TELETHON KIDS INSTITUTE

Northern Entrance
Perth Children's Hospital
15 Hospital Avenue
Nedlands WA 6009

PO Box 855,
West Perth WA 6872

ABN 86 009 278 755

T | 61 8 6319 1000
E | info@telethonkids.org.au
W | telethonkids.org.au

Proudly supported by the people of Western Australia through Channel 7's Telethon

29th November 2020

Dear ACFRT Board Trustees,

I am writing to provide a final report for my ACFRT Post Graduate Studentship Grant entitled: "Personalized small molecule therapy to correct CFTR function in Cystic Fibrosis (CF) patients".

Currently available drugs do not help all patients with CF. CF is caused by over 2000 different mutations, varying the pattern of disease for each person. Here, I aimed to develop new drugs for people with rare CF mutations. The therapies that I have investigated are a type of drug called antisense oligonucleotides (AOs). AOs can change the message that is sent from the gene to protein. AOs are made to target the specific site in the *CFTR* gene where the mutation is. AOs can either cut out a mutation site or put a gene part back in, depending on the type of mutation. In selected cases, AOs could improve CFTR function and reduce the severity of disease. AOs are designed to target specific splicing sites to alter the mRNA transcript, with the aim of restoring protein function in diseases caused by frameshift, nonsense and missense mutations. The AO can alter splicing by either strengthen or weaken exon selection for inclusion in the mRNA. By modifying the mRNA structure, the disease-causing mutation is negated, and it may be possible to improve the function of the resulting protein, and as a result alter the course of disease.This project assessed first identified a systematic approach that was employed to develop therapeutic compounds to reduce the severity of CF in patients with mutations that are potentially amenable to splice intervention and restoration of the open reading frame.

With limited data available on the use of AOs to induce splice alterations in the *CFTR* gene, I first aimed at identifying other in-frame exons that may be excised from the mature mRNA using AOs. Exons and flanking introns carry a variety of highly coordinated splicing regulatory elements essential to the normal pre-mRNA processing. The current assumption in inducing targeted exon skipping is that the AOs can mask exonic splicing enhancers so that the exon is

not recognised by the splicing machinery and excluded from the mature mRNA along with the flanking introns. The first and last exons of the CFTR gene (total 27 exons) are not considered amenable to AO induced splice modulation, leaving 25 exons. Using the National Centre for Biotechnology Information (NCBI) database, thirteen of these exons are in-frame; 4, 5, 7, 9, 10, 11, 12, 13, 15, 20, 22, 23 and 24. According to variant location hot spots (Molinski *et al.*, 2018) and the availability of CF patient pAECs, seven of these exons were selected 4, 7, 9, 10, 11,15, 23 for further study.

Initial optimisation of AOs was undertaken using modified bases on a phosphorothioate backbone (2′-O-methyl) which were then transfected using Lipofectamine 3000 into monolayer non-CF and CF pAECs. The transfected cells were incubated for 48 hours after which the cells were collected, and RNA extracted. Reverse transcription polymerase chain reaction (RT-PCR) used *CFTR* primers amplifying the *CFTR* exons of interest to determine the level of induced exon skipping. Resulting transcripts were measured using densitometry and compared to estimate the relative efficiency of exon skipping. Of the 56 2′-O-methyl AOs that were tested for targeted exon skipping, all but one resulted in some level of splice modulation.

Seven of the most effective 2′-O-methyl AO sequences were re-synthesised as the clinically more appropriate PMO chemistry. The AO chemistry can impact the splice modulation capacity, therefore before AO induced protein changes can be assessed with PMOs, the mRNA splicing must be assessed first. Each PMO was electroporated at several concentrations into pAECs and incubated for 3, 5, 7 or 10 days to ascertain dose and time effects. Each PMO induced exon skipping ranging from 18% to 100%. Since it has been shown that restoration of CFTR function in just 6-10% of epithelial cells results in comparable chloride ion transport to those with 100% restoration (Johnson *et al.*, 1992), data generated from these experiments suggest that if an exon is deemed partially redundant, complete exon skipping and protein restoration may not be required to have therapeutic benefit.

Modification of CFTR protein size and changes in expression levels resulting from PMO splice modulation was initially assessed by western blot analysis. Here, non-CF and CF pAECs grown in monolayer and at ALI were utilised for Ussing Chamber analysis which is routinely used to quantify CFTR function. For these experiments, PMOs were electroporated into pAECs immediately prior to establishment on culture inserts, however it was observed that this impeded their capacity to differentiate with cultures developing large gaps in the cellular layer accompanied with low Trans Epithelial Electrical Resistance values. Based on these findings, PMOs were then added directly to the apical compartment of a partially differentiate ALI

culture at 200 µM on days 18, 20 and 22 prior to Ussing chamber analysis on day 28 (Stein *et al.*, 2010). However, gymnotic delivery of these uncomplexed PMOs resulted in no functional changes, with subsequent RT PCR revealing that only 3-4% exon skipping was achieved, highlighting the need for improved PMO delivery to airway epithelial cells.

To summarise, assessment of various AO sequences was done utilising human pAECs, where it was proposed that exon skipping to restore the reading frame of the *CFTR* gene could result in improved CFTR protein production for selected disease-causing mutations. Seven PMO sequences were optimised with each successfully skipping the targeted exon considered potentially amenable for excision. Although effects on protein could not be characterised due insufficient delivery of PMOs to pAECs, future work could utilise cell penetrating peptides conjugated to these PMO sequences, therefore allowing effective PMO delivery to differentiated pAEC ALI cultures and functional assessment to be conducted. It is proposed that the resulting internally truncated CFTR protein would have improved function and/or become amenable to CFTR modifying drugs for amenable cases of CF.

I am very much honoured and privileged to be selected as the recipient for this scholarship. If given the opportunity to do so, I would have a great interest in pursuing future research in CF.

Thank you

Sincerely

Ms Kelly Mary Martinovich B.Sc.

PhD Student

Telethon Kids Institute

The University of Western Australia

Presentations arising from this project

International Conference papers

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Rescue of CFTR Function Impaired by Mutations in Exon 15 in Children with Cystic Fibrosis.

E-Poster presentation- European Respiratory Society Congress, Virtual event, 6th -9th September 2020

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Antisense oligonucleotide mediated splice modulation to improve CFTR function of intron 9 5T polymorphism. (2019), Poster Session. *Pediatr Pulmonol*, 54: S155-S480. doi:10.1002/ppul.22495

Poster presentation- North American Cystic Fibrosis Conference, Nashville, Tennessee, USA, 31st October- 3rd November 2019.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Antisense oligonucleotide mediated splice modulation to improve CFTR function of intron 9 5T polymorphism.

Poster presentation: Australasian Cystic Fibrosis Conference, Perth, Australia, 4th -6th August 2019.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Rescue of CFTR Function Impaired by Mutations in Exon 15 in Children with Cystic Fibrosis. (2018), Poster Session Abstracts. *Pediatr Pulmonol*, 53: S148-S456. doi:10.1002/ppul.24152

Oral and poster presentation- North American Cystic Fibrosis Conference, Denver, Colorado, USA, 18th -20th October 2018.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF: A personalised medicine approach to rescue CFTR function in children with cystic fibrosis caused by a mutation specific intron 9 polymorphism.

Oral presentation- Australasian Cystic Fibrosis Conference, Melbourne, Victoria, Australia, 6th- 8th August 2017.

K.M. Martinovich, S. Fletcher, S.D. Wilton, A. Kicic, and S.M. Stick: Antisense Oligonucleotide therapy to rescue CFTR function in Cystic Fibrosis patients.

Poster presentation- Australasian Gene and Cell Therapy Conference, Sydney, NSW, Australia, 24th- 26th May 2017.

National conference papers

K.M. Martinovich, S. Fletcher, S.D. Wilton, A. Kicic, and S.M. Stick on behalf of AREST-CF & WAERP: Rescue of CFTR Function Impaired by Mutations in Exon 15 in Children with Cystic Fibrosis. 2019. *Respirology*, 24: 22-102 doi:10.1111/resp.13491. TO 015

Oral presentation- Thoracic Society of Australia and New Zealand Annual Scientific Meeting, Gold Coast, QLD, Australia, March 30th- April 2nd 2019.

K.M. Martinovich, S. Fletcher, S.D. Wilton, A. Kicic, and S.M. Stick: Antisense Oligonucleotide therapy to rescue CFTR function in Cystic Fibrosis patients.

Poster presentation- Lorne Genome Conference, Lorne, Victoria, Australia, 13th-15th February 2017.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick: Antisense Oligonucleotide therapy to treat CF.

Oral presentation- National Epithelial Workshop, Subiaco, Perth, Australia, 31st March-1st April 2016.

Local conference papers

K.M. Martinovich: A novel treatment for Cystic Fibrosis.

Invited talk, Thoracic society of Australia and New Zealand Lung Club Dinner meeting 17th November 2020.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Rescue of *CFTR* reading frame by antisense oligonucleotide splice modulation.

Oral-Presentation-Wal-yan Respiratory Research Centre Annual Scientific Meeting, Rottnest Island, Perth, Australia, 13th November 2020.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Antisense oligonucleotide mediated splice modulation to improve CFTR function of intron 9 5T polymorphism.

Poster presentation- Institute for Respiratory Health: The Next 20 Years Symposium, Perth, Australia, 29th November 2019.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Antisense oligonucleotide mediated splice modulation to improve CFTR function of intron 9 5T polymorphism.

Poster presentation- Combined Biological Science Meeting, Perth, Australia, 30th August 2019.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Potential of antisense oligonucleotides as a therapy for people with CF.

Oral presentation- An evening with a CF scientist; AREST-CF community night, Telethon Kids Institute, Perth Children's Hospital, Nedlands, Perth, Australia, 1st May 2019.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Rescue of CFTR Function Impaired by Mutations in Exon 15 in Children with Cystic Fibrosis.

Oral presentation- Combined Biological Science Meeting, WA Department of Health New Investigator Session, Nedlands, Perth, Australia, 31st August 2018.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF: Rescuing CFTR Function Caused by Frameshift, Nonsense Mutations in Cystic Fibrosis Patients.

Oral presentation- Telethon Kids Student Symposium, Rottnest Island, Perth, Australia, 5th-6th September 2017,

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF: A personalised medicine approach to rescue CFTR function in children with Cystic Fibrosis caused by a mutation specific intron 9 polymorphism.

Poster presentation- Combined Biological Science Meeting WA, Nedlands, Perth, Australia, 25th August 2017.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF: Rescuing CFTR function caused by mutation specific polymorphisms in Cystic Fibrosis patients.

Poster presentation- Thoracic Society of Australia and New Zealand WA branch Conference, Floreat, Perth, Australia, 28th-29th July 2017.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick on behalf of AREST-CF & WAERP: Developing personalised therapy for people with CF.

Oral presentation- An evening with a CF scientist; AREST-CF community night, Telethon Kids Institute, Subiaco, Perth, Australia, 4th May 2017.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick: Antisense Oligonucleotide therapy to rescue CFTR function in Cystic Fibrosis patients.

Oral presentation- Telethon Kids Institute Scientific Retreat, Nedlands, Perth, Australia, 14th-15th November 2016.

K.M. Martinovich, A. Kicic, S. Fletcher, S.D. Wilton, S.M. Stick: Antisense Oligonucleotide therapy to rescue CFTR function in Cystic Fibrosis patients.

Oral presentation- Western Australian Respiratory Research Symposium- Rottnest Island, Perth, Australia, 3rd-4th November 2016.

New Investigator Awards

New Investigator Award

Wal-yan Respiratory Research Centre

Rottnest Island, Perth, Australia, 13th November 2020

WA Department of Health New Investigator Award

Combined Biological Science Meeting

Nedlands, Perth, Australia, 31st August 2018.

Poster awards

Institute for Respiratory Health: The Next 20 Years Symposium 2019, Best poster award.

Combined Biological Science Meeting, Perth, Australia, 2019, Best poster award- Biochemistry & Molecular Biology