Does Pregnenolone Reduce CLN2 Disease Severity Through Changes in StarD3 and LCAT Protein Levels?

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Introduction and Aims

Neuronal Ceroid Lipofuscinoses (NCL) are paediatric neurodegenerative diseases that also appear in adults[1]. This research focuses on CLN2 disease or classical late-infantile NCL, which is caused by a mutation in the gene that encodes for Tripeptidyl Peptidase 1 (TPP1)[2]. This mutation creates an accumulation of lipopigments, leading to neuronal and retinal cell death. Currently, there is an enzyme replacement therapy available, but this only slows CLN2 disease from worsening, so additional treatments are still needed[3].

Zebrafish animal models were chosen to study this neurodegenerative disease, having been mutated to have the CLN2 variant. In previous research in the Russell laboratory, through RNA sequencing (RNA-Seq), the mRNAs StarD3 and LCAT were identified as dysregulated and targets for further study. Treating the affected animals with the neurosteroid pregnenolone (PREG) improved the dysregulation in the mRNAs, which is thought to work by regulating cholesterol trafficking in such genes[4]. Research suggests that all NCLs are likely to have dysregulated cholesterol, so pregnenolone may have wider benefits.

Methods and Summary of the Work Undertaken

To determine the levels of the proteins encoded by these mRNAs with and without pregnenolone treatment, the western blotting (WB) technique was used[5]. Two groups of embryos were produced: wildtype (WT) and those with a mutation in TPP1. Adult heterozygous *tpp1+/-* zebrafish were bred together to produce fertilised eggs. A microscope was utilized to separate homozygous *tpp1-/-* and WT sibling embryos and to cull and discard the non-fertilised eggs and excess embryos (Figure 1).

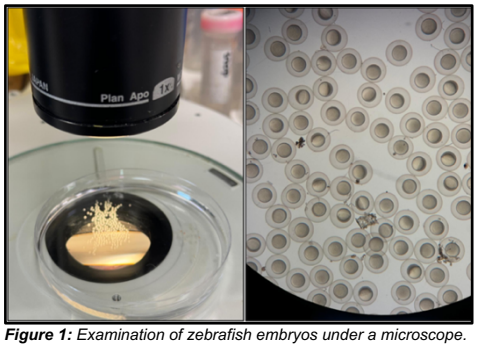

Figure 1: *Examination of zebrafish embryos under a microscope.*

There were a total of six groups of embryos separated into different wells (Figure 2).

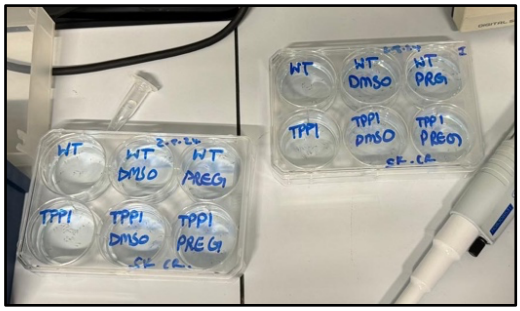

Figure 2: *The six groups of embryos: 20 WT, 20 WT dimethyl sulfoxide (DMSO), 20 WT pregnenolone, 20 TPP1, 20 TPP1 DMSO, and 20 TPP1 pregnenolone. DMSO was needed to dissolve the pregnenolone, so wells with only DMSO acted as negative controls to validify that changes were due to pregnenolone.*

At four days post-fertilization, the western blotting protocol commenced. Multiple western blots were done; the steps were sample preparation, protein quantification (BCA kit), electrophoresis, immunodetection, and then a chemiluminescent reagent was added to the membrane, which produces light that is captured by the iBright imaging system. The results of the western blotting can then be analysed, showing up as bands of protein separated due to their varying molecular masses.

Results and Outcomes

Only the latest blot will be shown in this results section. The protein ladder used to compare the bands of the blots was the PageRuler™ Plus Pre-stained Protein Ladder (Figure 3).

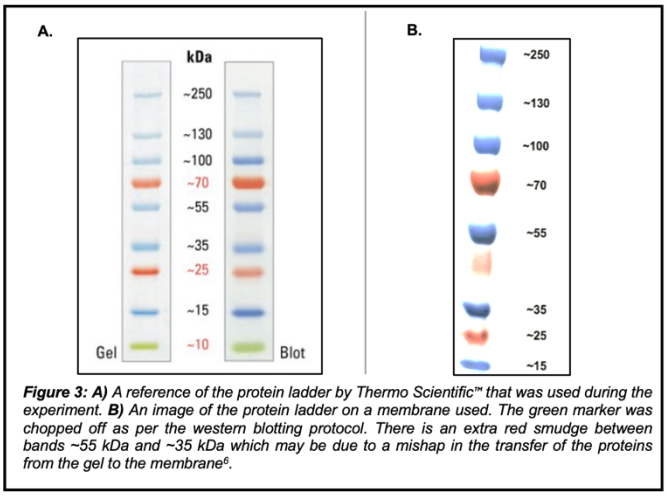

Figure 3: A) *A reference of the protein ladder by Thermo Scientific™ that was used during the experiment.* ***B)*** *An image of the protein ladder on a membrane used. The green marker was chopped off as per the western blotting protocol. There is an extra red smudge between bands ~55 kDa and ~35 kDa which may be due to a mishap in the transfer of the proteins from the gel to the membrane[6].*

β-actin is a housekeeping protein not expected to vary between samples. The β-actin blot shows that equivalent amounts of protein were loaded (except for the WT sample where there is no band) and the approximately correct size of protein was recognised (Figure 4). However, when the same blot was previously probed for LCAT, there were variations in band density, but the band sizes were incorrect. It is likely the LCAT antibody does not recognise zebrafish LCAT proteins.

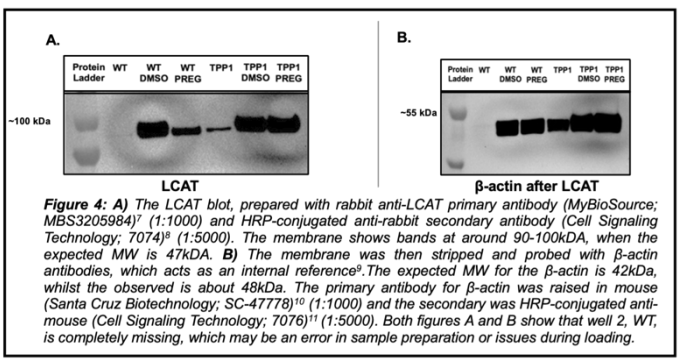

Figure 4: A) *The LCAT blot, prepared with rabbit anti-LCAT primary antibody (MyBioSource; MBS3205984)[7] (1:1000) and HRP-conjugated anti-rabbit secondary antibody (Cell Signaling Technology; 7074)[8] (1:5000). The membrane shows bands at around 90-100kDA, when the expected MW is 47kDA.* ***B)*** *The membrane was then stripped and probed with β-actin antibodies, which acts as an internal reference[9].The expected MW for the β-actin is 42kDa, whilst the observed is about 48kDa. The primary antibody for β-actin was raised in mouse (Santa Cruz Biotechnology; SC-47778)[10] (1:1000) and the secondary was HRP-conjugated anti-mouse (Cell Signaling Technology; 7076)[11] (1:5000). Both figures A and B show that well 2, WT, is completely missing, which may be an error in sample preparation or issues during loading.*

In the StarD3 blot (Figure 5), there is a lot of non-specific binding, and the results are not clear. This is unlikely to be due to insufficient use of the blocking agent, bovine serum albumin (BSA), as longer blocking times, lower primary antibody concentrations, and so on did not improve the specificity. The antibody, in all probability, does not recognize zebrafish StarD3.

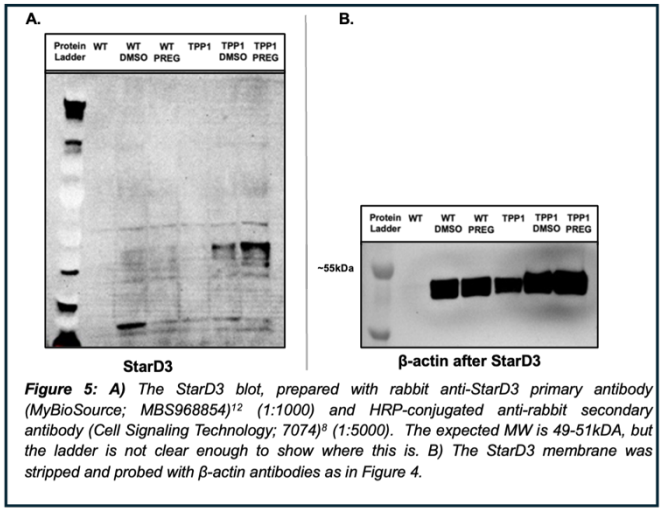

Figure 5: A) *The StarD3 blot, prepared with rabbit anti-StarD3 primary antibody (MyBioSource; MBS968854)[12] (1:1000) and HRP-conjugated anti-rabbit secondary antibody (Cell Signaling Technology; 7074)[8] (1:5000). The expected MW is 49-51kDA, but the ladder is not clear enough to show where this is. B) The StarD3 membrane was stripped and probed with β-actin antibodies as in Figure 4.*

Future Directions

Not all the results of the blots were as expected, and the membranes did not show all the bands clearly. This experiment has been repeated on three different occasions, but the same findings have kept arising. The issue may be that the antibodies used were not raised against zebrafish and may not have recognized the intended proteins. Therefore, using zebrafish-specific antibodies may be a good decision moving forward. In future experiments, further troubleshooting can be done to optimize the results. A knockout (KO) model would allow the verification of these findings, however, due to the high funds and time required, it is not an easy option to pursue.

Departures from the Original Project Plan

During the planning for the study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was originally the loading control proposed, but β-actin was used instead. Furthermore, DMSO was not initially going to have its own wells with WT and TPP1 but was later added as a negative control. StarD3 and LCAT immunohistochemistry were also intended but were not done as it was not clear if the antibodies recognized the zebrafish proteins.

Impact of the Work

This research could provide a greater insight into the mechanisms behind CLN2 disease and allow the Bioscience community to become closer to discovering appropriate treatment options. Finding a suitable therapy will help to ensure that children affected by this neurodegenerative disease are given an equal opportunity as others to maintain their cognitive intellect well into adulthood. Finally, sharing the results of such an experiment will be a big step forward for neurodegenerative disorders in current Bioscience research.

Value of the Studentship

To the student: This studentship has taught me extensive laboratory techniques such as pipetting, microscopy, zebrafish husbandry, mutant identification, western blotting, calculation and preparation of reagents, and protein extraction. I have also had training in other important technical skills including data analysis using the iBright, power calculations, statistical analysis, and the production of graphs using GraphPad Prism. I would attend weekly meetings displaying my work, accumulating both presentation skills and technical literacy in PowerPoint, Canva, and Excel. My experience with zebrafish allowed me to garner invaluable insights into in-vivo experimental research involving vertebrates and I gained much appreciation of the ethical considerations when working with live animals.

To the research group: Due to the multitude of western blots performed, the protocol has become more optimised to carry future experiments out successfully. The conditions that were tried will no longer be replicated, saving time as the team continues to carry out this project. Discovering that the LCAT and StarD3 antibodies used in this experiment do not recognise zebrafish proteins will allow the team to shift their focus to other potential avenues.

Acknowledgements

I could not have carried out this research without my supervisor, Claire Russell (Figure 6), and Nastassja Ramdeen, a researcher in the same lab who guided me through the course of my studentship. I would also like to express my utmost gratitude to the Royal Veterinary College for kindly hosting me in the Russell laboratory and to the Biochemical Society for providing me with the resources to carry out this research using different antibodies.

Without this opportunity, I would have completed my undergraduate degree without ever having the experience of a hands-on research study in a laboratory environment. Thanks to the Biochemical Society, I was able to gain immense insight into a career in research, all while learning from an incredible team of researchers. My ambition to pursue a career in the sciences has been solidified. I am certain all the skills and knowledge I gained throughout this journey will create a strong foundation for me to build upon in my future career.

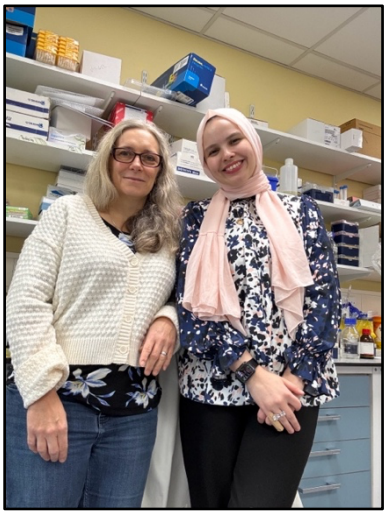

Figure 6: Claire Russell (project supervisor) and Shaima Kebabza (student) from left to right.

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