatonin; DOX: doxorubicin; DM: doxorubicin+melatonin; MI: melatonin+luzindole; DMI: doxorubicin+melatonin+luzindole.

4.2.4 The effect of melatonin and doxorubicin treatment on protein degradation through autophagy.

4.2.4.1 Autophagy

4.2.4.1.1 CYTO-ID detection kit

The DOX group showed a significantly increased response in chloroquine (** $p < 0.01$), indicating autophagy inhibition, and the rapamycin (autophagy inducer) + chloroquine (* $p < 0.05$) treatment, from control levels. Furthermore, rapamycin treatment was significantly decreased compared to both chloroquine (* $p < 0.05$) and the

rapamycin+chloroquine (* p < 0.05) treatment in this group. The DM group had a significantly (* p < 0.05) increased response in chloroquine treatment from control levels (* p < 0.05), whereas the rapamycin treatment was significantly decreased compared to chloroquine treatment (Fig. 4.2.5).
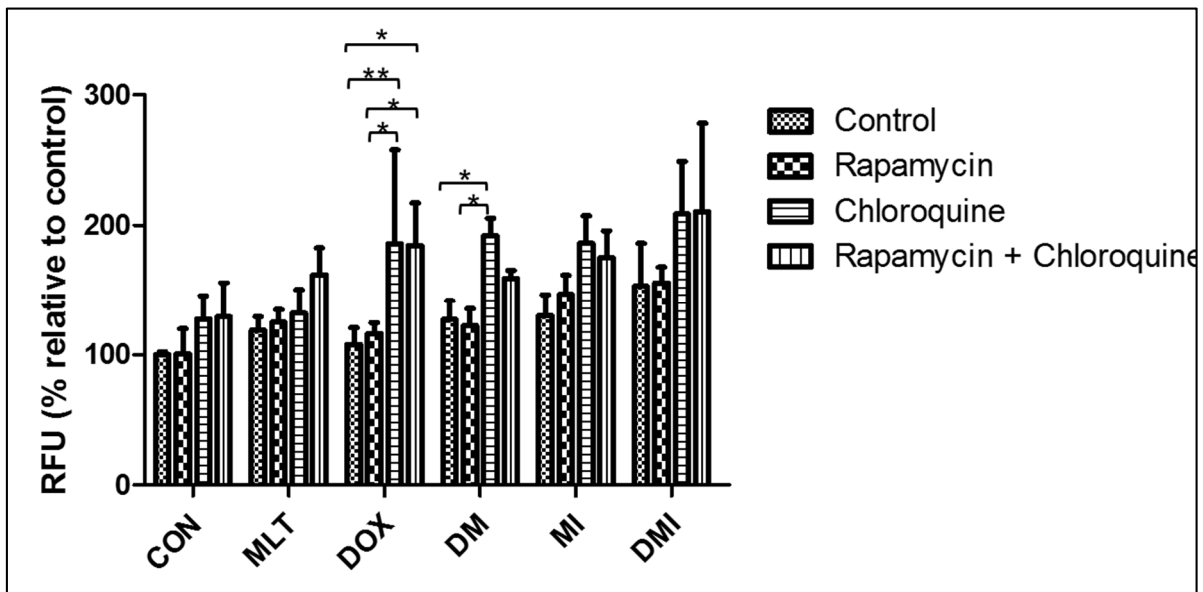
Figure 4.2.5: Detection of autophagy by microplate reader in myotubes. Myotubes were cultured with DMSO (control), 0.5 uM rapamycin, or 0.5 uM rapamycin+ 10 uM chloroquine (CLQ) for 18 hours, followed by staining with green detection reagent as described in the manual. Nuclei were counter stained with Hoechst 33342. The data indicate the relative green fluorescence intensity normalized by blue fluorescence intensity. Data represent the mean from three wells. Abbreviations- CON: control; VEH: vehicle; MLT: melatonin; DOX: doxorubicin; DM: doxorubicin+melatonin; MI: melatonin+luzindole; DMI: doxorubicin+melatonin+luzindole.

Chapter 5: Discussion

The role of melatonin (MLT) in doxorubicin (DOX)-induced skeletal muscle atrophy has not previously been investigated in a clinically relevant tumour-bearing model. There is currently a lack of information regarding the effect of MLT treatment on mitochondrial function during DOX-induced myotoxicity, as well as its role during muscle regeneration. This thesis aimed to provide insight into the effect of MLT on cell death, autophagy/mitophagy, mitochondrial fission and fusion, mitochondrial biogenesis, sirtuin levels, and muscle degradation and regeneration processes.

5.1 *In vivo study*

5.1.1 DOX treatment reduces body mass and skeletal muscle cross sectional area (CSA)

Cachexia is a syndrome of involuntary weight-loss, distinguished by progressive loss of fat mass, skeletal muscle and functional impairment (Ohnuma and Adigun 2017). The cachectic state can be induced by the condition of cancer (Mattox 2017) or due to chemotherapeutic toxicity (Wang et al. 2015, Rasanen et al. 2016). The *in vivo* model used in this study was modified from a xenograph mouse breast cancer tumour model previously established by our research group at Stellenbosch University (Dr Mark Thomas, PhD thesis, 2012). Results from that mouse model showed that mice in the tumour control group had significantly reduced body mass and reduced *soleus*, *plantaris* and *gastrocnemius* muscle weights and cross sectional areas compared to the group that bore no tumour. Although tumour growth over time was apparent in both models, no differences in body weight was observed between the control and tumour control groups in the current study (Fig. 4.1.1B).

It is now evident that muscle atrophy induced by cancer and chemotherapy follow similar signalling pathways (Barreto et al. 2016). However, the current results indicate that *soleus* (Fig. 4.1.3A), *plantaris* (Fig. 4.1.4A) and *gastrocnemius* (Fig. 4.1.5A) muscle weights and cross sectional areas (Fig. 4.1.3C, Fig. 4.1.4C and Fig. 4.1.6B) in the tumour control group were also not significantly different from the control group, except for a significant (* $p < 0.05$) reduction in Type I slow oxidative fibres of the *gastrocnemius* muscle (Fig. 4.1.6B). These results, taken together, indicate that cancer cachexia was not achieved with the current model. This could perhaps be due to the shorter time period in which the tumour was allowed to grow or the number of cells used to inoculate the rats. Muscle wasting has been well characterized in rodent models of cancer and a high incidence is also seen among patients with gastric, pancreatic, lung and colorectal tumours (Tan and Fearon 2008). Rat models include Walker 256 carcinosarcoma, Morris hepatoma 7777, Yoshida ascites hepatoma 130 (AH130); whilst murine models include the MCG 101, murine adenocarcinoma 16 (MAC16), human melanoma, C26 colorectal adenocarcinoma and the most commonly used Lewis lung carcinoma model (Mueller et al. 2016, Holecek et al. 2012). It was also previously shown that a MCF-7 xenograft model of breast cancer induced profound cachexia (Kumar et al. 2003). In order to test the effectiveness of anti-cachexia compounds, models should more closely mimic the clinical complexities by ideally including chemotherapy treatment.

In the current study, rats began DOX treatment 14 days after tumour inoculation. The rats treated with DOX demonstrated a 22.5%, 5.6%, 25.8% and 26.8% reductions in means of body mass at sacrifice (Fig. 4.1.1B), *soleus* (Fig. 4.1.3A), *plantaris* (Fig. 4.1.4A) and *gastrocnemius* (Fig. 4.1.5A) muscle weights respectively compared to control. This was despite only 9 days of DOX treatment. Our data thus, confirmed the

cachectic state, as defined by Strasser (2008): a loss of more than 5% of body mass in the previous 6 months. This is taken as a defining point for cachexia where death usually occurs in patients when there is 30% weight loss (Tisdale 2002). DOX-induced skeletal muscle atrophy in this model is further confirmed when determining muscle weight to body weight ratios. The muscle weight to body weight ratios for both *plantaris* (Fig. 4.1.4B) and *gastrocnemius* (Fig. 4.1.5B) muscles were significantly decreased when compared to controls. This finding is supported by qualitative (Haematoxylin and eosin staining, Fig. 4.1.2) and quantitative (CSA analysis of NADH-TR stained muscle fibres) histology of the general structure of gastrocnemius muscle which showed that both slow and fast twitch muscle fibre CSA's were significantly reduced by the chemotherapeutic agent (control Type I: 906.81±73.86 & Type II: 2073.±73.86) (DOX Type I: 504.11±92.78 & Type II: 1281.87±230.07) compared to CON (Fig. 4.1.6A&B). Hydock et al. (2011), showed that muscle mass of both *soleus* (mainly type I fibres) and *extensor digitorum longus* (mainly type II fibres) decreased with DOX treatment in rats, similar to what was found in the current study. The group was also able to show that the degree of dysfunction was greater in the *soleus* muscle (Hydock et al. 2011), which brings up the question why type II fibres would be more protected from DOX-induced myotoxicity? It might be due to the fact that DOX is more localised in type I fibres since mitochondrial densities are greater compared to type II fibres thus less ROS damage is experienced by type II fibres. Other possible reasons could be that type II fibres contain larger sized motor neurons or that antioxidant defence capacity is greater. This question and these proposed assertions remain to be fully elucidated.

Direct injection of DOX into skeletal muscle has been found to result in reduced cross sectional areas (CSA) of sternocleinomastoid muscle of rabbits (McLoon et al. 1998) and permanent muscle loss in eyelids of patients who suffer from hemi-facial and

blepharospasm (Wirtschafter and McLoon 1998). Moreover, it was shown in several studies that intravenous administration of DOX resulted in a reduction of body weight in two different rodent models (Hajjaji et al. 2012, Rasanen et al. 2016) which was shown in a different study to be mainly due to skeletal muscle tissue loss (Nissinen et al. 2016). These observed effects might have been due to a reduction in food intake since clinical side effects of the drug include nausea and loss of appetite (Braun et al. 2014); which induce a systemic hypercatabolic state (Baracos 2006). Although enforced reduction of food intake in other groups of rodents could control for DOX-induced effects on appetite, the current studies focussed on elucidating mechanisms of effect that were largely not proposed to be affected by caloric intake.

5.1.2 MLT and DOX treatment effects in circulation

When studying skeletal muscle wasting induced by cancer cachexia, DOX or both cancer cachexia and DOX as treatment, it is important to not only consider skeletal muscle in isolation, since the cardiovascular system is also affected due to systemic inflammation present during cancer cachexia and cardiotoxicity caused by DOX (Braun et al. 2014 and Kazemi-Bajestani et al. 2014). A compromised cardiovascular system has direct effects on skeletal muscle, since oxygen and nutrient supply to skeletal muscle would decline resulting in a hypoxic environment and reduced oxidative metabolism which may be directly related to cachectic patient muscle weakness and fatigue.

Myoglobin, the iron binding and oxygen carrier in muscle, was significantly increased in circulation with DOX treatment (Fig. 4.1.7). This indicates that muscle membrane structure was compromised resulting in the leakage of this marker into circulation (Macaluso et al. 2014). It was shown previously that the transcription factor Mef2C was downregulated in skeletal muscle of cachectic mice (Shum et al. 2012). This

transcription factor is important for myogenesis and since the myoglobin gene is also a target of Mef2C, Shum et al. (2012) speculated that it may be associated with decreased oxygen transport capacity and reduced ATP generation. There would thus be a double complication with regard to myoglobin for patients if it was found that myoglobin is also high in circulation of patients on DOX treatment, as was found here in rodents. As previously mentioned, DOX has a binding affinity for iron (Mjos et al. 2015) and in the centre of myoglobin's structure an iron atom resides in the ferrous oxidation state (Arcon et al. 2015) to which oxygen directly binds. Oxidation of this molecule results in the inability to normally bind oxygen. This could therefore be a third aspect of burden.

A hallmark of cancer cachexia is anaemia which presents with haemoglobin levels of < 12 g/dl, occurring in almost 40% of cancer patients (Knight et al. 2004). Anaemic patients present with symptoms of fatigue, paleness of the skin, shortness of breath (Jelani and Katz 2010), and it is thought that DOX may induce anaemia independently of cancer cachexia (Bhinge et al. 2012). Haemoglobin has a similar structure to myoglobin and transports oxygen in circulation; at muscle tissue it offloads its oxygen to myoglobin which further transports oxygen within muscle. Interestingly, haemoglobin levels were also significantly reduced in the DOX treated group (Table 3) which indicates that reduced oxygen is supplied to muscle tissue with potentially compromised myoglobin molecules. The oxygen transport burden from lungs to mitochondria could therefore be four-fold.

In the current study serum myoglobin levels were significantly increased in the DOX group alone (Fig. 4.1.7). MLT treatment prevented myoglobin leakage into circulation indicating a muscle membrane protective effect, since the DM group myoglobin levels were also at control levels (Fig. 4.1.7). Furthermore, haemoglobin levels (Table 3)

were significantly reduced in the DOX treatment group. MLT also prevented the DOX-induced haemoglobin (Table 3) reduction (Table 3), further sparing oxygen carrying capacity to and in the muscle. This might be due to its antioxidant effects. Free radical formation can be blunted due to oxygen oxidation of ferrous iron to ferric iron in both myoglobin and haemoglobin; or potentially by directly interacting with DOX in membranes, thus preventing damage to the myocyte membranes and structures (Costa et al. 1997, Alves et al. 2017). Further evidence for this is that the haematocrit levels and RBC number (Table 3) improved in MLT treated rats in opposition to the significant reductions observed in DOX treated animals. This might indicate that MLT is able to rescue the myelosuppressive effects of DOX (Chen et al. 2017). A study by Pin et al. (2015), who co-administered erythropoietin (to prevent anaemia) together with mild exercise in tumour-bearing mice, found that oxidative muscle fibres were spared from atrophy. The combination treatment was found to lessen muscle strength loss and prevented mitochondrial ultrastructural alterations, while it increased oxidative capacity and ATP content (Pin et al. 2015).

MLT treatment also increased WBC, neutrophil and eosinophil numbers compared with control levels (Table 3), while these parameters were significantly reduced with DOX. The eosinophil counts of the tumour control and vehicle groups were significantly greater than that of the DOX group and higher than control and MLT treated groups (Table 3), which could be explained by tumour-associated tissue eosinophilia and tumour-associated blood eosinophilia (associated with metastasis and a poor prognosis) which have been described in a variety of tumour types (Lowe et al. 1981). Both neutrophils and eosinophils are required for muscle regeneration, as these cells are recruited to muscle damage sites to rapidly clear cellular necrotic debris. It has also been shown that eosinophils are essential for muscle repair as it

secretes cytokines which activate the regenerative actions of muscle resident fibro/adipocyte progenitors thus forming an adaptive niche for proliferating satellite cells in regenerating muscles (Heredia et al. 2013). The basophil count was significantly increased in the MLT group alone compared to all other groups. Basophils release the vasodilator, histamine, which is able to increase blood flow to tissues to improve oxygen and nutrient delivery to muscle. The results of the peripheral WBC differential counts should be compared with results of intramuscular levels of WBC to make further conclusions which should be undertaken in future studies.

5.1.3 Improvement of muscle antioxidant capacity by MLT

MLT is an unique antioxidant since it is capable of scavenging a range of different radicals, while its metabolites also possess antioxidant properties (Zang et al. 1998, Zhang and Zhang 2014). MLT not only scavenges free radicals, but it was also shown to improve antioxidant defence systems in muscle (Leonardo-Mendonca et al. 2017).

Intramuscular muscle antioxidant capacity was significantly increased in the MLT treated groups compared to all other groups (Fig. 4.1.13), which highlights the free radical (Zhang and Zhang 2014) scavenging properties of MLT. It is important to note that ROS is important for normal cell function and should be modulated within homeostatic ranges. Unlike many other antioxidants, MLT not only reduces ROS but may act as 'conditional' pro-oxidant and increase ROS as demonstrated in some *in vitro* studies (Zhang and Zhang 2014), suggesting that MLT could potentially buffer ROS levels. This characteristic of MLT is concentration dependent, cell type dependent, is not correlated with cytotoxicity and is mostly observed in cancer cells.

ROS stimulated catabolism of myotubes has been shown to result in reduced myosin and sarcomeric actin *in vitro* (Gilliam et al. 2012). Increased ROS levels can directly

damage myocytes or indirectly via the stimulation of catabolic signalling. The mitochondria are major sights of ROS generation and MLT and its metabolites have been shown to protect the electron transport chain and mitochondrial DNA from oxidative damage thus preserving energy production (Coto-Montes et al. 2016).

MLT may improve antioxidant capacity since one MLT molecule is capable of scavenging multiple reactive oxygen species (Tan et al. 2002). Moreover, MLT may be improving intramuscular antioxidant defence capacity by supporting several other local antioxidant enzymes, as it has previously been shown to stimulate the production of the antioxidant, glutathione (Korkmaz et al. 2008), the most abundant non-protein thiol in cells with intracellular concentrations of 1–10 mM (Smith et al. 1996). This capability of MLT to enhance tissue resident antioxidant defences is crucial since a growing body of evidence show that antioxidant supplementation may induce muscle atrophy by downregulating endogenous antioxidant defences (Espinosa et al. 2016). This was demonstrated in a C26-bearing mouse model of cancer cachexia in which cancer cachexia was enhanced following supplementation with a combination of antioxidants, rich in catechins, quercetin and vitamin C (Assi et al. 2016). Their result may have been influenced by the combination of multiple antioxidants or the additive dosage, but the results of this thesis confirm MLT's primary antioxidant capacity enhancing effect.

5.1.4 The role of melatonin in markers of mitochondrial biogenesis; mitophagy fission and fusion

The peroxisome proliferator-activated receptor-γ coactivators (PGC-1) are the master regulators of mitochondrial biogenesis. DOX treatment significantly reduced PGC1-α protein levels from control levels, whereas MLT pre-treatment restored PGC1-α protein levels to control levels (Fig. 4.1.14), which indicates that MLT protects

mitochondria which is consistent with literature where MLT was found to restore PGC1-α protein levels to control levels in pinealectomized rats (Teodoro et al. 2014).

PGC-1 coactivators are enriched in tissues such as skeletal muscle which has a high capacity for mitochondrial respiratory function (Baar et al. 2002, Pilegaard et al. 2003). Growing evidence suggest that PGC-1 signalling plays an important role in skeletal muscle structure and function (Lin et al. 2002, Arany et al. 2007, Handschin et al. 2007), and ROS induced PGC1-α signalling forms a key axis in the regulation of skeletal muscle mitochondrial function (Liao 2012). With regard to DOX myotoxicity, DOX treatment has been shown to induce an oxidative-to-glycolytic metabolic shift in normal skeletal muscle (Fabris and MacLean 2015). In contrast, PGC-1α is implicated in muscle fibre-type switching and its over-expression in transgenic mice has been shown to increase the proportion of oxidative type I fibres (Zhang et al, 2017). Interestingly, MLT has also been shown to modulate PGC1-α signalling in different tissue types (Guo et al. 2014, Teodoro et al. 2014, Yu et al. 2017), therefore the effect of MLT on PGC1-α expression was investigated during DOX-induced myotoxicity.

A study conducted by Kavazis et al. (2014) demonstrated that short term endurance exercise was protective against acute DOX-induced FoxO transcription in skeletal muscle by increasing PGC1-α in the *soleus* muscle. In support of the findings of this thesis, a recent study demonstrated that MLT reduced ROS production, enhanced mitochondrial biogenesis and preserved mitochondrial function thus improving myocardial ischemia/reperfusion injury in type 1 diabetic rats (Yu et al. 2017). The authors concluded that the AMPK-PGC1α-SIRT3 axis was essential in this process. In further support of the findings presented here, Teodoro et al. (2014) demonstrated that MLT was able to restore mitochondrial function and insulin resistance via

activation of CREB- PGC-1α pathway in an *in vitro* model of insulin resistance in rat skeletal muscle primary cells and *in vivo* in pinealectomized rats.

Mfn2 is a target of PGC-1α that enhances mitochondrial fusion (Zorzano 2009). Mfn2, specifically aids the merger of neighbouring mitochondrial membranes resulting in mitochondrial fusion (Papanicolaou et al. 2012) and its decreased expression in muscle is associated with cachexia-induced muscle loss (Xi et al. 2016). However, literature on fission and fusion events in skeletal muscle is limited and needs to be explored further.

Mfn2 protein expression in the DM group was significantly (* $p < 0.05$) reduced from the average control group's level (Fig. 4.1.16B). This may indicate that DOX treatment decreased fusion and MLT pre-treatment was not able to recover Mfn2 to control levels. Previously it was shown that acute administration of DOX (20 mg/kg body for 24 h) did not alter Mfn2 protein levels in rat *soleus* muscle (Kavazis et al. 2014). Furthermore, in a recent study by Tang et al. (2017) it was found that DOX decreased cardiomyocyte Mfn2 expression which stimulated mitochondrial fission and ROS production leading to cardiomyocyte cell death. A reduction in Mfn2 may represent another mechanism by which DOX potentially induces atrophy since decreased Mfn2 expression is associated with muscle loss in cancer cachectic mice (Xi et al. 2016).

No significant differences in Mfn1 protein expression were observed, however the MLT group showed a trend (* $p < 0.065$) toward an increase compared to the DOX group (Fig. 4.1.16A). Mfn1 interacts with Mfn2 at the mitochondrial membrane and its expression has been shown to increase mitochondrial length and interconnectivity (Legros et al. 2002), which might indicate that MLT may aid in promoting mitochondrial fusion.

Mitophagy in skeletal muscle has been receiving increased attention from researchers, especially within the context of muscle atrophy (Wu et al. 2009, Romanello et al. 2010). One major pathway of mitophagy is the PINK1/Parkin pathway. PINK1, a mitochondrial serine/threonine kinase, becomes stabilized at the outer mitochondrial membrane (OMM) following depolarization of the OMM. PINK1 also phosphorylates ubiquitin, since Parkin requires ubiquitin ligase activity (Moyzis et al. 2015). PINK1 next binds to Parkin aiding its translocation to the mitochondria where Parkin ubiquitinates mitochondrial surface proteins (Koleini and Kardami 2017). Parkin-dependent ubiquitination of mitochondrial surface proteins results in their proteasome degradation, mitochondrial fragmentation and thus promotes mitophagy (Wrighton 2011, Moyzis et al. 2015, Saito and Sadoshima 2015). In the current study MLT treatment alone significantly (* $p < 0.05$) increased the mitophagy marker, PINK1 compared to all other groups, while the DM group remained at control levels (Fig. 4.1.17). This finding indicates that MLT could potentially enhance mitophagy, which is in line with literature where evidence is provided for mitophagy enhancement with MLT to promote *de novo* mitochondrial biogenesis (Tan et al. 2016). Various studies using different tissue types have shown that MLT treatment was able to exert protective effects by increasing PINK1 protein levels in order to clear damaged mitochondria (Kang et al. 2016, Cao et al. 2017). MLT's ability to increase PGC1- α levels and to promote mitophagy relates well with MLT's anti-anaemic effects discussed in section 5.1.2, since Pin et al. (2015), showed that mild exercise and treatment with erythropoietin prevented atrophy in tumour-bearing mice and that these benefits relied on PGC-1α induction and the promotion of mitophagy.

5.1.5 The effects of MLT and DOX on muscle degradation processes

In line with MLT's oncostatic effects, it is able to induce apoptosis in tumour cells but can also inhibit apoptosis in other tissue (Hill et al, 2011, Hu et al. 2016, Proietti et al. 2013, Sheen et al. 2016, Slominski et al. 2012). It is well established that DOX-induced cardiotoxicity results in the loss of cardiomyocytes through cell death pathways. Skeletal muscle however is multi-nucleated and the extent to which it is affected by cell death mechanisms is debatable, since apoptosis of single cells cannot account for the complexity of the programmed death of multinucleated myofibres. Numerous articles though reported on myonuclei loss by mechanisms commonly associated directly with apoptosis when rodents hindlimbs were suspended (Bennett et al. 2013, Hao et al. 2011).

In the current study, cCaspase3, a marker of apoptosis was significantly increased in the DOX only group compared to controls. Although the DOX with MLT co-treatment group tended to have reduced cCaspase3 compared to DOX alone, it was not significantly different (4.1.8A). No significant differences were observed in the apoptotic marker cPARP (4.1.8B).

Conflicting evidence for DOX-induced cell death has been reported. An acute high dose (20 mg/kg body weight) administration of DOX in mice did not change in caspase activity over a period of nine days in mixed fibre type *gastrocnemius* muscle (Campbell and Quadrilatero 2016). However, in a similar model of acute high dose DOX administration (15 mg/kg body weight), DOX induced DNA fragmentation of myonuclei as well as Bax activation in *gastrocnemius* muscle (Yu et al. 2014). It has been demonstrated that MLT decreased the apoptotic marker cCaspase3 in C2C12 myoblasts exposed to oxidative stress (Kim et al. 2011). *In vivo* studies further confirmed this action of MLT where a significant reduction in cCaspase3 was observed

4 days after skeletal muscle contusion injury in MLT supplemented rats (Stratos et al. 2012). The selective properties of MLT have been demonstrated in studies where caspase cleavage was induced in melanoma and breast cancer cells, demonstrating its pro-apoptotic ability in cancer cells (Gatti et al. 2017).

It is hypothesised that muscle fibres maintain their myonuclear domains in conditions of atrophy (Tews 2005), where evidence exists that only a subset of myonuclei undergo apoptosis during skeletal muscle atrophy, while the muscle fibre remains intact (Liu and Ahearn 2001). However this notion has been challenged by experiments which demonstrated that myonuclear loss is negligent during atrophy where nuclei in the same muscle fibres were monitored *in vivo* for up to one month. The authors provided data that nuclei undergoing apoptosis during atrophy were derived from mononucleated stromal or satellite cells, but not myonuclei from the muscle fibre (Bruusgaard and Gundersen 2008). Therefore the current thesis research also investigated markers of atrophy without apoptosis.

The effects of Doxorubicin and Melatonin on the Ubiquitin ligases

The FoxO family of transcription factors is important for MuRF1 and MAFbx gene expression, along with other regulators including AMPK and PGC1-α. FoxO1 followed a similar protein expression pattern to FoxO3a but no significant differences were observed between groups (4.1.9C). FoxO3a protein levels were significantly increased in the DOX treated group compared to control and the DM group (4.1.9D). A significant increase in the atrophy marker MURF1 was observed in DOX treated groups compared to the control group and the MLT only group (4.1.9B), while MAFbx protein levels in the DM group were significantly reduced compared to all other groups, excluding the DOX treated group (4.1.9A).

It has previously been demonstrated that acute exposure (24 h) of *rats* to DOX (20 mg/kg) resulted in increased FoxO3, MuRF-1 and BNIP3 expression in *soleus* muscle (Kavazis et al. 2014). It was also demonstrated that DOX administration (15 mg/kg body weight, 48 h) resulted in increased mRNA expression of MAFbx and MURF1 in skeletal muscle *in vivo* independent of FoxO activation (Yamamoto et al. 2008). On the other hand, both MAFbx and MuRF1 mRNA levels which are normally very high in the hindlimb muscle of spinal cord injured rats, decreased in response to the exogenous MLT treatment (Park et al. 2012). Similarly, muscle atrophy-related genes, MAFbx and MuRF1 were down-regulated in *gastrocnemius* muscle of MLT-administered rats after stroke-induced muscle atrophy although only MAFbx at the mRNA level was attenuated in the *soleus* of these rats (Lee et al. 2012). The MURF1 and MAFbx protein expression patterns are quite different but it is important to note that MURF1 and MAFbx transcriptional control is complex and variable expression patterns are not uncommon, especially since multiple transcription factors can activate these atrogenes (Bodine and Baehr 2014). The results of this thesis seem to indicate that DOX may be inducing atrophy via FOX3a activation and subsequently MURF1 protein levels, while MLT may reduce atrophy signalling by decreasing MAFbx protein levels. Downstream from atrophy signalling no significant differences were observed in protein levels of two markers of the UPP, namely K48 and K63 (4.1.10).

Melatonin regulates autophagy

Phospho-mTOR levels were significantly increased in the DOX group compared to control levels (Fig. 4.1.11A). Increased mTOR protein levels are associated with the inhibition of autophagy and lysosome biogenesis (Kim and Guan 2015). Moreover, p62 protein levels in the DOX group were significantly increased in comparison to the DM group which indicates that it is not being cleared through autophagic degradation

(Fig. 4.1.11B). LC3-1 to LC3-IIB conversion levels in the DOX group were significantly reduced compared to all other groups which is indicative of lack of autophagosome formation (Fig. 4.1.11C). These results taken together indicate that the autophagy process may have been inhibited by DOX administration in the current study and thus potentially resulted in the accumulation of dysfunctional organelles. However, other studies have reported that DOX induced upregulation of LC3-II levels (Kobayashi et al. 2010), and increased autophagic vacuoles in both mice (Kawaguchi et al. 2012) and rats (Wu et al. 2014).

In contrast, the DM group had significantly reduced protein levels of phospho-mTOR and p62 compared the DOX group, while LC3-1 to LC3II-B conversion levels were at baseline levels indicating that autophagy was not being inhibited, p62 and its cargo were being cleared and autophagy degradation was functional. These findings suggest that MLT may prevent skeletal muscle atrophy by regulating autophagy positively. But, several other studies have reported that MLT can attenuate autophagy *in vivo* in response to different stressors (Feng et al. 2017, Nopparat et al. 2017, San-Miguel et al. 2015, Trivedi et al. 2016 and Zhang et al. 2016).

A number of studies have demonstrated that stimulating autophagy (starvation, moderate diet restriction and Rapamycin administration) prior to DOX treatment is protective, since this would aid the elimination of dysfunctional mitochondria which are a source of ROS (Tal et al. 2009, Lee et al. 2012). Furthermore, data from other *in vivo* models suggested a protective reduction of LC3-II in response to DOX administration and showed that autophagy upregulation promoted cardiomyocyte survival (Sishi et al. 2013, Bartlett et al. 2016). It was demonstrated in another study that DOX was able to block autophagic flux through the prevention of lysosome acidification (Li et al. 2016). In the current thesis research autophagic flux was not

assessed since the appropriate groups of autophagy inhibitors and inducers were not included in the study design.

DOX treatment increases Calpain-3 protein levels

Increased intracellular calcium levels activate calpains which cleave important structural proteins. Min et al. (2015) designated both mitochondrial ROS generation and calpain activation as major contributors to DOX-induced muscle dysfunction. Moreover, calpains inhibit autophagy by preventing fusion of lysosomes and autophagosomes, and thus the maturation of autophagosomes (Sarkar, 2013). In line with literature we found that DOX induced an increase in calpain in this study.

The protein expression patterns of ubiquitously expressed calpain -1 & -2 were similar to that of calpain-3 but no significant differences in ubiquitous calpains were observed between groups (Fig. 4.1.12A&B). However a significant increase in calpain-3 protein levels was observed in the DOX treated group compared to the MLT treated groups (Fig. 4.2.12C). To the best of my knowledge, this is the first evidence suggesting a role for calpain 3 activation with DOX treatment. On the other hand, a lack of calpain-3 results in the most severe phenotypic form of muscle degeneration which is characterised by oxidative stress, mitochondrial abnormalities and energy deficits (Kramerova et al. 2009). Calpain-3 is thus associated with sarcomeric remodelling and maturation following degradation. This result may indicate that the DOX treated group may be undergoing early stage regeneration. In many models the effects of DOX-induced muscle atrophy were studied in acute models, where high doses of DOX were administered which gives little information with regard to what happens in a clinical setting where patients receive intermittent and lower doses of DOX treatment. Finally, it has been shown previously that MLT treatment both *in vitro* (Suwanjang et al. 2013)

and *in vivo* (Samantaray et al. 2008) attenuated calpain degradation, which is consistent with findings in this study.

5.1.6 Effects of DOX and MLT on muscle regeneration

Muscle regeneration is an important homeostatic process of the adult skeletal muscle, which maintains muscle health throughout life in response to stimuli, such as injury by activating muscle satellite cells (Joanisse et al. 2017).

In the current study satellite cell number (Fig. 4.1.18B) and PAX7 protein levels (Fig. 4.1.18C) did not decrease from control values with DOX treatment but tended towards being slightly higher than control levels (not significant). There was evidence of membrane damage as seen by Mb leakage into circulation with DOX treatment and membrane damage alone could be a cue for satellite cells to become active. Indeed, MyoD (Fig. 4.1.19B) protein levels in the DOX group were significantly higher compared to the MLT group suggesting activation. DOX treatment has previously been shown to decrease satellite cell number by inducing apoptosis (Bruusgaard and Gundersen 2008) which may explain the lack of increase in Pax7+ cells despite activation.

Little evidence exists for MLT's actions during muscle regeneration following injury but an *in vivo* study showed that MLT may improve muscle regeneration following crush injury in rats by increasing satellite cell numbers (Stratos et al. 2012). MLT treated groups (MLT and DM) had significantly increased satellite cell numbers (Fig. 4.1.18B) and PAX7 protein levels (Fig. 4.1.18C) compared to control groups, indicating stimulation of proliferation which is in agreement with the results of Stratos et al. (2012) in their muscle crush injury model. But, no differences in protein levels of myogenin

(Fig. 4.2.19A) were observed between groups, indicating little or no effect on differentiation.

MLT treatment has been shown to increase IGF-1 levels of satellite cells in an atrophy model of rat castration (Oner et al. 2008), as well as IGF-1 receptor expression in a model of atrophy subsequent to stroke (Lee et al. 2012). IGF-1 is a positive regulator of both proliferation and differentiation of skeletal muscle cells (Miyake et al. 2007). In reviews by Kurabayashi et al. (1993 and 1994) it was discussed that MyoD and myogenin gene transcripts decreased in C2C12 myoblasts following DOX exposure. Furthermore, it has been determined previously that MyoD is directly involved with DNA repair following genotoxic stress (Kobayashi et al. 2004) which could explain the higher protein levels of MyoD observed in the DOX group compared to MLT group. ROS levels have been shown to increase with age (Romano et al. 2010) and conversely MLT has been shown to decline with age (Hardeland 2012, Song et al. 2016). Increasing evidence shows that MLT protected against age-related mitochondrial oxidative damage (Song et al. 2016). Sarcopenia is the gradual degenerative loss of skeletal muscle mass with aging and is associated with reduced satellite cell number (Coto-Montes et al. 2016). In a recent review by Coto-Montes et al. (2016) the authors speculated that MLT may be able to increase satellite cell number and improve regenerative processes in the sarcopenic condition. The current result showed that MLT may increase satellite cell number (4.1.19) after DOX-induced skeletal muscle atrophy.

Studies that have investigated the effects of MLT on satellite cells and skeletal muscle regenerative processes remain limited. However, the protective and regulatory effects of MLT on the drive to commitment and differentiation of mesenchymal stem cells (MSCs) into osteogenic, chondrogenic, adipogenic or myogenic lineages may provide

some insight (Luchetti et al. 2014). A role for MLT in the control of survival and differentiation of mesenchymal stem cells (multipotent progenitor cells capable of transdifferentiating into various cell lineages and primarily involved in developmental and regeneration processes) has been proposed (Luchetti et al. 2014). MSCs possess strong paracrine capacity by secreting cytokines and growth factors capable of suppressing the immune system, inhibiting fibrosis and apoptosis, augmenting angiogenesis and stimulating differentiation of tissue specific stem cells (Gnecchi and Melo 2009). MLT has been shown to confer cell protective and regulatory effects in an array of immune and non-immune cell subsets (Luchetti et al. 2010). These effects are believed to extend to MSCs as well, since MLT pre-treatment of MSCs in an *ex vivo* model of ischemic kidney injury promoted the secretion of proangiogenic/mitogenic factors and prevented cell apoptosis (Mias et al. 2008). Moreover, it has been revealed that MSCs express MLT-receptors on their surface further strengthening support for the role of MLT as a protective factor for MSCs (Radio et al. 2006, Mias et al. 2008, Sethi et al. 2010, and Wang et al. 2013). It was also demonstrated that MLT protected MSCs following exposure to H_2O_2 (Mias et al. 2008). Specifically, acting via the MLT receptors it conferred anti-apoptotic activity and regulated MSC antioxidant genes. This was achieved by MLT inducing the overexpression of the antioxidant enzymes catalase and superoxide dismutase-1. These effects of MLT on MSCs were confirmed by studies which showed that transplanted MSCs were protected against oxidative stress in the microenvironment of injured tissue (Liu et al. 2013, Wang et al. 2013).

5.1.7 Effects of DOX and MLT on sirtuin levels

DOX cardiotoxicity studies showed that SIRT1 down-regulation is accompanied by cell apoptosis and increased oxidative stress both *in vitro* and *in vivo* (Ruan et al. 2015, Sin et al. 2015, Du et al. 2017). Furthermore, it is becoming evident that MLT is

potentially modulating expression and/or activity of SIRTs, which is the subject of a recent review by Mayo et al. (2017). However, there are no studies to my knowledge where the role of MLT on SIRT activity in the context of DOX-induced myotoxicity has been investigated.

In the current study, mitochondrial specific SIRT3 protein levels were significantly increased in DOX treated (DOX and DM) groups (Fig. 4.1.20B). While, SIRT1 levels remained relatively unchanged (Fig. 4.2.20A). Cheung et al. (2015), demonstrated that SIRT3 protein overexpression attenuated DOX-induced ROS production *in vitro* and could thus potentially be used as therapy for DOX-induced cardiac dysfunction. Moreover, SIRT3 was found to attenuate DOX-induced cardiac hypertrophy, mitochondrial dysfunction (Du et al. 2017) and cardiomyopathy in mice (Pillai et al. 2017). Sin et al. (2016) demonstrated that DOX-induced myotoxicity in aged skeletal muscle was alleviated via SIRT1-dependent mechanisms following acute resveratrol treatment. In another acute model (20 mg/kg for 24 h) of DOX-induced myotoxicity, F344 rats were administered DOX and immediately starved of water and food for 24 hrs. Treatment did not affect the expression of SIRT1 and SIRT3 in *soleus* muscle of fasted animals which is in contrast to the aforementioned studies (Dirks-Naylor et al. 2013). In the current study, increased SIRT3 protein levels in the DOX treated groups may represent a form of compensatory adaptation by mitochondria to increase SIRT3 protein levels to combat DOX myotoxicity, perhaps in an attempt to increase PGC1-α activity which is a target of SIRT, as shown by Guo et al. (2014).

5.2 *In vitro* study

The current data revealed that DOX acted directly on differentiated C2C12 myotubes to decrease myotube width, and that this change was preceded by a rise in mitochondrial ROS. By treating the increase in ROS using MLT as antioxidant, DOX-stimulated myotube atrophy was attenuated. These findings identify a causal role for free radical mediated signalling in DOX-induced catabolism.

DOX and muscle catabolism

In the clinical setting, muscle atrophy is a significant contributor to mortality in cancer patients (Burckart et al. 2010). This state of catabolism remains while patients undergo treatment, affecting muscle function and resulting in poor quality of life (Knobel et al. 2001). Skeletal muscle atrophy is evident in patients receiving DOX treatment (Bonifati et al. 2000, Tozer et al. 2008), an effect recognised in *in vivo* models of chemotherapy (McLoon et al. 1998, Falkenberg et al. 2002, Gilliam et al. 2011). Here, a similar catabolic response in C2C12 myotubes was observed following DOX exposure. The present study adds to literature and demonstrates that direct exposure to DOX reduces myotube size.

DOX-induced mitochondrial ROS

DOX, an anthracycline, is highly effective against a variety of solid tumours but is limited by cytotoxic side effects on non-cancerous tissue via oxidative stress (Gilliam and St Clair 2011). It has been shown to increase ROS in both cardiac and skeletal muscle (Doroshow 1983, Gilliam et al. 2011, Smuder et al. 2011). The mitochondria are believed to be the main source of DOX-induced free radicals and to constitute the main source of superoxide anion in most tissues (Yen et al. 1996, Turrens 2003, Chandran et al. 2009). In a recent study where the progression of cancer cachexia

over time in a tumour bearing mouse model was investigated, the authors showed that ROS levels increased after a week of tumour inoculation and that mitochondrial dysfunction preceded the cachectic phenotype (Brown et al. 2017). DOX accumulates in the inner mitochondrial membrane (IMM), where it can be reduced by complex I of the electron transport chain into an unstable semiquinone. When oxidized, an electron is transferred to oxygen producing superoxide anion (Davies and Doroshow 1986, Chen et al. 2007, Monsuez et al. 2010). In the current study mitochondrial specific free radical activity was found to be significantly elevated in C2C12 myotubes following DOX exposure, providing evidence for the mitochondria as a potential source of ROS. Exposure of myotubes to MLT, a naturally produced antioxidant, inhibited the increase in superoxide levels caused by DOX. Interestingly, the inhibition of MT1 receptor by luzindole, did not alter superoxide levels from control levels, indicating that MLT was still able to confer protection. This could be due to the following reasons: 1) luzindole is mainly a MT1 receptor antagonist and only partial inhibitor of the MT2 receptor, thus MLT could be reducing ROS via MT2 receptor signalling; 2) due to MLT's amphiphilic properties it is capable of directly crossing lipid bilayers and could thus exert its effects in this way (Nosjean et al. 2001), since MLT's biological activity may be mediated via non-receptor mediated antioxidant activity (Dubocovich et al. 2003 & 2010); or/and 3) MLT may be reducing free radicals through the formation of its metabolites, which are also potent antioxidants.

Furthermore, there are three potential mechanisms by which MLT can decrease mitochondrial ROS production. The chemical backbone of the MLT molecule contains an indole moiety which interacts with oxidants, giving the molecule the capability to directly scavenge hydrogen peroxide, as it accumulates in the IMM (Szeto and Schiller 2011). MLT has been shown to promote mitochondrial respiration, which can reduce

ROS formation by increasing ATP production (Reiter et al. 2002). Finally, MLT attenuates the depolarization of mitochondrial membrane potential and the release of the apoptotic initiator protein cytochrome c, which is an enzyme of the electron transport chain. Cytochrome c release decreases ATP synthesis and increases electron leak, resulting in ROS production (Szeto and Schiller 2011) from the inner mitochondrial membrane (Wang et al. 2011).

The disruption of oxidative stress can induce catabolic signalling and protein degradation in skeletal muscle (Moylan and Reid 2007). MLT, which has a high affinity for mitochondria where it accumulates in high concentrations (Tan et al. 2016), negated changes caused by DOX, thus preserving myotube size by stabilizing cytosolic anti-oxidant activity. This finding suggests that increased mitochondrial ROS may cause DOX-induced atrophy.

Sirtuins

SIRT3, a NAD^+ dependant deacetylase, is the main mitochondrial specific protein deacetylase (Lombard et al. 2007) and is expressed in response to fasting or caloric restriction (Shi et al. 2005, Palacios et al. 2009, Hallows et al. 2011). SIRT3 deacetylates numerous mitochondrial enzymes to coordinate metabolic alteration and regulate mitochondrial respiration and oxidative stress resistance (Cheung et al. 2015). SIRT3-deficient mice presents with significantly reduced tissue ATP levels (Ahn et al. 2008), and are also vulnerable to cardiac hypertrophy (Sundaresan et al. 2009, Hafner et al. 2010). A recent review by Mayo et al. (2017) describes the MLT/SIRT interaction as dichotomous since different actions may be affected in normal and in cancer cells. The function of SIRT3 in skeletal muscle, however is not well characterized.

In the current in vitro study SIRT3 was significantly increased in the DOX (consistent with the in vivo result, Fig. 4.1.20B) and MI groups compared to control levels (Fig. 4.2.4). Cardiotoxicity studies have shown that increased SIRT3 expression attenuated DOX-induced oxidative stress and improved mitochondrial respiration (Cheung et al. 2015). Moreover, the overexpression of SIRT3 protected mice from DOX-induced cardiomyopathy by preventing mitochondrial DNA damage (Pillai et al. 2016). SIRT3 levels could be increased in the DOX group as compensatory mechanism to protect the mitochondria from DOX-induced free radical damage (Jing et al. 2011) and to preserve skeletal muscle oxidative capacity (Lin et al. 2014), since DM group was not changed from control levels (4.2.4). SIRT3 expression has been shown to be increased in response to fasting (Cheung et al. 2015) which could be another reason why SIRT3 levels are increased in the DOX group since DOX treated animals reduced their food and water intake by more than half within the 24 h period following initial administration, a behaviour which persisted for several days (Dirks-Naylor et al. 2013). On the other hand, in an attempt to better regulate mitochondrial energy production efficiency, SIRT3 may be contributing to myotube atrophy since a previous study has reported a 30% reduction in muscle mass via transgenic expression of SIRT3M3 (an isoform of SIRT3) in skeletal muscle (Lin et al. 2014). They found that SIRT3 regulated the formation of oxidative muscle fibres, enhanced muscle metabolic function, and decreased muscle mass (Lin et al. 2014). SIRT3 however, may have different functionalities via its other protein isoforms, SIRT3M1 and SIRT3M2. The finding that SIRT3 can reduce muscle mass, could also be the reason why its protein levels are significantly increased in the MI group (4.2.4) which suggests that MLT may be regulating SIRT3 expression via the MT1 receptor. This has previously been shown in hepatoxicity studies where MLT upregulated SIRT3 via MT1 receptor-PI3K/AKT-PGC-

1α signalling pathway (Song et al. 2017) and SIRT3 inhibition blocked protective antioxidant effects of MLT in hepatocytes (Chen et al. 2015, Pi et al. 2015). MLT's ability to influence mitochondrial sirtuin activity is a relatively unexplored research field in relation to skeletal muscle which may provide interesting new therapeutic avenues.

Autophagy

Due to the interesting *in vivo* findings on MLT's role in mediating the autophagy process, it was decided to investigate autophagic flux *in vitro*. The CYTO-ID fluorescent detection kit was used to assess autophagic flux. The assay weakly labels lysosomes and assesses both autophagosome and autolysosomes, further down the autophagic process. An increase in fluorescent signal indicates both autophagosomal and autolysosomal abundance which is usually the case if there is autophagy or lysosomal dysfunction.

Basal autophagic flux seemed to be present in all groups (Fig. 4.2.5) since chloroquine levels were increased compared to control levels. Hence, all groups displayed functional autophagy, with no autophagy dysfunction due to the treatment intervention. The DOX (** $p < 0.01$) and DM (* $p < 0.05$) treatment groups had significantly greater levels in the chloroquine compared to control group, therefore, these cells had high and functional autophagic flux. The rapamycin+chloroquine group was not significantly higher compared to the chloroquine group, suggesting that rapamycin treatment had not further increased flux. This indicates that either the cells were not majorly responsive to rapamycin treatment (different rapamycin concentrations and incubation times) or their basal flux was (due to the treatments) already so high that it could not increase further. Li et al. (2016) demonstrated that autophagy flux is blocked due to DOX treatment in cardiac muscle of mice and in cardiomyocytes in culture. The

authors observed the inhibition of flux was accompanied by an increase in undegraded autolysosomes. In the same study no alteration in autophagic flux was found in DOX treated C2C12 skeletal muscle myoblasts. This result is similar to what was observed in this thesis research in C2C12 myotubes. To further make conclusions on the autophagy flux data, protein levels of key autophagy markers together with appropriate groups should be assessed and compared.

Chapter 6: Summation

In summary, the studies in this thesis clearly demonstrated the detrimental effects of DOX on skeletal muscle. In particular, sarcolemma damage was demonstrated indirectly by leakage of Mb into circulation; calpain homeostasis was altered and atrogenes activated. However, administration of MLT provided effective treatment and allowed for elucidation of possible mechanisms involved in the detrimental effects of DOX.

Possible mechanisms of DOX-induced protein degradation and MLT-induced protection

As DOX enters the cell it accumulates in the nucleus, mitochondria and cell membrane. In the nucleus and mitochondria, it intercalates with DNA and interferes with topoisomerase-II-mediated DNA repair thus increasing cell death. DOX is further oxidized to an unstable metabolite and again converted to DOX in a process which generates reactive oxygen species. ROS can then damage myocyte membranes which results in the leakage of proteins such as Mb into circulation.

Moreover, ROS can damage organelles such as the mitochondria and sarcoplasmic reticulum via lipid peroxidation, further damaging DNA, increasing oxidative stress and triggering apoptotic pathways. Damage to mitochondria and the sarcoplasmic reticulum may result in the leakage of calcium ions into the cell; free cytosolic calcium in turn activates degradative calpains which further damages the sarcolemma and triggers myofibrillar damage. ROS signalling may also dephosphorylate FoxO transcription factors by modifying protein kinases or modulating phosphatases, which then translocate to the nucleus where it transcribes atrogenes and impairs autophagy degradation. These processes then lead to protein degradation and muscle wasting.
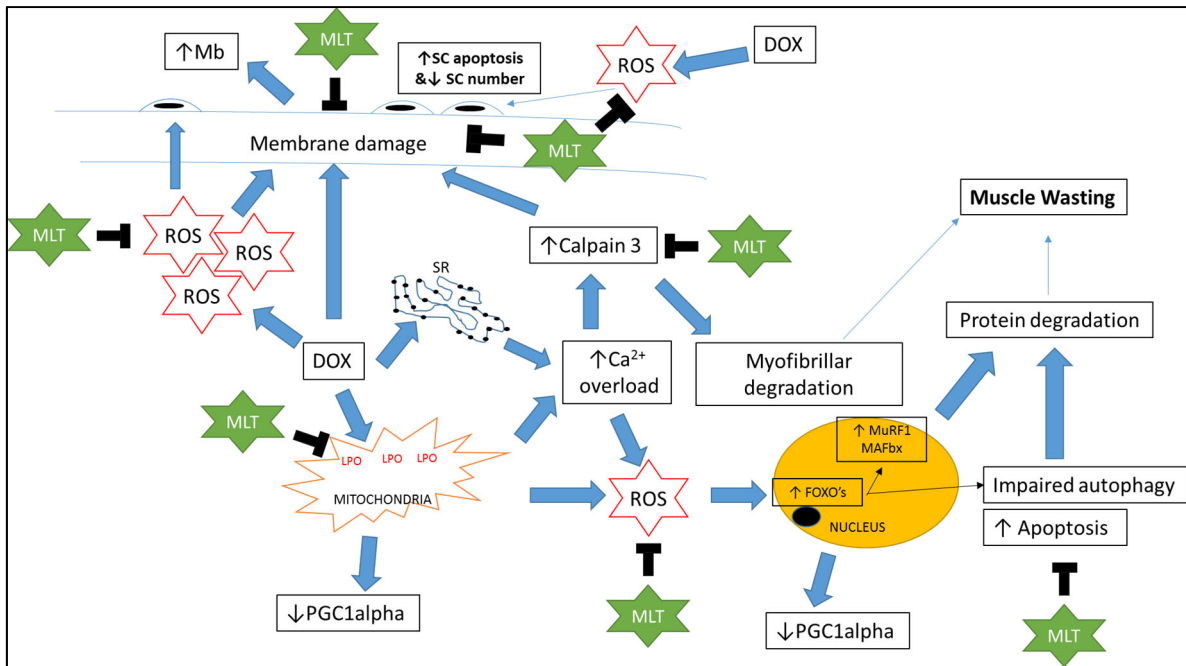
Figure 6.1: Proposed signalling cascade in DOX and ROS mediated mechanisms of skeletal atrophy and MLT protection as described in this study. Abbreviations: Mb, myoglobin; DOX, doxorubicin; MLT, melatonin; MURF-1, muscle ring-finger 1; ROS, reactive oxygen species; Ca^{2+}, calcium; SR, sarcoplasmic reticulum; LPO, lipid peroxidation; FoxO's, Forkhead box O.

MLT and its metabolites are potent antioxidants and act to decrease ROS production to prevent membrane damage and protein leakage, by inhibiting mitochondrial damage and dysfunction, resulting in a nett increased mitochondrial biogenesis. Free cytosolic calcium ions are reduced due to the protection of mitochondria and the sarcoplasmic reticulum which then attenuates calpain activation and myofibrillar damage. MLT also prevents cytosolic FoxO from being dephosphorylated by intervening in ROS signalling and may thus reduce transcription of atrophy proteins and decrease autophagy degradation and apoptosis. Furthermore, MLT attenuated skeletal muscle atrophy by reducing cell apoptosis and increasing satellite cell number.

Based on the literature and the research of this thesis it is worth investigating the effects of MLT supplementation with the aim of attenuating skeletal muscle atrophy in

cancer patients. It is however, important to do so in a relevant model. The results of the current study indicate that exogenous MLT supplementation can inhibit skeletal muscle wasting induced by DOX in a pre-cachectic tumour-bearing rat model.

The prevention of cancer cachexia remains a challenge and only a few drug therapies have been demonstrated to be beneficial. MLT supplementation to cancer patients is a low cost; low risk intervention. It is time that clinicians, researchers and policymakers give increased attention to the beneficial effects of MLT and translate this considerable body of experimental, epidemiological, and clinical evidence into further clinical trials and ultimately clinical practice.

Limitations and recommendations for future studies

Two limitations of the *in vivo* research include not having measured muscle function which would have added great value to the thesis and that tumour effects could not be described since cancer cachexia was not achieved. Therefore it is unclear what effect the cancer contributed to the cellular effects described. A significant decrease in CSA of type 1 muscle fibres of the *gastrocnemius* muscle in the TCON group was however observed. This may indicate the start of metabolic alterations and thus pre-cachexia. Furthermore, food intake could also have added value to the study.

Furthermore, limitations of the *in vitro* model included not using and inhibitor of the MT2 receptor, since Luzindole is only a partial inhibitor of the MT2 receptor. The study could further have benefited from the addition of a Luzindole only control group to probe whether Luzindole alone had any effects on C2C12 myotubes.

Future studies should include measures of muscle function and food intake. Moreover, a different rodent model in which cancer cachexia has been established should be used in combination with DOX treatment in order to assess whether MLT confers

protection to skeletal muscle. Clinical trials should next be considered due to the burden of DOX-induced muscle toxicity in cancer patients and the safe and cost-effective role of MLT as a possible adjuvant therapy to limit this burden.

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