Final Report

Author: Dr Michelle Farrar
Qualification: MBBS (Hons) FRACP PhD
Institution: Sydney Children's Hospital, Randwick NSW
School of Women's and Children's Health, UNSW

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Title of Project: Establishing an Australian paediatric peripheral neuropathy biobank: a rich future scientific research resource

Summary: *(approximately 1,000 words)*

Peripheral nerve diseases result in progressive muscle weakness and wasting, and many with childhood onset may lead to significant disability and reduced survival. Spinal muscular atrophy (SMA) is a noteworthy exemplar, characterized by progressive muscle weakness and is the leading inherited cause of infant mortality. Following PBS listing in Australia in 2018, the first disease modifying agent, nusinersen, altered the therapeutic landscape for SMA leading to considerable improvements in survival, reduction in morbidity and attainment of functional motor skills across the phenotypic spectrum in treated patients. With a focus on the challenges and questions that remain open, an Australian paediatric peripheral neuropathy biobank provides a rich future scientific research resource

Funding was utilised to support a research co-ordinator within the neuromuscular research team to establish the collaborative biobanking programme to support long-term biomarker studies. The goal was to implement the infrastructure, capability and standard operative procedures to initiate a long-term programme of paediatric peripheral neuropathy biological sample collection and storage. The ultimate aim is to utilise these biospecimens and develop a biomarker platform to allow early detection and characterization of various paediatric neuropathies and its genetic subtypes and assist with monitoring future mechanistic therapies.

Ethics approval was obtained for collection and storage of biospecimens at Sydney Children's hospital Network, including Standard Operating Procedures. So far, more than 200 biospecimens have been collected and stored until experimental analyses in children with SMA who underwent procedures as part of standard care (e.g. venipuncture, lumbar puncture), alongside clinical assessments (aged 1 month – 18 years).

This will provide an ongoing rich resource for future studies, to expand scientific knowledge regarding pathophysiology and treatment responses, extending our clinical

and neurophysiological prospective ‘new natural history’ studies. The present project is built upon years of strong collaborative work and represents a key step essential to further interdisciplinary innovative research.

Hypothesis vs Findings:

Hypothesis: Establishing a data set of biological samples (blood, CSF, saliva, & urine), linked to clinical records from paediatric patients with SMA/peripheral neuropathy, will provide a rich resource for scientific discovery.

Findings: The Brain Foundation research Grant has greatly enabled us to establish a biobank of SMA at Sydney Children’s Hospital, Randwick. Routine CSF specimens of patients with genetically proven 5q-SMA who were treated with nusinersen at the Department of Neurology between Dec 3rd, 2018 - October 11th, 2019 were banked. In a period of 11 months, we collected 209 biological samples (CSF, serum, saliva, urine) from a well-characterised cohort of forty-two SMA patients who underwent repeated lumbar punctures for nusinersen administration. The samples were collected and banked at induction phase and maintenance phase of nusinersen. The cohort includes various SMA phenotypes, ranging from pre-symptomatic newborns to adolescents with SMA type 3. This has enabled new collaborations with the Ramaciotti centre UNSW Sydney to apply innovative genetic technologies for the discovery of novel biomarkers in this cohort.

Preliminary molecular findings:

For initial molecular analyses, CSF was chosen as it is in direct contact with the central nervous system (CNS) and is a promising source for finding biomarkers for diseases in the CNS. Preliminary work included method development using a small subset of stored samples for subsequent proteomic and RNA analyses. The method development phase is crucial to determine if the standard operating procedures currently used are accurate to efficiently extract proteins and RNA from the stored biological specimens.

CSF proteomics:

Protein concentration of CSF can vary between 0.3 – 0.7 mgs/ml. Additionally, low-abundance proteins, carrying great diagnostic potential, are often obscured by the presence of high-abundance proteins. The large dynamic range of proteins within the CSF (over 10 orders of magnitude) is a serious challenge for new biomarker discovery. This problem can be overcome by depleting over-represented proteins to go deeper into the proteome in order to identify putative biomarkers of disease.

Seppro SuperMix Depletion System was utilised to deplete CSF of highly abundant proteins such as albumin and immunoglobulins. These initial analyses identified a significant gain in the number of proteins in the depleted fraction compared to the non-

depleted CSF fraction. Compared to the 403 proteins identified in the non-depleted CSF fraction, 471 proteins were detected in the depleted CSF, representing an enrichment of 14% (Table 1).

Table 1: Comparison of protein concentration and number of proteins using Seppro SuperMix Depletion System

	Protein concentration	Number of proteins identified
Non-depleted fraction	0.025 µg/ µL	403
Depleted fraction	0.25 µg/ µL	471

Liquid chromatography–mass spectrometry data coupled with Mascot Daemon searches of SwissProt and LudwigNR databases yielded high-quality identifications of select protein species. Several were related species of the same 'parent' protein, differing in molecular weight and pI. It is interesting to note that several protein species have been identified in the literature as candidate biomarkers for neuropathies including amyotrophic lateral sclerosis and SMA. These protein species include Cadherin 13, Lumican, Follistatin, Cystatin C, Reticulon, Gelsolin, Neuronal cell adhesion molecule 1, Park 7, Aspartate aminotransferase, Fructose bisphosphate aldolase, Ubiquitin-40s ribsosomal protein amongst several others. String analysis revealed association between Lumican, gelsolin, cystatin 3 and Fructose bisphosphate aldolase. According to the molecular function analysis, most of the identified protein species were related to protease binding, lyase activity, cysteine-type endopeptidase inhibitor activity. The identified protein species were linked to several KEGG and Reactome pathways: carbon metabolism, gluconeogenesis, carbohydrate metabolism, neutrophil degranulation, amyloid fiber formation.

CSF RNA work:

We examined RNA composition of CSF using Total RNA Purification Kit (Norgen Biotek Corp). RNA isolations were carried out on two CSF samples using different starting volumes (100 µL and 200 µL), according to the manufacturer's protocol. We used the Agilent Small RNA Assay (Agilent Technologies 2100 Bioanalyzer with 2100 Expert Software) to determine the yield and purity of RNA.

Table 2: RNA concentrations using Bioanalyzer

ID	Initial vol for RNA extraction (µL)	RNA Conc Nanodrop (ng/ µL)	260/280 Nanodrop	260/230 Nanodrop	RIN Bioanalyzer Pico
Sample 1	100	12.33	1.88	0.7	1.6
Sample 1	200	12.4	1.68	0.32	1.6
Sample 2	200	9.98	1.85	0.74	2

Our preliminary RNA work confirmed absence of larger sized RNAs (28s and 18s) as expected given that CSF samples have a negligible number of cells (Figure 1). We found presence of small RNAs; micro RNA (miRNA), small-interfering RNA (siRNA), piwi-interacting RNAs (piRNA) in CSF (Figure 1). Our CSF RNA concentrations and RNA integrity number (RIN) are in line to what has been previously published in the literature. These preliminary results have been confirmed with the manufacturers (Norgen Biotek) for accuracy and reproducibility.

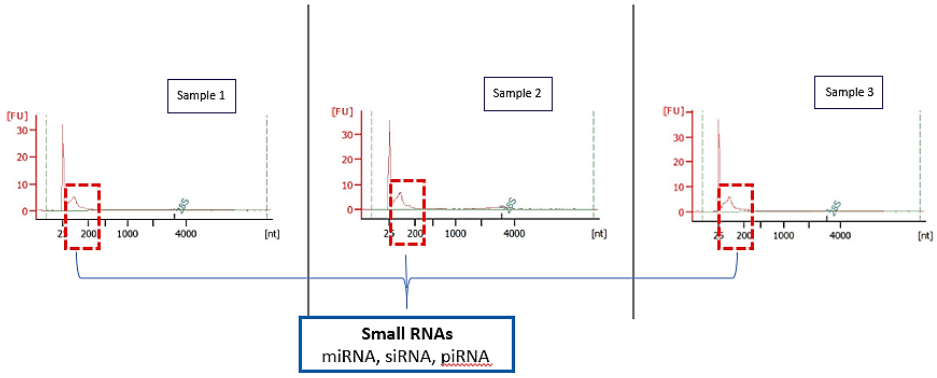

Figure 1: Total RNA composition of CSF showing presence of small non-coding RNAs.

Unanswered Questions: We continue to use these banked samples to determine proteomic and RNA markers predictive of disease onset, disease progression and response to treatment.

What these research outcomes mean: To continue to improve understanding of paediatric SMA, it is important to collect long-term data to enhance our ability to better understand factors influencing outcomes. Our preliminary data will assist in planning our ongoing research studies. We will promote ongoing collection of biological data and utilise banked samples to carry out molecular work to expand scientific knowledge regarding pathophysiology and treatment responses. Taken together, this will provide a rich resource for future studies and enhance collaboration for SMA research nationally. The present project represents a key step essential to translate research into clinical practice and to drive improved clinical outcomes and achieve best care for children with SMA and neuropathy.