Statistical Analysis Plan	
Clinical Investigation Plan Title	New Imaging Biomarkers for Muscular Diseases - Multispectral Optoacoustic Imaging in Spinal Muscular Atrophy (MSOT_SMA)
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1. Version History

Version	Changes	Authors, Title
v1.0	SAP generated	Adrian P. Regensburger, M.D. Ferdinand Knieling, M.D.
v.1.1	SAP under review (11/07/2019)	external Biostatistician

2. List of Abbreviations and Definitions of Terms

Abbreviation	Definition
SMN1	Survival motor neuron 1
SMN2	Survival motor neuron 2
OAI	Optoacoustic imaging
MSOT	Multispectral optoacoustic tomography
HINE	Hammersmith Infant Neurological Examination
CHOP Intend	The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders
HFMSE	expanded Hammersmith functional motor scale
ULM/RULM	Revised Upper Limb Module
6-MWT	6-minute walking test
RUCT	reflected ultrasound computed tomography
ROC	Receiver operator characteristics
HV	healthy volunteers
SMA	spinal muscular atrophy patients

3. Introduction

SMA is an autosomal-recessive disorder, characterized by progressive muscle weakness and atrophy with an incidence of 1/10,000. The condition is caused by a homozygous deletion or mutation in the survival motor neuron 1 (SMN1), resulting in reduced expression of the survival motor neuron (SMN) protein. This leads to the degeneration of motor neurons in the spinal cord and brain stem. A nearby related gene, survival motor neuron 2 (SMN2), could partially compensate the loss of SMN1. Individuals with a higher copy number of SMN2 do in general have a milder phenotype. New therapeutic approaches, e.g. nusinersen (spinraza©), an antisense oligonucleotide medication that modulates pre-messenger RNA splicing of the survival motor neuron 2 (SMN2) gene, are promising to help the formerly incurable children. However, most clinical trials lack primary outcomes other than clinical testing. At the moment there are no prospective, quantitative biomarkers available to detect muscle atrophy at an early age, and to follow up disease progression.

As a new imaging modality, optoacoustic imaging (OAI) combines benefits of optical (high contrast) and acoustic (high resolution) imaging. Multispectral optoacoustic tomography (MSOT) is therefore capable of visualizing the distribution of endogenous absorbers by initiating laser-induced thermoelastic expansion and detection of resulting pressure waves. This imaging technique enables the label-free detection and quantification of different endogenous chromophores, such as melanin, hemoglobin, deoxyhemoglobin and lipids. Previously, it was demonstrated that MSOT is capable to monitor disease severity in Crohn's disease by detecting different signal levels of hemoglobin as markers of intestinal inflammatory activity.

In this study we want to refine the capability of MSOT to characterize muscle tissue and to determine a non-invasive, quantitative biomarker for the disease assessment in SMA patients from birth using MSOT.

4. Study Objectives and endpoints

The study population consists of patients with spinal muscular atrophy and healthy volunteers.

Objectives

Primary objective

- Comparison of the optoacoustic spectrum determined by MSOT in patients with SMA and healthy volunteers

Secondary objectives

- Comparison of the quantitative lipid signal fraction determined by MSOT in patients with SMA and healthy volunteers
- Comparison of the quantitative fraction of collagen signal determined by MSOT in patients with SMA and healthy volunteers
- Comparison of the quantitative fraction of hemo-/myoglobin signal determined by MSOT in patients with SMA and healthy volunteers
- Comparison of the quantitative fraction of oxygenated/deoxygenated hemoglobin determined by MSOT in patients with SMA and healthy volunteers
- Correlation of lipid content determined with MSOT with disease duration/patient age
- Correlation of collagen determined by MSOT with disease duration/patient age
- Correlation of haemoglobin/myoglobin content determined by MSOT with duration of disease/patient age
- Correlation of oxygenated/deoxygenated hemoglobin determined by MSOT with duration of disease / patient age
- Correlation of lipid content determined with MSOT with age-related functional muscle tests (Hammersmith Infant Neurological Examination (HINE)/ The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP Intend)/expanded Hammersmith functional motor scale (HFMSE)/ Revised Upper Limb Module (RULM)/ &-Minute-Walk Test (6-MWT)))
- Correlation of collagen determined with MSOT with age-dependent functional muscle tests (HINE/ CHOP Intend/ HFMSE/Revised Upper Limb Module/6-MWT)
- Correlation of hemo-/myoglobin content determined with MSOT with age-dependent functional muscle tests (HINE/ CHOP Intend/ HFMSE/Revised Upper Limb Module/6-MWT)
- Correlation of oxygenated/deoxygenated hemoglobin determined with MSOT with age-related functional muscle tests (HINE/ CHOP Intend/ HFMSE/Revised Upper Limb Module/6-MWT)
- Measurement of signal differences in right / left comparison

- Correlation of MSOT ultrasound image (RUCT) with standard sonography

Endpoints

Primary Endpoint

- Spectral profile of muscle tissue [Time Frame: Single time point (1 day)]: Spectral profile of muscle tissue determined by multispectral optoacoustic tomography (MSOT) of patients with spinal muscular atrophy compared to healthy volunteers units

Secondary Endpoints

- Muscular lipid content [Time Frame: Single time point (1 day)]: Quantitative lipid signal derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA compared to healthy control Units: arbitrary units (a.u.)
- Muscular collagen content [Time Frame: Single time point (1 day)]: Quantitative collagen signal derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA compared to healthy control Units: arbitrary units (a.u.)
- Muscular myo-/hemoglobin content [Time Frame: Single time point (1 day)]: Quantitative myo-/hemoglobin signal derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA compared to healthy control Units: arbitrary units (a.u.)
- Muscular de-/oxygenated myo-/hemoglobin content [Time Frame: Single time point (1 day)]: Quantitative de-/oxygenated myo-/hemoglobin signal derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA compared to healthy control Units: arbitrary units (a.u.)
- Correlation of lipid signal with clinical data (age/disease duration) [Time Frame: Single time point (1 day)]: Quantitative lipid signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA correlated with individual clinical data (disease duration/age (in month))
- Correlation of collagen signal with clinical data (age/disease duration) [Time Frame: Single time point (1 day)]: Quantitative collagen signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA correlated with individual clinical data (disease duration/age (in month))
- Correlation of myo-/hemoglobin signal with clinical data (age/disease duration) [Time Frame: Single time point (1 day)]: Quantitative myo-/hemoglobin signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in

patients with SMA correlated with individual clinical data (disease duration/age (in month))

- Correlation of de-/oxygenated myo-/hemoglobin signal with clinical data (age/disease duration) [Time Frame: Single time point (1 day)]: Quantitative de-/oxygenated myo-/hemoglobin signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA correlated with individual clinical data (disease duration/age (in month))
- Correlation of lipid signal with physical assessment (HINE/HFMSE/CHOP INTEND/ULM) [Time Frame: Single time point (1 day)]: Quantitative lipid signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA correlated with individual physical assessment (HINE/HFMSE/CHOP INTEND/ULM)
- Correlation of collagen signal with physical assessment (HINE/HFMSE/CHOP INTEND/ULM) [Time Frame: Single time point (1 day)]: Quantitative collagen signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA correlated with individual physical assessment (HINE/HFMSE/CHOP INTEND/ULM)
- Correlation of myo-/hemoglobin signal with physical assessment (HINE/HFMSE/CHOP INTEND/ULM) [Time Frame: Single time point (1 day)]: Quantitative myo-/hemoglobin signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA correlated with individual physical assessment (HINE/HFMSE/CHOP INTEND/ULM)
- Correlation of de-/oxygenated myo-/hemoglobin signal with physical assessment (HINE/HFMSE/CHOP INTEND/ULM) [Time Frame: Single time point (1 day)]: Quantitative de-/oxygenated myo-/hemoglobin signal (Units: arbitrary units (a.u.)) derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA correlated with individual physical assessment (HINE/HFMSE/CHOP INTEND/ULM)
- Side differences of MSOT signals [Time Frame: Single time point (1 day)]: Quantitative collagen signal derived by transcutaneous Multispectral Optoacoustic Tomography (MSOT) in patients with SMA compared between sides Units: arbitrary units (a.u.)
- Correlation of RUCT and B-Mode Ultrasound [Time Frame: Single time point (1 day)]: Quantitative grey scale signal derived by reflection mode ultrasound computed tomography (RUCT) correlated with grey scale B-Mode Ultrasound

5. Investigation Plan

- This is a mono-centric, open-labeled study, which aims to compare the optoacoustic spectra and optoacoustic signals between healthy volunteers (HV) and SMA patients.
- The study will include 10 HV and 10 SMA patients.
- Each subject will be examined by MSOT, ultrasound and physical examinations. Clinical data will be obtained from the medical record and during the study.

Inclusion Criteria:

- genetically proven SMA

Exclusion Criteria:

- Pregnancy
- Tattoo on skin to be examined
- For healthy volunteers only: suspected muscular disease/myopathia

6. Determination of Sample Size

Due to lack of clinical trials in this area, the comparative changes of MSOT spectra and signals in patients with SMA are unknown. Therefore, no sample size calculation was performed. This study will also serve as a basis for power calculations for future trials.

7. Target parameters

All measurements with MSOT are performed over the proximal and distal limb muscles in a right-left comparison (leg proximal: Musculus quadriceps, distal: Musculus triceps surae; arm proximal: Musculus biceps, distal: Forearm flexors) in healthy subjects compared to patients with SMA.

Primary target:

Optoacoustic Absorption Spectrum of Muscle.

This target is measured non-invasively by MSOT

Secondary targets:

Quantitative lipid signal (in arbitrary units)
Quantitative collagen signal (in arbitrary units)
Quantitative hemo/myoglobin signal (in arbitrary units)
Muscle oxygenation (in %)
Ratio of lipid to hemo/myoglobin signal or collagen to hemo/myoglobin signal
These target values are collected non-invasively using MSOT.

Muscle texture (Heckmatt score, echogenicity)
These target values are determined by means of B-image sonography.

Clinical scores for determining muscle strength consisting of:
Patients < 2 years and patients ≥ 2 years with inability to sit:

- Hammersmith Infant Neurological Examination (HINE)/ The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP Intend)

at age ≥ 2 years and ability to sit in a wheelchair additionally:

- Revised Upper Limb Module (RULM)

Patients ≥ 2 years and sitting ability:

- expanded Hammersmith functional motor scale (HFMSE)/ Revised Upper Limb Module (RULM)

in case of ability to walk additionally:

- 6-minute walking test

<table>
<tr><td>< 2 years</td><td>≥ 2 years with inability to sit</td><td>≥ 2 years and ability to sit in a wheelchair</td><td>≥ 2 years and ability to sit</td><td>≥ 3 years and ability to walk</td></tr>
<tr><td>HINE Section 2</td><td>HINE Section 2</td><td>HINE Section 2</td><td colspan="2" rowspan="2"></td></tr>
<tr><td>CHOP Intend</td><td>CHOP Intend</td><td>CHOP Intend</td></tr>
<tr><td colspan="2" rowspan="3"></td><td>RULM</td><td>RULM</td><td>RULM</td></tr>
<tr><td></td><td>HFMSE</td><td>HFMSE</td></tr>
<tr><td></td><td></td><td>6MWT</td></tr>
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These target values are clinically determined at presentation:

Age

Sex

Weight

Skin color

Ethnic Background

Disease duration

Current medication

7. Statistical Methods

7.1. Study Subjects

We will describe all screened and enrolled patients.

7.2. Clinical Investigation Plan (CIP) Deviations

Data will be analyzed according to the SAP; any further/additional/deviation from the SAP will be reported as such. Further post-hoc analysis will be performed, if necessary.

7.3 Analysis Sets

One analysis set will be created for study purpose.

7.4 General Methodology

Continuous variables are given as means and standard deviations; categorical variables are provided as numbers and percentages. If appropriate, descriptive statistics will be provided using Tables.

MSOT spectra are compared between HV and SMA patients. An area under the curve (AUC) of individuals/per group spectra will be compared. Furthermore, single wavelength of individuals/groups will be compared.

Between group analyses regarding unmixed and single wavelength MSOT signals will be analyzed as follows: Data are tested for normal distribution using Shapiro-Wilk test prior to inferential analysis. MSOT signals are compared between cohorts in a pairwise manner (matched for age) using dependent samples t-tests. If the assumption of normal distribution is violated Wilcoxon signed-rank tests is used. Receiver operator characteristics (ROC) analysis between muscles of HV and SMA-patients is performed. As gold standard genotyping is used. All inferential tests are two-tailed, p values ≤ 0.05 are considered statistically significant. Bonferroni-Holm adjustment is used to control type I error, if appropriate. All analyses are performed using GraphPad Prism (Version 7.00 or newer, GraphPad Software, La Jolla, CA, USA) and/or IBM SPSS Statistics, version 25 or newer (IBM Corp., N.Y., USA).

7.5 Handling of Missing Data and Dropouts

No method of imputation will be used for missing data.

7.6 Safety Evaluation

During the study adverse events and serious adverse events will be monitored. The investigator is available for study subjects at any time, in case of any events.