PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVI and XXXVII cycles)

Name of PhD student: Lauren Millichap

Title of PhD research: Assessment of mitochondrial dysfunction in neurodegeneration of inherited and non-communicable diseases: causes, consequences and treatment.

Name of PhD supervisor: Luca Tiano and Elisabetta Damiani
Research lab name: Biochemistry of Oxidative Stress and Aging.

Cycle:
[X] XXXVI
[ ] XXXVII

PhD Curriculum:
[ ] Marine biology and ecology
[X] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[X] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[ ] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate: ....

ABSTRACT (1000 characters, including spaces):
Mitochondrial dysfunction has been implicated as one of the key events leading to neurodegeneration. Mitochondria are essential for efficient neuronal function in order to maintain neuronal integrity and survival, however these organelles are major sources of reactive oxygen species and are vulnerable targets for oxidative stress-induced damage. Several mitochondrial-associated molecular pathways are often compromised in the pathophysiology of neurodegeneration, including Nrf2, PGC1α and PPARγ pathways. Mitochondrial dysfunction has become an attractive therapeutic target, and these molecular pathways can be pharmacologically targeted by bioactive quinones, including coenzyme Q10, pyrroloquinoline quinone and melatonin. These compounds have been reported to remove dysfunctional mitochondria by inducing several processes, such as mitochondrial biogenesis, mitophagy and antioxidant systems, in order to prevent the development and progression of neurodegeneration. The aim of this research project is to assess the causes and consequences of mitochondrial dysfunction and oxidative stress in a neuronal cell model of neurodegeneration, in addition to assessing the effects of bioactive quinones in the rescue and/or prevention of neurodegeneration.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

Recent evidence suggests that progressive impairments to mitochondrial function and activity have a fundamental role in the development of neurodegeneration, however the cause of mitochondrial dysfunction and the degree to which it contributes to cellular morbidity in neurodegeneration has not been fully elucidated [1, 2]. Mitochondrial-associated molecular pathways are often compromised in the pathophysiology of neurodegeneration, including proteins that are

frequently dysregulated in mitophagy and mitochondrial biogenesis, including peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1α) and peroxisome proliferator-activated receptor-gamma (PPARγ) [3, 4, 5]. This affects the removal of damaged and dysfunctional mitochondria, contributing to cellular distress and damage, and has therefore been suggested as a cause of neuronal damage and degeneration [3]. Evidence of a CoQ_{10} deficiency has also been reported in several neurodegenerative disorders, and may contribute to disease pathophysiology by causing disruptions to energy metabolism and the cellular antioxidant status [6]. New evidence has revealed that mitochondrial-targeted therapies represent important novel therapeutic candidates for restoration of mitochondrial function by ameliorating mitochondrial respiratory chain (MRC) dysfunction and oxidative stress, and inducing processes such as mitochondrial biogenesis and mitophagy, in order to maintain neuronal cell integrity and survival in several disorders that involve neurodevelopment disruptions, such as Parkinson's disease [7]. Several therapeutic compounds have been reported in the literature as potential therapeutic targets in order to slow down and prevent the development and progression of neurodegeneration in these disorders, including bioactive quinones, such as coenzyme Q10 (CoQ_{10}), pyrroloquinoline quinone (PQQ) and melatonin. [6, 8-10].

- SCIENTIFIC AIMS

This project aims to investigate the causes and consequences of mitochondrial dysfunction and oxidative stress associated with neurodegeneration; and to elucidate the mechanisms by which this impairment is caused and how it contributes to cellular morbidity; and to establish appropriate treatment strategies to target these parameters.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Establishment of a rotenone-induced Parkinson's disease model and validation of readouts protocol using the SH-SY5Y human neuroblastoma cell line.

Objective.

- To establish an *in vitro* model of Parkinson's disease in an SH-SY5Y human neuroblastoma cell line.
- To validate readout protocols to assess the effects of induced stress and neurodegeneration in the Parkinson's disease neuronal cell model.
- To produce an *in vitro* Parkinson's disease model using rotenone in order to assess the effects on mitochondrial function and oxidative stress in the SH-SY5Y human neuroblastoma cell line.

Methods.

Cell culture: SH-SY5Y neuroblastoma cells, derived from SK-N-SH, were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The SH-SY5Y cells were maintained at 37ºC in an incubator containing 5% carbon dioxide. SH-SY5Y cells were grown to 70-80% confluence before they were seeded into 96-well plates for statin treatment and analysis.

***In vitro* model of rotenone-induced Parkinson's disease:** SH-SY5Y cells were incubated with increasing concentrations of rotenone (0.5µM – 2 µM) to mimic neuronal damage observed in Parkinson's disease. Rotenone induces Parkinsonian-like features as it destroys dopaminergic neurons, a key pathological feature of Parkinson's disease. Rotenone is mitochondrial respiratory chain (MRC) complex I inhibitor, resulting in electron transfer inhibition from the iron-sulphur (Fe-S) clusters, located in complex I, to ubiquinone (CoQ_{10}), causing increased oxidative stress and neuronal dysfunction. SH-SY5Y cell incubation with rotenone has a dose-dependent effect, causing increased oxidative stress and mitochondrial dysfunction. SH-SY5Y cells were treated with increasing concentrations of rotenone based upon levels reported in the literature to produce an *in vitro* neuronal cell model of Parkinson's disease. The treated SH-SY5Y cells were incubated for 24 hours at 37 ºC in 5% CO_2. The rotenone concentrations were prepared from a stock concentration of 0.1 M rotenone. After incubation, end-point analysis was carried out and the effects on mitochondrial functionality and oxidative stress was analysed.

Analytical methods

- Cell viability assay using PrestoBlue reagent and measured using a plate reader.
- Oxidative stress assessment

- Measurement of mitochondrial superoxide production using the fluorescent probe, MitoSOX and measured using the flow cytometer.
- Measurement of intracellular reactive oxygen species (ROS) production using the fluorescent probe, CM-H2DCFDA and measured using the flow cytometer.

- Cellular CoQ_{10} content measured via HPLC detection.
- Mitochondrial membrane potential
  - Measurement of reductions in the mitochondrial membrane potential using the fluorescent mitoprobe, Dilc1(5) and measured using the flow cytometer.

Obtained Results.

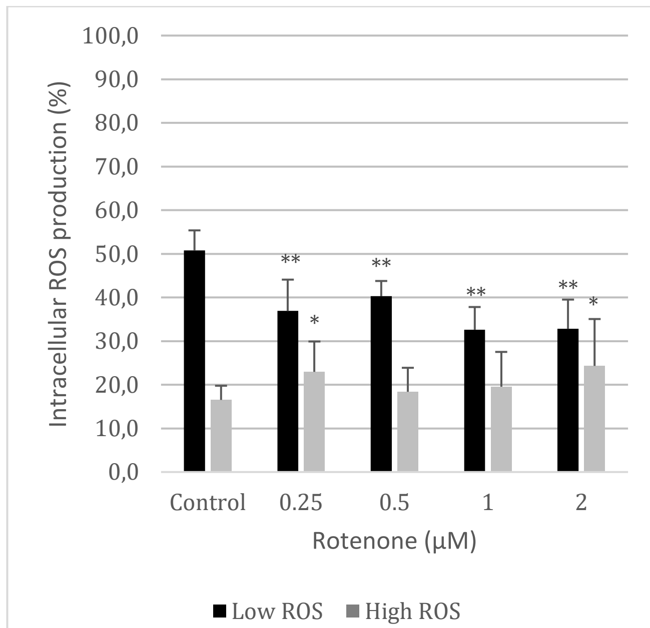


Figure 1. Intracellular reactive oxygen species (ROS) production (%) in SH-SY5Y cells stained with $DCFH_2$-DA (10 µM) probe following exposure to increasing concentrations of rotenone for 24 hours. The black bars represent cells producing a low level of ROS and the grey bars represent cells producing a high level of ROS in response to rotenone exposure. * P < 0.05, ** P < 0.01

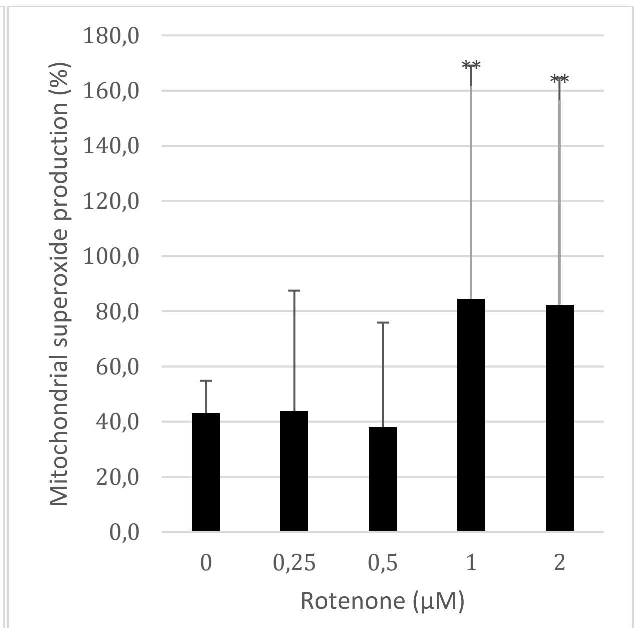


Figure 2. Mitochondrial superoxide production, $O_2^{\bullet-}$, (%) in SH-SY5Y cells stained with MitoSOX (5 µM) probe following exposure to increasing concentrations of rotenone for 24 hours. * P < 0.05, ** P < 0.01

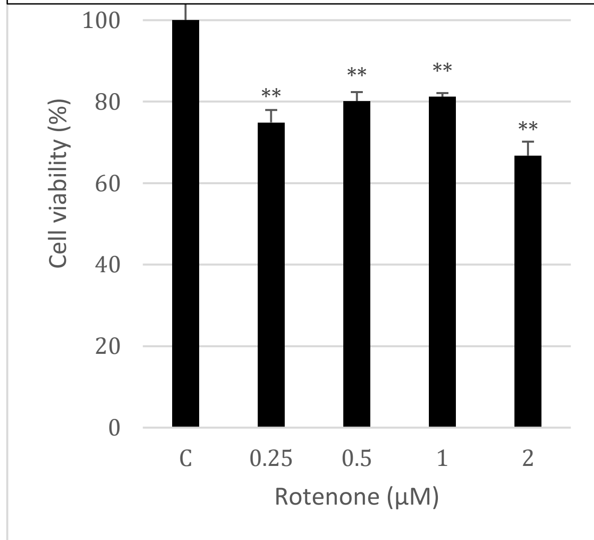


Figure 3. Percentage viability following rotenone exposure in SH-SY5Y cells incubated for 24 hours. * P < 0.05, ** P < 0.01

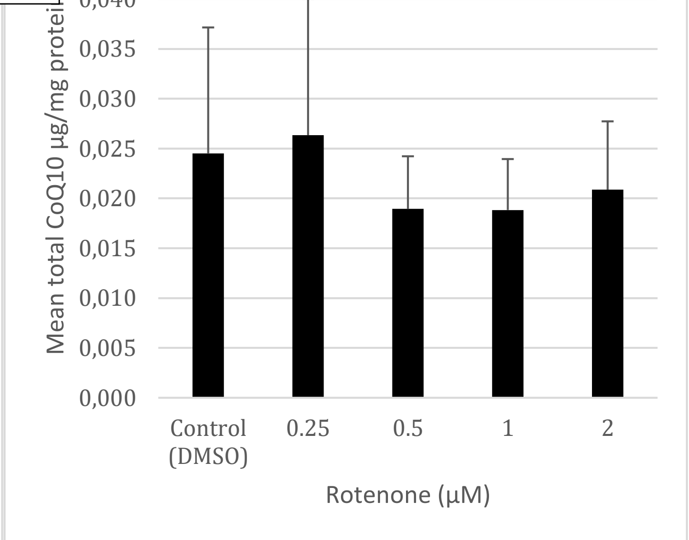


Figure 4. Cellular coenzyme Q_{10} (CoQ_{10}) content standardised to cellular protein content following rotenone exposure in SH-SY5Y cells incubated for 24 hours. * P < 0.05, ** P < 0.01

The data from this study demonstrates that rotenone contributes to neuronal dysfunction and death as shown by increased intracellular ROS (Fig 1) and mitochondrial superoxide production (Fig 2), in addition to a reduction in SH-SY5Y viability (Fig 3) and a decrease in the cellular CoQ_{10} content (Fig 4). This data was used to establish a rescue and prevention treatment model using bioactive compounds that aim to reduce rotenone-induced ROS production, preventing mitochondrial dysfunction and oxidative damage in order to promote neuronal survival.

WP 2. Establishment of a simvastatin-induced coenzyme Q10 (CoQ_{10}) deprivation model and validation of readouts protocol using the SH-SY5Y human neuroblastoma cell line.

Objective.

- To establish an *in vitro* model of CoQ_{10} deprivation in an SH-SY5Y human neuroblastoma cell line.
- To validate readout protocols to assess the effects of CoQ_{10} deprivation on neurodegeneration in the SH-SY5Y human neuroblastoma cell line.
- To produce a CoQ_{10} deprivation model using statins in order to assess the effects on mitochondrial function and oxidative stress in the SH-SY5Y human neuroblastoma cell line.

Methods.

Cell culture: As described in WP1.

***In vitro* model of neurodegeneration and CoQ_{10} deprivation:** SH-SY5Y cells were incubated with increasing concentrations of simvastatin (0.25µM – 5 µM) to induce CoQ_{10} depletion. CoQ_{10} is an important electron carrier in the mitochondrial respiratory chain (MRC) and is an important antioxidant. Simvastatin inhibits CoQ_{10} biosynthesis, resulting in altered mitochondrial function and increasing oxidative stress. SH-SY5Y cell incubation with simvastatin has a dose-dependent effect, causing increased oxidative stress and mitochondrial dysfunction. SH-SY5Y cells were treated with increasing concentrations of simvastatin based upon levels reported in the literature to produce a CoQ_{10} deprivation model. The treated SH-SY5Y cells were incubated for 48 hours at 37 ºC in 5% CO_2. The simvastatin concentrations were prepared from a stock concentration of 25 mM simvastatin. After incubation, end-point analysis was carried out and the effects on mitochondrial functionality and oxidative stress was analysed.

Obtained Results.

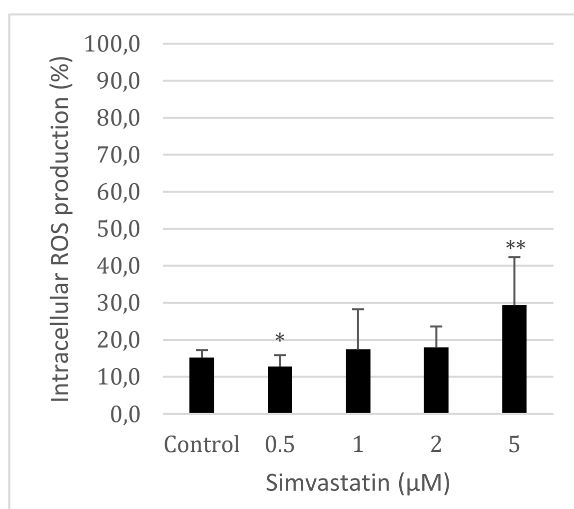


Figure 5. Intracellular reactive oxygen species (ROS) production (%) in SH-SY5Y cells stained with $DCFH_2$-DA (10 µM) probe following exposure to increasing concentrations of simvastatin for 48 hours. * $P < 0.05$, ** $P < 0.01$

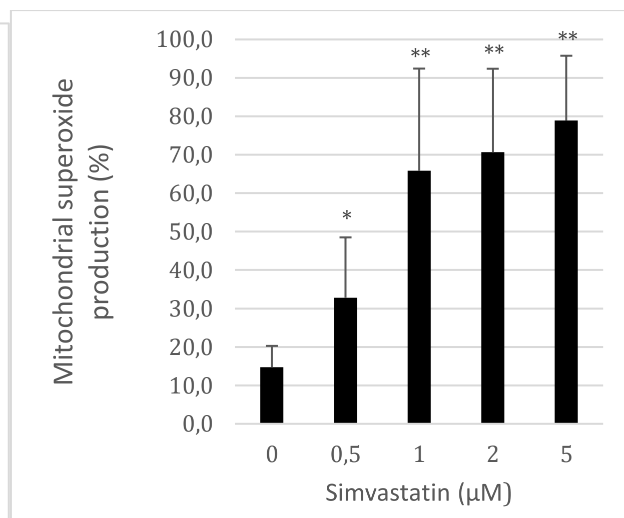
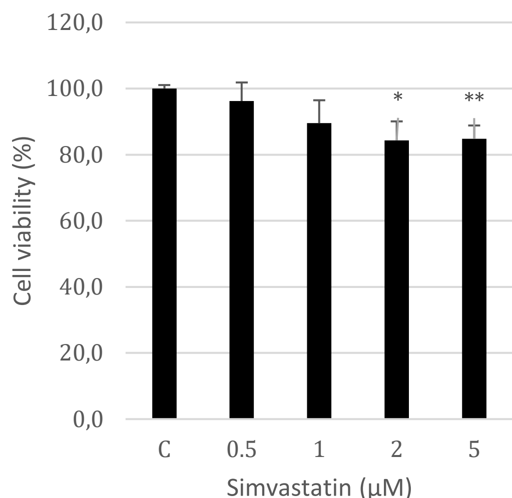


Figure 6. Mitochondrial superoxide production, $O_2^{\bullet-}$, (%) in SH-SY5Y cells stained with MitoSOX (5 µM) probe following exposure to increasing concentrations of simvastatin for 48 hours. * $P < 0.05$, ** $P < 0.01$



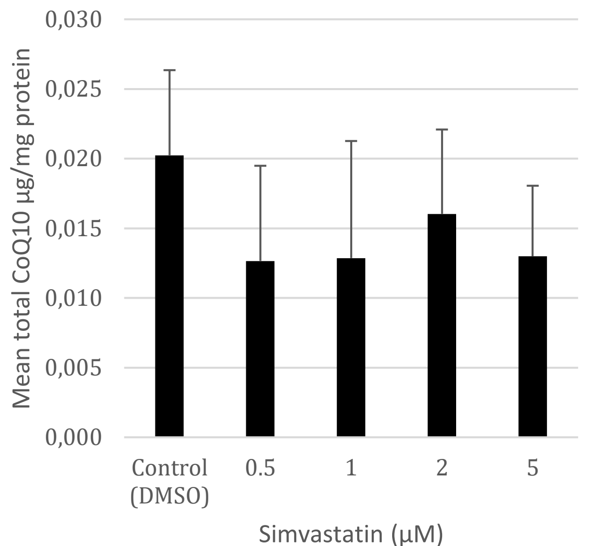


Figure 7. Percentage viability following simvastatin exposure in SH-SY5Y cells incubated for 48 hours. * $P < 0.05$, ** $P < 0.01$

Figure 8. Cellular coenzyme Q_{10} (CoQ_{10}) content ratioed to cellular protein content following simvastatin exposure in SH-SY5Y cells incubated for 48 hours. * $P < 0.05$, ** $P < 0.01$

The data from this study demonstrates that simvastatin contributes to neuronal dysfunction and death as shown by increased intracellular ROS (Fig 5) and mitochondrial superoxide production (Fig 6), in addition to a reduction in SH-SY5Y viability (Fig 7) and a decrease in the cellular CoQ_{10} content (Fig 8). This data was used to establish a rescue and prevention treatment model using bioactive compounds that aim to reduce simvastatin-induced CoQ_{10} deprivation leading to increased ROS production, therefore preventing mitochondrial dysfunction and oxidative damage in order to promote neuronal survival.

WP 3. Establishment of the rescue and/or prevention of mitochondrial dysfunction and oxidative stress in neuronal cell models of neurodegeneration using bioactive quinones and validation of readout protocols in the SH-SY5Y human neuroblastoma cell line.

Objective.

- To establish treatments that aim to rescue and/or prevent mitochondrial dysfunction and oxidative stress in an *in vitro* model of neurodegeneration in the SH-SY5Y human neuroblastoma cell line.
- To determine the effectiveness of several bioactive quinones in the rescue and/or prevention of mitochondrial dysfunction and oxidative stress in the SH-SY5Y *in vitro* model of neurodegeneration.
- To validate readout protocols to assess the effects of induced mitochondrial dysfunction and oxidative stress in an *in vitro* model of neurodegeneration in the SH-SY5Y human neuroblastoma cell line.

Methods.

Cell culture: As described in WP1.

***In vitro* treatment model to rescue and/or prevent mitochondrial dysfunction and oxidative stress in neurodegeneration:** For the simvastatin-induced CoQ_{10} deprivation model, SH-SY5Y cells were incubated with simvastatin (1 μM and 2 μM) for 48 hours to induce CoQ_{10} depletion. For the rotenone-induced Parkinson's disease model, SH-SY5Y cells were incubated with rotenone (0.5 μM and 1 μM) for 24 hours to mimic an *in vitro* model of neurodegeneration. After the incubation period, the SH-SY5Y cells were treated with two different concentrations of ubiquinone, the oxidised form of CoQ_{10} (5 μM and 10 μM) and ubiquinol, the reduced form of CoQ_{10} (5 μM and 10 μM) for 24 hours at 37 ºC in 5% CO_2. CoQ_{10} has been demonstrated to improve mitochondrial function by reducing ROS production, in addition to improving cognitive function in neurodegenerative patients. For the rescue model, SH-SY5Y cells were firstly treated with the stressor (simvastatin or rotenone) for 48 hours and 24 hours, respectively, and then treated with ubiquinone or ubiquinol for 24 hours at 37 ºC in 5% CO_2. For the prevention model, SH-SY5Y cells were firstly treated with ubiquinone or ubiquinol for 24 hours, and then incubated with either rotenone (24 hours) or simvastatin (48 hours) at 37 ºC in 5% CO_2. The *in vitro* rescue and prevention models mimic early-onset and late progression of neurodegeneration. After incubation, end-point analysis was carried out and the effects on mitochondrial functionality

and oxidative stress were analysed. Other *in vitro* rescue and/or prevention models of neurodegeneration will be assessed two other bioactive quinones, such as PQQ, which has been demonstrated to upregulate mitochondrial biogenesis to improve mitochondrial function, and melatonin, which has been found to restore mitochondrial function by scavenging ROS and maintain the mitochondrial membrane potential.

Expected/Obtained Results: The data from this study is expected to show that rotenone-induced mitochondrial dysfunction leading to an increase in oxidative stress can be rescued by both oxidised and reduced CoQ_{10}. CoQ_{10} has a neuroprotective effect by reducing mitochondrial ROS generation and preventing ROS-induced neuronal damage. The measurement of ROS production and CoQ_{10} content of the SH-SY5Y cells will be assessed, were reductions in ROS production and increases in CoQ_{10} cellular content and cell viability are expected following SH-SY5Y treatment with oxidised and reduced CoQ_{10}. Moreover, the same end-point analyses will be carried out with simvastatin-treated cells. These experiments will be repeated following treatment with other bioactive quinones known to improve mitochondrial functionality, reduce oxidative stress-induced damage and promote neuronal survival, including melatonin and PQQ.

WP 4. Biochemical assessment of mitochondrial dysfunction in the SH-SY5Y human neuroblastoma cell model of neurodegeneration.

Objective.

- To measure the activity of mitochondrial respiratory chain (MRC) complex I and complex II-III activity in an *in vitro* model of neurodegeneration in the SH-SY5Y human neuroblastoma cell line.
- To measure the activity of citrate synthase in an *in vitro* model of neurodegeneration in the SH-SY5Y human neuroblastoma cell line.
- To assess and analyse the biochemical effects of neurodegeneration on the functioning of the mitochondria via spectrophotometric analysis.

Methods.

Cell culture: As described in WP1.

Spectrophotometric analysis of mitochondrial respiratory chain (MRC) complex enzyme activity in an *in vitro* model of neurodegeneration: SH-SY5Y cells were treated with rotenone and simvastatin as described in WP3. The enzyme kinetics of complex I (NADH-ubiquinone oxidoreductase) and complex II-III (succinate dehydrogenase cytochrome C reductase) of the rotenone and simvastatin treated samples were assessed via spectrophotometry. The activity of complex I is measured spectrophotometrically by the rotenone-sensitive decrease in NADH and a decrease in the absorbance at λ=340 nm is measured, as a result of the oxidation of NADH catalysed by the enzyme, NADH-ubiquinone oxidoreductase. After reading for 5 minutes, rotenone (1 mM) was added to the sample cuvettes to remove any interferences. The activity of complex II-III is also measured spectrophotometrically by the succinate-dependent antimycin A sensitive reduction of cytochrome *c* and an increase in the absorbance at λ=550 nm is measured, as a result of the reduction in cytochrome *c* catalysed by the enzyme, ubiquinol-cytochrome *c* oxidoreductase. After 7 minutes of reading, antimycin A was added to the sample cuvettes to remove any interferences. The measurement of the MRC enzyme complex kinetics was assayed at 30°C on the Uvikon 941 plus spectrophotometer. The absorbance values were read every 30 seconds using the auto rate assay setting. The samples were ratioed to the activity of citrate synthase (CS).

Spectrophotometric analysis of citrate synthase (CS) activity in an *in vitro* model of neurodegeneration: SH-SY5Y cells were treated with rotenone and simvastatin as described in WP3. The activity of citrate synthase (CS) is measured spectrophotometrically by the reaction of free coenzyme A (CoA) with DTNB at 412 nm. CS is a biomarker of mitochondrial content. The absorbance measurement of CS activity was assayed at 30°C on the Uvikon 941 plus spectrophotometer. The absorbance values were read every 30 seconds using the auto rate assay setting. The samples were ratioed to the cellular protein content.

Obtained Results.

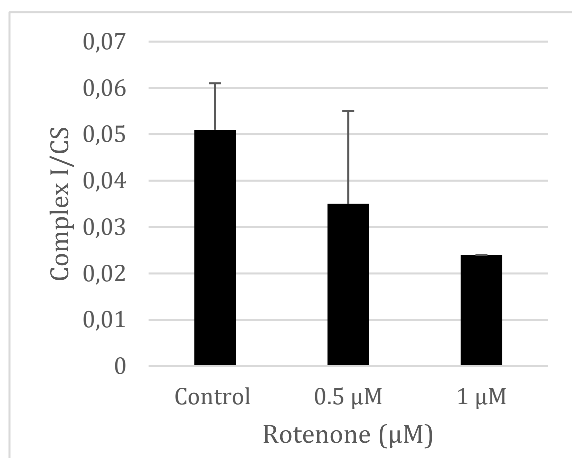


Figure 11. Mitochondrial respiratory chain (MRC) complex I (NADH-ubiquinone oxidoreductase) activity ratioed to citrate synthase activity (CS) in SH-SY5Y cells incubated with rotenone for 24 hours.

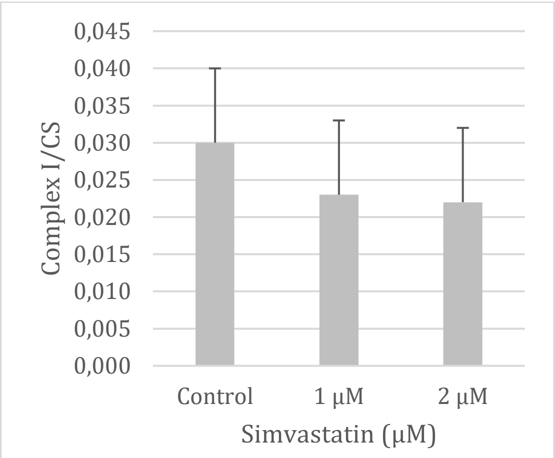


Figure 12. Mitochondrial respiratory chain (MRC) complex I (NADH-ubiquinone oxidoreductase) activity ratioed to citrate synthase activity (CS) in SH-SY5Y cells incubated with simvastatin for 48 hours.

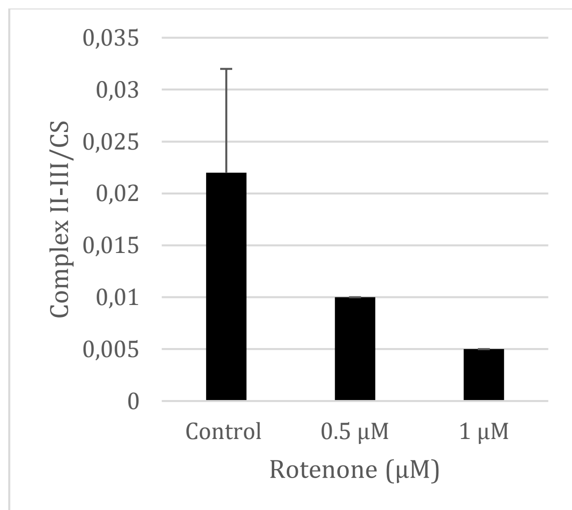


Figure 13. Mitochondrial respiratory chain (MRC) complex II-III (succinate dehydrogenase cytochrome C reductase) activity ratioed to citrate synthase activity (CS) in SH-SY5Y cells incubated with rotenone for 24 hours.

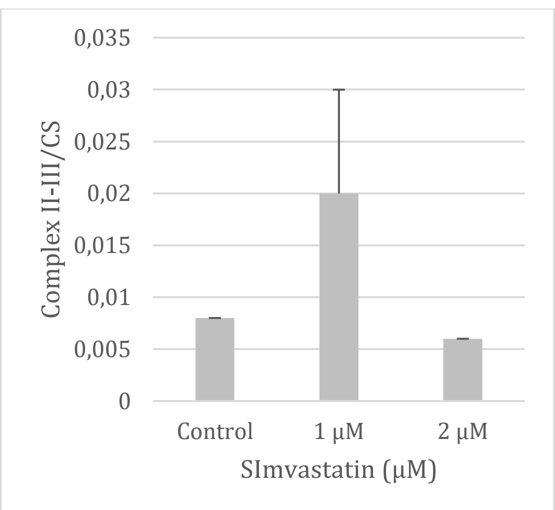


Figure 14. Mitochondrial respiratory chain (MRC) complex II-III (succinate dehydrogenase cytochrome C reductase) activity ratioed to citrate synthase activity (CS) in SH-SY5Y cells incubated with simvastatin for 48 hours.

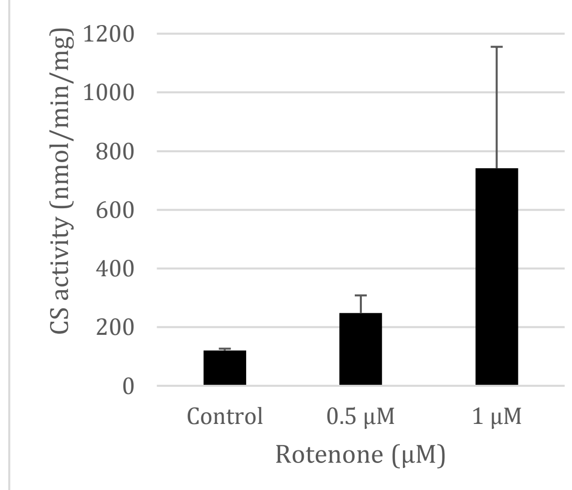


Figure 15. Citrate synthase activity (CS) (nmol/min/mg) in SH-SY5Y cells incubated with rotenone for 24 hours.

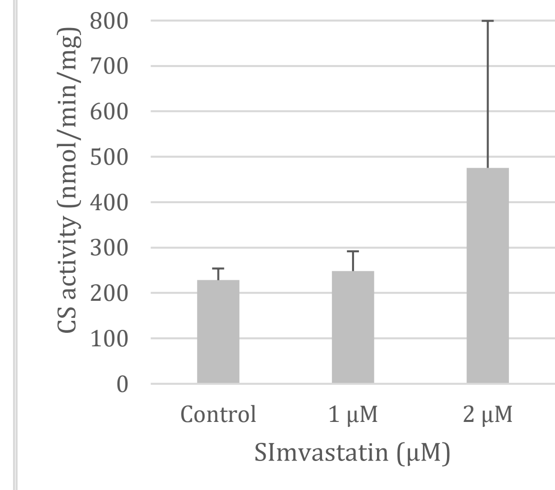


Figure 16. Citrate synthase activity (CS) (nmol/min/mg) in SH-SY5Y cells incubated with simvastatin for 48 hours.

The data from this study demonstrates that there is a decrease in the activity of both complex I and complex II-III of the mitochondrial respiratory chain, therefore leading to mitochondrial dysfunction and increased oxidative stress, which as a result contributes to neuronal dysfunction and death (Fig 11 -14). Moreover, this study also demonstrates that there is an increase in the activity of citrate synthase (CS), a biomarker for mitochondrial density within the cell, suggesting upregulation of the process of mitochondrial biogenesis (Fig 15; Fig 16). An increase in the activity of CS demonstrates that the mitochondria are dysfunctional and this is a compensatory mechanism where the cell upregulates the synthesis of new mitochondria in order to prevent energy failure and neuronal cell death. This data will be used to establish a rescue and prevention treatment model using PQQ, which aims to upregulate mitochondrial biogenesis, to prevent rotenone and simvastatin-induced mitochondrial dysfunction and oxidative stress in the SH-SY5Y neuronal cell model of neurodegeneration.

- REFERENCES

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4. Li, P.A.; Hou, X; and Hao, S. Mitochondrial biogenesis in neurodegeneration. J Neurosci Res. 2017. 95(10); 2025-29. *[Review]*
5. Wang, Y; Xu, E; Musich, P.R.; and Lin, F. Mitochondrial dysfunction in neurodegenerative diseases and the potential countermeasure. CNS Neurosci Ther. 2019. 25(7); 816-24. *[Review]*
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10. Zhi, S.M.; Fang, G.X.; Xie, X.M.; Liu, L.H.; Yan, J.; Liu, D.B.; and Yu, H.Y. Melatonin reduces OGD/R-induced neuron injury by regulating redox/inflammation/apoptosis signaling. Eur Rev Pharmacol Sci. 2020. 24(3): 1524-1536.
11. Brischigliaro, M.; Frigo, E.; Fernandez-Vizarra, E.; Bernardi, P.; and Viscomi, C. Measurement of mitochondrial respiratory chain enzymatic activities in *Drosophila melanogaster* samples. STAR Protoc. 2022. 3(2): 101322.

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Advanced Molecular Modelling Applied to Drug Discovery

2. Assessing oxidative stress in biological systems

3. Technology Transfer and Innovation

4. Theory and application of complex networks

5. DiSVA Shot of Science Seminars

List of periods spent abroad

1. Liverpool, United Kingdom (10 months; November 2020 – August 2021)

2. Liverpool John Moores University, United Kingdom (2 months; August – October 2022)

List of conferences/workshops attended and of contributions eventually presented

1. The MaSBiC-DiVSA Annual Symposium – 'From structure to function, unveiling the role of proteins in health and disease' (16th-17th September 2021)
2. 2nd International Conference on Neuroprotection by Drugs, Nutraceuticals, and Physical Activity – Virtual Edition. (9th-10th December 2021)
3. 10th Conference of the International Coenzyme Q10 Association (12th-15th May 2022) – with a poster entitled 'Stimulation of mitochondrial biogenesis facilitates increases of cellular Q10 bioavailability in mouse muscle cells.
4. The Future of Redox Biology (17th-19th June 2022)

Part 3. PhD student information on publications

If not yet published, please indicate the publication status (submitted, accepted, in preparation...)

List of publications on international journals

J1. Cirilli, I., Damiani, E., Dludla, P.V., Hargreaves, I., Marcheggiani, F., Millichap, L.E., Orlando, P., Silvestri, S., Tiano, L. Role of Coenzyme Q_{10} in Health and Disease: An Update on the Last 10 Years (2010-2020). Antioxidants (Basel). 2021. 10(8), 1325. DOI: 10.3390/antiox10081325

J2. Millichap, L., Damiani, E., Tiano, L., Hargreaves, I. Targetable pathways for alleviating mitochondrial dysfunction in neurodegeneration of metabolic and non-metabolic diseases. International Journal of Molecular Sciences. 2021. 22(21):11444. doi: 10.3390/ijms222111444

List of publications on conference proceedings

C1. ...

C2. ...

List of other publications (books, book chapters, patents)

B1. ...

B2. ...

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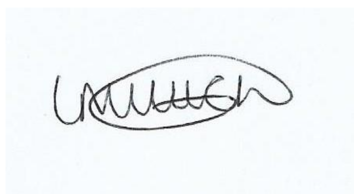

Supervisor signature