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TITLE: Biomarker Identification by Conjoint Analysis of Metabolites and Gut Microbiome in ALS Patients and Animal Models to Monitor Disease Stages of ALS

PRINCIPAL INVESTIGATOR: Dr. John Nieland, PhD

CONTRACTING ORGANIZATION: 2N Pharma ApS, Aalborg East, Denmark

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2N Pharma ApS
Niels Jernes Vej 10,
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9220 Aalborg East
Denmark

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This grant proposes to develop a disease modifying treatment of Amyotrophic Lateral Sclerosis (ALS), targeting mitochondrial dysfunction as well as identify biomarkers to be used to screen potential ALS patients, differentiate diagnosed patients into subgroups and assess therapeutic efficacy. By combining the 2N Pharma (2N) team´s expertise in neurodegenerative disease research and the expertise of our co-applicant, Dr. Barmada in the research area of ALS/FTD with our innovative conceptualization, 2N can bring a novel treatment and a novel biomarker concept into a clinical market.

For over a decade, Dr. Nieland and collaborators have researched the connection between impaired energy metabolism and CNS diseases. Carnitine palmitoyl transferase 1 (CPT1) is a rate-limiting enzyme located in the outer mitochondrial membrane and is responsible for the conversion of long-chain acyl-CoAs into long-chain acyl-carnitines in the mitochondria. Based on findings reporting dysregulated metabolism in ALS patients and animal models of ALS, we hypothesize that inhibiting lipid metabolism by targeting CPT1 can halt progression or prevent induction of ALS.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. Introduction

2N Pharma is developing a disease-modifying drug for the treatment of Amyotrophic Lateral Sclerosis (ALS) based on our unique neurometabolic approach. By targeting the key enzyme in beta-oxidation, CPT1, with our small molecule, reversible partial fatty acid oxidation inhibitors (pFOXis), named Mitometin, we modulate the cellular energy generation by indirectly promoting glucose metabolism, which then restores the energy balance. The purpose of this project is to develop a disease modifying therapy for ALS and to identify biomarkers that can be used for screening of potential ALS patients, stratification of ALS patients as well as to assess the therapeutic efficacy of ALS drugs. The scope this research project is divided into three sections: A) In vitro studies, where we will assess Mitometin´s effect on reversing deficiencies in human iPSC-derived motor neurons from sporadic and familial ALS patients. B) In vivo studies, where we will evaluate the efficacy of Mitometin in SOD1 and TDP-43 mouse models. C) Machine learning tool, where we will combine data from ALS mouse models and data from humans (both ALS patients and healthy controls) to generate an AI platform to identify biomarkers that can predict disease onset and disease progression.

2. Keywords

ALS
Mitochondrial dysfunction
Cellular energy generation
Neurometabolic approach
Carnitine palmitoyl transferase 1
Human iPSC
Biomarkers
Metabolomics
Gut microbiome
Machine learning

3. Accomplishments

a. **What were the major goals of the project?**

The scientific content of the project is unchanged, but the timeline has been updated.

The original planned start-date was June 1, 2022, but the actual start data was January 1, 2023. The table below shows the original and revised expected completion dates.

Specific Aim	Description	Timeline (month)	Original planned completion date	Revised expected completion date	Percent completed
AIM 1	**Evaluate the efficacy of Mitometin in human iPSCs from ALS patients (in vitro)**	**5-21**	**Feb 2024**	**Sep 2024**	**In progress**
Task 1.1	HRPO approval plan	1-5	Feb 2023	May 2023	35%
Task 1.2	Bioenergetic analysis of human iPSCs	10-15	Aug 2023	Mar 2024	Not started
Task 1.3	LC-MS metabolomics	16-21	Feb 2024	Sep 2024	Not started
AIM 2	**Evaluate the efficacy of Mitometin in SOD1 and TDP-43 mouse models (in vivo) and investigate metabolites in serum**	**5-19**	**Aug 2023**	**Jul 2024**	**In progress**
Task 2.1	ACURO approval plan (SOD1 model)	5-7	Aug 2022	Jul 2023	10%
Task 2.2	SOD1 mouse model	8-13	Feb 2023	Jan 2024	Not started
Task 2.3	ACURO approval plan (TDP43 model)	5-7	Aug 2022	Jul 2023	10%
Task 2.4	TDP43 mouse model	8-13	Feb 2023	Jan 2024	Not started
Task 2.5	Metabolomic analysis (mouse samples)	14-19	Aug 2023	Jul 2024	Not started
AIM 3	**Investigate the gut microbiome in SOD1 and TDP43 mice**	**14-18**	**Apr 2023**	**Jun 2024**	**Not started**
Task 3.1	Microbiome analysis (mouse samples)	14-18	Apr 2023	Jun 2024	Not started
AIM 4	**Conduct a conjoint analysis of metabolomic and microbiome data from in vitro and in vivo studies**	**16-22**	**Nov 2023**	**Oct 2024**	**Not started**
Task 4.1	Bioinformatic analysis of metabolomic (2.5) and microbiome (3.1) data	16-22	Feb 2024	Oct 2024	Not started
AIM 5	**Investigate metabolites in serum and CSF from ALS patients and combine this into a machine learning tool for identification of human biomarkers in ALS patients**	**7-24**	**May 2024**	**Dec 2024**	**In progress**
Task 5.1	HRPO approval plan	1-8	May 2023	Aug 2023	50%
Task 5.2	Metabolomic analysis (human samples)	13-16	Sep 2023	Apr 2024	Not started
Task 5.3	Bioinformatic analysis of all data generated throughout the project (cell, animal and human data)	17-24	May 2024	Dec 2024	Not started

b. **What was accomplished under these goals?**

The project start was delayed for 7 months and the actual project start date was January 1, 2023.

We used the time between the original planned start date, June 1, 2022, and the actual start date, January 1, 2023, to select the optimal compound by screening and characterizing 2N Pharma's pFOXi molecules in-house and with external partners. After selecting a molecule, we engaged Syngene International to synthesize a new batch of the compound and perform a range of ADME assays.

We have been in contact with the subcontractors (University of Michigan, DNA Sense, Metabolon, JAX), who are all informed about the delay.

Major activities during the reporting period:

- **Synthesis of a new batch.**

  Objective: To synthesize the selected 2N molecule (2N050) for the *in vitro* and *in vivo* studies.

  Result: We engaged Syngene International to synthesize a batch of 2N050 and conduct DMPK studies. This will be finalized in May 2023.

- **Preparation of HRPO documents.**

  Objective: To submit the HRPO documents related to Task 1 and Task 5.

  Result: We are soon finalizing the documents for Task 1, whereas we are still working on the documents for Task 5. We expect to submit these documents in August 2023.

- **Planning of animal studies.**

  Objective: To plan the ALS mouse models (SOD1 and TDP43 model).

  Result: We have had meetings with both the original intended service provider and alternative providers to discuss the details of the TDP43 mouse model (experimental setup, dosing, timeline etc.). We will need to design the study in more details before initiating, and the selected service provider will need to complete the ACURO documents before initiating any activities. This activity is therefore still ongoing.

c. **What opportunities for training and professional development has the project provided?**

Preben Bruun-Nyzell (CEO) and Anne Skøttrup Mørkholt (Project Manager) are participating in the Hyperloop Neuroscience 2023 program, organized by the Danish ministry of foreign affairs, and will attend the World Medical Innovation Forum in Boston and visit the Spaulding Neuro-Rehabilitation Center at Mass General Brigham and Boston Children's Hospital Neuroscience Center.

John Nieland (PI) has presented 2N Pharma's research at national and international conferences/seminars:

- Precision in Drug Discovery & Preclinical Summit (PDDP) in Amsterdam, The Netherlands
- New research on brain diseases – Parkinson´s disease, Multiple sclerosis and Alzheimer´s at Folkeuniversitetet, Denmark
- Other presentations in Denmark for Parkinson's patient organizations.
- Dr. Nieland's work and 2N Pharma was mentioned in an article in the Washington Post: https://www.washingtonpost.com/climate-environment/2022/11/30/shrews-shrink-regrow-own-brains/

- Dr. Nieland was interviewed by CBC Radio-Canada based on the article in Washington Post.

d. **How were the results disseminated to communities of interest?**

Nothing to report.

e. **What do you plan to do during the next reporting period to accomplish the goals?**

We intend to initiate several activities within the next reporting period (5/16/2023 to 5/15-2024).

Dr. Sami Barmada, University of Michigan will complete Task 1.2. When we receive the compound from Syngene International, he can start the work related to this task.

2N Pharma and JAX (or other service provider) will complete Task 2.1, 2.2, 2.3 and 2.4.

2N Pharma will complete Task 5.1, which allows us to start collecting samples from ALS patients and healthy controls.

4. Impact

a. **What was the impact on the development of the principal discipline(s) of the project?**

b. **What was the impact on other disciplines?**

c. **What was the impact on technology transfer?**

d. **What was the impact on society beyond science and technology?**

Nothing to report in section 4. Impact.

5. Changes/Problems

a. **Changes in approach and reasons for change**

Nothing to report.

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

The project start data was delayed 7 months, from the original planned start date of June 1, 2022 to the actual start data of January 1, 2023. The cause was technical issues with selecting the optimal pFOXi molecule for the project.

Despite the initial problems and delay during our first reporting period, we intend to complete the project as originally proposed, though we have an adjusted timeline. This is a large and complex project, which involve tasks with both cell work, animal experiments and serum and CSF samples from ALS patients, which is why we are not worried about the delay as this is normally a part of doing research. We will work hard to complete this interesting project and fulfill our milestones.

Chemistry resources

The major reason of delayed project is due to selection/synthesis of 2N compound.

Uddybes. Hvor meget vil vi fortæller her?

Cell studies

The cell studies in Task 1.2 and Task 1.3 are delayed due to the delay with synthesis of compound. We have completed the synthesis and plan to initiate Task 1.2 in October 2023.

Animal studies

The animal studies in Task 2.2 and Task 2.4 are delayed due to the delay with synthesis of compound and preparation of ACURO documents. We have completed the synthesis now and are in the planning phase of the study design. We initiated Task 2.1 and Task 2.3. We plan to initiate Task 2.2 and Task 2.4 in August 2023.

HRPO documents

Task 1.1: We have finalized the HRPO form but are awaiting the determination letter and protocol for Task 1.2 and 1.3. We expect to submit the documents for review to HRPO in May 2023.

Task 5.1: We have submitted an application to the Danish Health and Ethics Committee in April 2023. We are now waiting for final approval. After this, we expect to submit the documents for review to HRPO in August 2023.

c. **Changes that had a significant impact on expenditures**

Nothing to report.

d. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

e. **Significant changes in use or care of human subjects**

Nothing to report.

f. **Significant changes in use or care of vertebrate animals.**

Nothing to report.

g. **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. Products

a. **Publications, conference papers, and presentations**

b. **Website(s) or other Internet site(s)**

c. **Technologies or techniques**

d. **Inventions, patent applications, and/or licenses**

e. **Other Products**

Nothing to report in section 6. Products.

7. Participants & Other Collaborating Organizations

a. **What individuals have worked on the project?**

Name:	*John Nieland*
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-7423-0122
Nearest person month worked:	1
Contribution to Project:	Dr. Nieland has been overseeing and guiding the project activities.
Funding Support:	None

Name:	*Anne Skøttrup Mørkholt*
Project Role:	Project Manager
Researcher Identifier (e.g. ORCID ID):	0000-0002-7718-6312
Nearest person month worked:	5
Contribution to Project:	Dr. Mørkholt has been planning and managing the activities in the project. She has been into contact with subcontractors and held meetings. She has prepared the Danish Health and Ethics Committee application related to Task 5.1
Funding Support:	None

Name:	*Steinunn Sara Helgudottir*
Project Role:	Senior Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0003-2452-7504
Nearest person month worked:	2
Contribution to Project:	Dr. Helgudottir has been planning and managing the activities related to Task 2. She has been into

	contact with subcontractors and held meetings. She has contributed to the Danish Health and Ethics Committee application related to Task 5.1
Funding Support:	None

b. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Lisa Juul Routhe has left the company and has been replaced by Dr. Steinunn Sara Helgudottir. Biosketch is attached.

c. **What other organizations were involved as partners?**

Syngene International, Biocon Park, Plot No. 2 & 3, Bommasandra Jigani Link Road, Bangalore 560 099, India. Syngene was hired to synthesize a batch of 2N Pharma´s compound for *in vitro* and *in vivo* studies and conduct DMPK studies.

8. Special Reporting Requirements

No special reporting requirements.

9. Appendices

BIOGRAPHICAL SKETCH

NAME: Steinunn Sara **Helgudottir,** Ph.D

POSITION TITLE: Head of Biology

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
Aalborg University, Aalborg, Denmark	B.Sc	06/2015	Medicine with Industrial Specialization
Aalborg University, Aalborg, Denmark	M.Sc	06/2017	Medicine with Industrial Specialization
Aalborg University, Aalborg, Denmark	Ph.D	06/2021	Faculty of Medicine

A. Personal Statement

I am the Head of Biology at 2N Pharma and I will serve as a senior scientist.

I am specialized in the research area of neurodegenerative diseases and targeting of the blood-brain barrier and will make use of this knowledge gained by years of research in this field.

During my years at Aalborg University as a research assistant and PhD Fellow, I conducted research in the Neurobiology and Drug delivery group at Aalborg University lead by Dr. Torben Moos. I have gained expertise in the area of targeting the pathological blood-brain barrier, conducting multiple transport studies as well as investigating the pathological alterations in the blood-brain barrier during neurodegeneration. I have years of experience in optimization of cellular-based *in vitro* assays and in fundamental laboratory techniques in molecular biology, such as isolation of cells, establishing models of the blood-brain barrier, transport studies, probe-based qPCR, ELISA, cell viability assays, immunohistochemistry, immunocytochemistry, epigenetic analysis and more. Thus, I bring substantial knowledge and experience to carry out my role successfully.

Publications and Research Products

Thomsen.M, Kostrikov.S, Routhe.LJ, Johnsen. K.B, Helgudóttir SS, Gudbergsson. J, Andreasen.L, Moos. T. *Preservation and substantial remodeling of the brain angioarchitecture in experimental chronic neurodegeneration* (2023) Submitted

Rasmussen. CM, Olsen.E., Routhe.LJ, Körbelin.J, Helgudóttir. SS, Thomsen. L, Schwaninger.M, Burthart.A, Moos. T. Journal of Neurochemistry 2023-01
A novel strategy for delivering Niemann‐Pick type C2 proteins across the blood–brain barrier using the brain endothelial‐specific AAV‐BR1 virus (2022)

Helgudóttir. SS
Expressional prerequisites for targeted drug delivery to the pathological brain (2021)

Helgudóttir. SS, Routhe. LJ, Burkhart. A, Jønsson. K, Pedersen. I, Lichota. J, Moos. T.
Molecular Neurobiology volume 57, pages 3526–3539 (2020)
Epigenetic regulation of ferroportin expression in the blood-brain barrier

Helgudóttir. SS, Lichota. J, Burkhart. A, Moos. T.
Molecular Neurobiology volume 56, pages 2362–2374 (2019)
Hepcidin mediates transcriptional alteration of ferroportin in differentiated neuronal-like PC12 cells subjected to iron challenge

B. Positions and Honors

Positions and Employment

2016 – 2017	Student assistant at the Medical School of Health, Aalborg University, Denmark
2017 – 2019	Research Assistant, Department of Health Science and Technology, Aalborg University, Denmark
2019 – 2021	PhD Fellow at the Neurobiology and Drug delivery group, Department of Health Science and Technology, Aalborg University, Denmark
2021 – 2022	Project Manager at 2N Pharma, NOVI Science Park, Aalborg, Denmark
2023 – now	Head of Biology at 2N Pharma, NOVI Science Park, Aalborg, Denmark

Professional Memberships, Licensures and Board Certifications

2019 **Laboratory Animal Science (corresponds to FELASA AD license), Aarhus University, Denmark**

C. Contributions to Science

How to target the pathological blood-brain barrier

Most therapeutics developed to treat neurodegenerative diseases fail in clinical trials, often due to limited accumulation within the brain parenchyma. It is therefore crucial to understand the blood-brain barrier and the alterations that occur during neurodegeneration, which can affect the transport and thereby response to a given drug. My research has contributed with data displaying that receptors present on the brain capillary endothelial cells are susceptible to epigenetic alterations during neuroinflammation, resulting in altered expression. I have conducted substantial amount of research in this area, which will be published later this year:

a) *Epigenetic induction of transferrin receptor expression on brain capillary endothelial cells*
Helgudóttir. S.S, Johnsen. K, Routhe. L, Thomsen. M. S, Rasmussen. C, Karamehmedovic. A, Moos. T.
b) *A novel Neuroinflammatory Blood-Brain barrier model using primary mouse brain capillary endothelial cells*
Helgudóttir. S.S, Burkhart. A, Routhe. L, Haraldsdóttir. H, Holm-Jacobsen. J, Dahl. S, Pretzmann. F, Lambertsen. K, Thomsen. M. S, Moos. T.
c) *Neuroinflammation-induced expressional alterations of targets for drug delivery*
Helgudóttir. S.S, Routhe. L, Rasmussen. C, Thomsen. M. S, Moos. T.

D. Additional Information: Research Support and/or Scholastic Performance

2020	
Multiple Sclerosis foundation, research grant	125,000 DKK
2019	
Multiple Sclerosis foundation, research grant	150,000 DKK
2018	
Multiple Sclerosis foundation, research grant	150,000 DKK
Aase og Ejnar Danielsens fond	83,000 DKK
Kong Christian den Tiendes fond	25,000 DKK
Iron, myelin and the brain traveling grant	5,000 DKK
2017	
Multiple Sclerosis foundation, research grant	50,000 DKK
BioIron Society traveling grant	5,000 DKK
2015	
A.P. Møller foundation recipient	25,000 DKK