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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
DRE	**Digital Research Environment**
EGA	**European Genome-phenome Archive (EGA)**
EU	**European Union**
GCP	**Good Clinical Practice**
HPO	**Human Phenotype Ontology**
IC	**Informed Consent**
IEM	**Inborn Error of Metabolism**
IMP	**Investigational Medicinal Product**
METC	**Medical research ethics committee (MREC); (in Dutch: medisch ethische toetsing commissie (METC))**
NVK	**Dutch Society for Pediatrics; (in Dutch: Nederlandse Vereniging Kindergeneeskunde)**
PGD	**Preimplantation Genomic Diagnosis**
RD	**Rare Disease**
VKGL	**Dutch Society for Laboratory Scientists in Clinical Genetics; (in Dutch: Verenging Klinische Genetica voor Laboratoriumspecialisten)**
VKGN	**Dutch Society for Clinical Geneticists; (in Dutch: Vereniging Klinische Genetica Nederland)**
VUS	**Variant of Unknown Significance**
WES	**Whole Exome Sequencing**
WGS	**Whole Genome Sequencing**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**
ZOEMBA	**Zoektocht naar Erfelijke MetaBole Aandoening**

SUMMARY

Rationale: Inborn Errors of Metabolism (IEM) are monogenic conditions in which the impairment of a biochemical pathway is intrinsic to the pathophysiology of the disease. Organ dysfunction results from intoxication and/or storage of metabolites, as well as a shortage of energy and building blocks. Rapid diagnosis of IEM enables initiation of targeted treatment (e.g. diet) slowing down or stopping the degenerative nature of the disease, resulting in significantly reduction of morbidity and mortality. A diagnosis also enables prognostication, access to community services and accurate genetic counselling for the patient and his/her family. Diagnosing IEM can be a major challenge, because of phenotypic heterogeneity and complex, expensive, diagnostic tests. Whole exome/ genome sequencing (WES/WGS) has revolutionized diagnostics of rare diseases and IEM, but still gives a negative or inconclusive result in >50% of cases. Addition of other omics technologies (metabolomics, glycomics, lipidomics, epigenomics, transcriptomics, proteomics) with integrated bioinformatics increases diagnostic yield, as it may point to the defective pathway allowing scrutinizing genes in genomic data or vice versa: it generates evidence of the deleterious functional impact of a DNA variants of unknown significance (VUS). In this study we will unite our national expertises and apply a multi-omics approach to solve the unsolved genetic basis of patients with a metabolic phenotype on a larger scale.

Objective: Integrating genomic (WES/WGS) and other -omics technologies in order to find the genetic causes, in 500 patients (children and adults) with an unexplained metabolic phenotype in whom standard care (genetic and metabolic evaluation) did not provide a diagnosis.

Study design: A prospective, diagnostic (deep phenotyping, WES/WGS and pan-omics) multicenter cohort study.

Study population: (In)capacitated patients (all ages/both genders) with a clinical (and/or family) history and abnormal additional examination (physical (neurological)/ biochemical/ radiological/ genetic) suspicious for an IEM, without diagnosis.

Main study parameters/endpoints: 1) identification of a genetic variant and alignment with its biochemical and phenotypical abnormalities; 2) evaluating the diagnostic yield of combined WES/WGS and omics techniques

Methods used: Patients with unexplained metabolic phenotypes are referred (on paper) and discussed by the ZOEMBA (Zoektocht naar Erfelijke MetaBole Aandoening) team. Clinical phenotyping, bioinformatic reanalysis of WES data and additional metabolomics will be performed in all participants. In case still no diagnosis is made, a tailormade diagnostic plan is made combining deep WES, WGS, glycomics, lipidomics, epigenomics, transcriptomics and/or proteomics leading to: a known IEM, a candidate variant or no diagnosis. In case of a

variant, additional functional studies (enzymatic assays, targeted omics, CRISPR/CAS, cell lines) will be performed to confirm the effect of the genetic variant on protein function. When still no diagnosis is established, matchmaking (genetic/phenotypical) through international databases might lead to a diagnosis.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:

The study involves collection of clinical data, reanalysis of previously analysed genetic data, additional “omics” and functional testing. All participants will have between 1 and 3 clinical visits for this study (at the UMC of referral) and a maximum of 2 telephone appointments with the arts-onderzoeker. Whenever possible study visits will be combined with regular hospital visits. Clinical data (clinical history, family history, physical examination, consultations, additional laboratory and/or radiological investigations) will be collected. A physical examination and blood and urine sampling will be performed in all participants at their first study visit. Any other already available biological samples (eg stored cell lines, dried blood spots, cerebrospinal fluid (CSF)) will be collected for re-analysis. For a selection of patients a skin biopsy will be performed at the 2nd clinical study visit for the use of functional studies. Potential burdens for participants are: the additional study visit(s), diagnostic procedures (e.g. blood, urine sampling and skin biopsy), as well as renewed (false) hope/uncertainty about finding a diagnosis. The potential benefit for all participants include: the opportunity to establish a diagnosis providing information on prognosis, (refinement of) management, genetic counselling with precise recurrence risk and option(s) for prenatal diagnosis.

1. Introduction and Rationale

Background: Rare Diseases (RD) and Inborn Errors of Metabolism (IEM)

The more than 7000 known rare genetic diseases collectively affect over 1,4 million persons in the Netherlands. The statistics on RDs are devastating: two-thirds of RDs are serious and disabling, three-quarters manifest in childhood, over half are life-limiting, most have no treatment and almost all have an enormous negative impact on family well-being (Boycott et al., 2013). Clearly, RDs represent a major healthcare challenge, contributing significantly to morbidity, mortality, and Dutch healthcare costs.

With the introduction of whole exome sequencing (WES), the targeted interrogation of the protein-coding exons (20,000 genes) of the genome in 2010, the identification of genes for many RD's, that were previously intractable to conventional gene discovery approaches, was facilitated. The diagnostic yield of WES has been shown to range from 25-30% in several large studies comprising ~5,000 patients (Lee et al., 2014; Yang et al., 2014; Retterer et al., 2016). Although very high compared to other tests, this clinical sensitivity still leaves >50% of patients with suspected genetic RDs without a conclusive diagnosis.

The focus of this study is to diagnose RD's caused by IEM presenting with systemic and/or neurologic findings. Inborn Errors of Metabolism (IEM) are monogenic conditions in which the impairment of a biochemical pathway is intrinsic to the pathophysiology of the disease. Intoxication and/or storage of metabolites with organ degeneration may be the result, as well as a shortage of energy and building blocks. More than 1000 IEMs are known, the genetic basis for many RD's remains to be discovered (Ferreira et al, 2018). In contrast to most rare genetic diseases with a degenerative phenotype, rapid diagnosis of IEM enables initiation of targeted treatment (e.g. diet, co-factor/vitamin supplements, substrate inhibition, enzyme replacement or stem cell transplantation) slowing down or stopping the degenerative nature of the disease, resulting in significantly reduction of morbidity and mortality (Coene et al., 2018; Graham et al., 2018; Horvath et al., 2018; van Karnebeek et al., 2016; van Karnebeek et al., 2016; Tarailo-Graovac et al., 2016). In addition, a diagnosis enables prognostication, access to community services and accurate genetic counselling for the patient and his/her family including prenatal options such as Preimplantation Genetic Diagnostics (PGD). Diagnosing patients with an IEM can be a major challenge, because of phenotypic heterogeneity and complex diagnostic tests. As a result, individuals suffering from IEM undergo very long and expensive diagnostic trajectories (often over a decade) and suffer health loss that may be prevented or ameliorated by timely diagnosis.

Current diagnostic trajectories of patients suspected of having an IEM

In general, symptoms of patients with an IEM are heterogeneous, affecting one or multiple organ systems and are often progressive in nature. Patients can have neurological (repeated rhabdomyolysis, exercise intolerance, neuropathy, myopathy, ataxia), ophthalmological (retinitis pigmentosa (RP)), otological (hearing loss and/or deafness) and/or endocrine abnormalities (hypoparathyroidism, hypoglycemia) as consequence of energy deficiency. In other IEMs endogenous intoxication causes neurological signs (encephalopathy, regression, movement disorder, psychiatric symptoms), ophthalmological (lens luxation) or organic abnormalities (liver and kidney function abnormalities. Storage associated IEMs, caused by enzyme deficiencies, can lead to yet different neurological (regression, psychiatric symptoms), ophthalmological (cataract/corneal clouding) and organ abnormalities (hepatosplenomegaly, cardiac hypertrophy, skeletal abnormalities, short stature, coarse facial features, umbilical/inguinal hernia). Many of the above mentioned features are disabling. Patients and family members hope to find therapies. They are often worried about the risk of recurrence of the disorder and future prospects. Without an accurate genetic diagnosis, these worries and questions cannot be addressed.

In current daily practice, patients with an unexplained metabolic phenotype, as outlined above, are seen by different medical specialists (e.g. ophthalmologists, endocrinologists, psychiatrists, neurologists, paediatricians, clinical geneticists). There is no standardized diagnostic evaluation of these patients; the presentation at the first specialist often determines the order in which diagnostic tests take place, which can vary from an eye examination, a cerebral MRI scan, cytogenetics to an exome or metabolomic testing.

Commonly, there are 2 main diagnostic trajectories that can be applied:

1) *targeted metabolic screening in plasma, urine and/or cerebral spinal fluid (CSF).* When an abnormal metabolite pattern is identified, fitting with a known IEM, targeted DNA diagnostics are performed, to confirm the clinical diagnosis.

2) *targeted genetic testing through Sanger sequencing (in case of a strong clinical suspicion), gene panels consisting of several genes associated with a certain phenotype (e.g. movement disorder), targeted exome sequencing (exome-based panels) or true open trio-WES analysis (often analysis of the clinome which comprises all genes published associated with a human disease, correlating the variants found in the affected person to his/her parents to identify Mendelian disorders).*

There are several potential outcomes of this trajectory:

1) A known pathogenic IEM profile or mutation in a gene previously reported to cause a an IEM, fitting with the patient's features leading to a diagnosis.
2) In the remaining 50% (or more) no etiologic diagnosis is established, with two possible scenarios:
   - A DNA variant of unknown significance (VUS) in the WES and/or a metabolic alteration in metabolic investigations is/are identified, which cannot be unified into a confirmed diagnosis.
   - No clues arise from (both) the above mentioned diagnostic trajectories

Causes for inconclusive results in undiagnosed patients with metabolic phenotypes

The reasons why a patient remains undiagnosed is that certain phenomena are not being picked up by the current standard test used in clinical care. Using WES it is known that certain types of genetic alterations (e.g. repeat expansions, mutations in the mitochondrial DNA, intronic mutations) are not identified. In addition, some parts of some genes have low coverage (for instance due to CG-enriched genes or mosaicism) (Pena et al., 2018; Zeiger et al., 2018). Metabolic analysis is based on a broad set of screening tests. In these tests not all metabolites are detected. Untargeted Metabolomics is relatively new and has not been implemented in clinical care settings.

With the combined approach to **identify** potential disease-causing DNA variants and **functional validation** of these variants to support their pathogenicity (e.g., data sharing and biological studies) new IEM's are elucidated, paving the way for (future) treatment options and genetic counseling. The success of the combined application of exome sequencing and extended metabolic analysis with omics technologies (metabolomics, glycomics, lipidomics, epigenomics, transcriptomics, proteomics) has recently been shown in several studies (Haijes et al., 2019; Coene et al., 2018; Graham et al., 2018; Horvath et al., 2018; van Karnebeek et al., 2016; van Karnebeek et al., 2016; Tarailo-Graovac et al., 2016).

New diagnostic possibilities for undiagnosed patients with metabolic phenotypes

To increase the diagnostic yield in patients with an unexplained metabolic phenotype, the following approaches can be used:

1) *Reanalysis of exome sequencing data.* There is now emerging data to suggest that re-analysis of exomes initially reported as negative is of significant diagnostic value. As this reanalysis takes time and resources, not currently available in daily clinical care, currently reanalysis is mostly done on research base. A recent study described 10% diagnostic yield of re-analysis of 40 clinical exome analyses, 1-3 years after the original report (Wenger et al., 2017).

In addition, analysis of data in a research setting allows for novel gene discovery, potentially doubling the solve rate (Beaulieu et al., 2014; Eldomery et al., 2017). A cohort analysis of undiagnosed patients with similar biochemical features, suspected of having an IEM, might reveal rare variants in the same gene in different patients which would be missed when sequencing data were analysed on a patient-by-patient basis (Massink et al., 2015; van Hasselt et al., 2014).

2) *Deep sequencing*. Typically, laboratories using next-generation sequencing technologies strive to cover each base of DNA with an average of 100 reads. Recent studies have shown, however, that additional read depth coverage (500x vs 100x) can aid in the identification of somatic mosaicism (mutations that are only present in a subset of cells) as a cause of a RD (Qin et al., 2015).
3) *Whole Genome Sequencing (WGS).* This targets the entire genome, providing more complete coverage compared to exome sequencing, while significantly outperforming with respect to other types of genomic variation such as insertions/deletions, copy number variants, chromosomal rearrangements, or causative variants in regulatory regions. It is therefore not surprising that WGS has been shown to increase the diagnostic and discovery yield from 15 to 40% in several cohorts (e.g., Gilissen et al., 2014; Taylor et al., 2015; Carss et al., 2017).
4) *RNA-sequencing.* Certain genetic mutations, such as non-coding variants, are not readily assessed by genome wide sequencing. Recently, RNA sequencing (RNAseq) has been identified as an innovative technology with the power to assess the functional impact (pathogenicity) of noncoding variants on genome-wide basis (Pauli et al. 2018). Moreover, Kremer et al., 2018, showed how systematic "transcriptomics" via RNA seq lead to a molecular diagnosis in 10-35% of patients in whom WES failed to do so. There has also been an increased appreciation for the importance of mRNA splicing for human health; estimates suggest that splicing defects account for approximately 15% of genetic diseases (Krawczak et al. 1992). In line with this, recent studies in our lab using patient-derived dermal fibroblasts indicated that RNA-seq analysis of these cells has the power to reveal splicing defects such as lost splice junctions and retained intronic sequences resulting from pathogenic variants affecting the spliceosome machinery (manuscript in revision).
5) *Metabolomics. Me*tabolomics allows for simultaneous profiling of thousands of metabolites and can pinpoint specific proteins in a pathway/biological process as candidates for disease causation (Graham et al., 2018).
6) *Glycomics*. In addition to metabolomics, glycomics provides complementary functional information on the consequences of potential disease mutations. In recent years, a wide variety of disease processes has been associated with abnormal

protein glycosylation, including ion homeostasis, Golgi trafficking and intracellular pH (Abu Bakar et al., 2018; Ashikov et al., 2018). In case of identifying disease mutations in such genes or related pathways, together accounting for >1000 genes, glycomics analyses can be performed.

7) *Lipidomics*. Lipidomics aims at the holistic characterization of lipid metabolism, predominantly by using high resolution mass spectrometry to perform semi-targeted identification of more than 1200 lipid species from many lipid classes. This technique has already been successfully applied to identify biomarkers/mechanisms in inborn errors of metabolism and is ideally suited to characterize disorders involving complex lipid metabolism (Herzog et al., 2016; Herzog et al., 2018; Herzog et al., 2018).
8) *Epigenomics:* Epigenetics is modification of DNA that does not alter its base sequence, but alters its expression and thereby alters phenotype (Jaenisch et al., 2003). With the introduction of whole epigenome analysis technology it had become feasible to look for aberrant DNA methylation patterns in patients that lead to mutations in underlying genes (Meeks et al., 2018). SOTOS syndrome (NSD1 mutations) for instance, is a classic example. It is known that especially in RD's associated with ID (Intellectual Deficits) genes associated with chromatin state are very much enriched. Since in many patients with RD (and ID) no genetic cuase of their disease has yet been identified, epigenetic changes might well be involved. As an example, within the Amsterdam UMC, location AMC, we have recently discovered a new syndrome that is caused by mutation within the SETDB1 gene, demonstrating a classical recognizable DNA methylation pattern (article in progress Mannens et al.).

   One challenging problem in diagnostics is the classification of DNA variants. Are these variants disease causing or merely harmless DNA variants? Studying the effect of DNA variants in epi-enzymes (proteins associated with the assembly of the chromatin structure) with whole epigenome technology discriminates between pathogenic variants and harmless DNA variants. This technology therefore serves not only as a new diagnostic test but also as a very valuable functional test. The genome diagnostics laboratory Amsterdam UMC (AMC) has recently set up this technology included an analysis pipeline as one of the first laboratories worldwide.
9) *Proteomics.* Other omics approaches have been developed that can aid RD discovery by pointing to a particular pathway or biological process for further interrogation.

Functional studies

Often IEM are rare with only few patients for each gene defect. Moreover, genotype-phenotype correlations can be poor. Therefore, when encountering genetic variants in individual patients, functional studies in (patient) cells can provide crucial evidence for pathogenicity of these variants and thus confirm a molecular diagnosis.
If, with use of the above mentioned techniques, a good candidate gene has been revealed, further alignment with its biochemical and clinical phenotype is necessary. Fibroblast cell cultures, generated from patient skin biopsies, are often used as test material for functional studies for diagnostic purposes. These tests usually focus on the (suspected or known) function of the protein encoded by the gene of interest, and are designed to determine the metabolic and cell biological phenotype of the patient cells. Measurements may consist of (for example) specific enzyme measurements, analysis of metabolic fluxes, or specific metabolite tests. Cultured patient fibroblasts are also desirable for genetic rescue experiments in order to show that the molecular/cellular phenotype observed in the patient fibroblasts can be rescued by introducing the wild type version of the gene, providing further evidence for a role of the gene in the disease of the patient. For some IEM, the affected protein or enzyme might be more apparent in blood cells compared to fibroblasts and therefore various blood cells will be isolated for analysis of the enzymatic activity and protein levels.

Sharing pheno- and genotypic and metabolic data

Due to the rarity of RD phenotypes, elucidation of the genetic disease cause often depends onmatch making in national and international databases . identifying other patients with a similar geno- and/or (metabolic) phenotype. The combination of different patients harboring mutations in the same gene combined with *cell studies* (derived from fibroblast cell lines) or cell models generated by *CRISPR/CAS* technology*,* can be used to show the specific effect of the mutation on protein function and phenotype (Chatzispyrou et al., 2015; van der Crabben et al., 2016; Rumping et al., 2018; Tessadori et al., 2018).
Standardized collection and labelling of patient data enables sharing data with cooperation partners. Moreover, after identification of a candidate gene and functional studies, multiple unrelated patients with mutations in the same gene are needed to establish phenotypical width of a (new) genetic disorder. Moreover, data sharing speeds up the diagnostic process diagnosis.

This study: implementing new diagnostic technologies in undiagnosed patients

The benefit of combining (deep) exome/genome sequencing, RNA sequencing and “omics” technologies, followed by functional studies to diagnose a single or a small group of patient(s), has convincingly been demonstrated. However, this approach is not standard insurance paid care. In addition, detailed and systematic clinical phenotyping, crucial for future recognition of patients (with late onset disease) has not been implemented in patient care. Based on WES, recent progress of RD gene discovery has been substantial, but peaked in 2012, whereafter both the Online Mendelian Inheritance in Man (OMIM) and Orphanet databases show a decreasing trend in gene discoveries (Boycott et al., 2017). Thus, with the more straightforward RDs having been solved, we must address bottlenecks to maintain, or even accelerate, the current pace of discoveries.

The Amsterdam UMC, Erasmus MC, UMC Groningen, Maastricht UMC, Radboudumc (delegated WGS to commercial facility “BGI Genomics”. After sequencing, WGS data analysis is performed by Radboudumc employed specialists) and UMC Utrecht will combine all necessary expertise and tools to establish exact etiological diagnoses in children and adults. Together we have very experienced pediatric and adult IEM teams consisting of clinicians (pediatricians, internists, neurologists and clinical geneticists) and laboratory specialists (genetic metabolic diseases, molecular geneticists and research groups) that work closely together for deep phenotyping, and integrative genomics and metabolomics. These specialists have united in a consortium, United for Metabolic Diseases (UMD).

We aim to include 500 undiagnosed patients suspected of having and IEM from all six clinical centers. The study will last at least 5 years; we expect to enrol on average 100 patients per year. The overall aim of this study is to diagnose patients with an unknown metabolic phenotype. In addition, we want to provide evidence that the combination of approaches and techniques used in this study will increase diagnostic yield compared to current separated approaches. With increasing diagnosis in patients with IEM, more knowledge on natural disease history and pathophysiological background will be gained, paving way for future therapeutics. In addition, families can receive proper counselling and will have options for reproductive choices.

2. OBJECTIVES

Primary Objective

With this study, in patients with an unknown metabolic phenotype, we want to identify the genetic cause and align it with its biochemical and phenotypical abnormalities. Moreover, with joined forces of the UMD we would like to pave way for implementation of these extended genetic and omics techniques in clinical care. Only with a solid genetic diagnosis patients will be able to receive (future) treatment, prognostication and patient's families can receive proper counselling, including reproductive options.

Secondary Objective(s):

1) identification and validation of novel, IEM related, genes/phenotypes/pathways
2) to study how omics and genomics complement each other in diagnosing rare disease (genetic cause, biomarker, pathophysiology, natural disease history)

3. STUDY DESIGN

This is a national multicenter prospective diagnostic study. The study is expected to run for at least 5 years.

4. STUDY POPULATION

4.1 Population

A total of 500 (in)capacitated patients (any age/gender) will be included, with an unexplained metabolic phenotype (see inclusion criteria), in whom no conclusive diagnosis has been established after extensive 'standard of care' clinical work-up (see inclusion criteria). Patients will be referred from the outpatient clinics of all participating university medical centres in the Netherlands: paediatrics, internal medicine, neurology, clinical genetics and psychiatry.

4.2 Inclusion criteria

Patients with an unexplained metabolic phenotype defined as: neurological symptoms and/or abnormalities on (physical) examination suggestive of an inborn error of metabolism (energy deficiency, intoxication type or storage type):
Energy deficiency: neurological (repeated rhabdomyolysis, verified exercise intolerance, neuropathy, myopathy, ataxia), ophthalmological (retinitis pigmentosa (RP)), otological (hearing loss, deafness), endocrine (hypoparathyroidism, hypoglycemia)
Intoxication: neurological (encephalopathy, regression, movement disorder, psychiatric symptoms), ophthalmological (lens luxation), organic (liver and kidney function abnormalities)
Storage: neurological (regression, psychiatric symptoms), ophthalmological (cataract/corneal clouding), skin (angiokeratomas), blood (cytopenias), organic (hepatosplenomegaly, cardiac hypertrophy, skeletal abnormalities, short stature, coarse facial features, umbilical/inguinal hernia)

AND / OR

one or more of the following suggesting a deficient metabolic pathway or process:

- abnormal metabolites in body fluids (CSF, urine, blood)
- functional studies at a biochemical/cellular level indicative of a metabolic deficiency (e.g. respiratory chain complex analysis)
- organ dysfunction (e.g. liver or kidney failure)
- an abnormal clinical function test (protein loading test, fasting test, meal test, validated exercise test, non-ischaemic underarm test)
- abnormalities on imaging (neuro-imaging (including spectroscopy); X-rays (dysostoses or other bone abnormalities); ultrasound (enlarged liver/spleen))
- a VUS (variant of unknown significance) in a gene involved in metabolism

AND

no diagnosis despite extensive clinical, metabolic and genetic investigations

- SNP-array/array-CGH: inconclusive results
- metabolic screening according to up to date clinical protocols: inconclusive results
- WES (open or gene panel): no class 4 or 5 variants in a known (OMIM annotated) disease related gene that can fully explain the phenotype of the patient

4.3 Exclusion criteria

A patient will be excluded from participation in this study if:

- after discussion by the ZOEMBA team (see Methods) he/she is suspected to have:
    - a genetic condition for which there is a simpler and more cost-effective test available for diagnosis
    - a complex genetic disorder (caused by a combination of multiple genes and/or environmental influences)
    - a condition that is thought to be caused by factors that are non-genetic, such as infection, injury or toxic exposure
- he/she is unable to follow the study protocol (e.g. additional blood samples)

4.4 Sample size calculation

A previous study in 47 (predominantly pediatric) patients with unexplained ID plus metabolic phenotypes (executed by the principal and coordinating investigator of the current study) showed a high diagnostic and discovery yield using an integrated approach with deep phenotyping, WES and metabolomics followed by functional analyses; a diagnosis was established in 68% of all patients plus a novel human disease gene identified in 11 patients. 44% of these diagnoses were amenable to causal therapies, including specific dietary restriction, supplementation or pharmacologic interventions.
In the proposed study, which focuses on unsolved cases after standard diagnostic trajectories and with inclusion of adults with more diverse phenotypes, we expect the yield to be lower. We make a conservative estimate of a diagnostic yield of 33% (comparable to findings from the NIH Undiagnosed Diseases Program of 34% (Gahl et al., 2016; Splinter et al., 2018)), and an expected discovery rate of a novel candidate gene 1 in 25.
Thus, we estimate to diagnose 170 patients, including 20 with novel candidate disease genes. With an inclusion period of at least 5 years, we expect to enrol on average 100 patients per year. The number of patients referred and / or enrolled per year will likely differ per UMC based on expertise and focus. A recent investigative query among the 6 UMCs has shown that our aim of enrolling 500 undiagnosed patients suspected to have an IEM is realistic based on their annual patient numbers and diagnostic trajectories.

5. TREATMENT OF SUBJECTS

Not applicable

6. INVESTIGATIONAL PRODUCT

Not applicable

7. NON-INVESTIGATIONAL PRODUCT

Not applicable

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

The primary endpoint of this study is the identification of genetic variant(s) in an affected patient and establishment that this/these genetic alteration(s) is/are (likely) pathogenic (with a deleterious impact on protein function; ACMG class IV or V) and causal of disease.

8.1.2 Secondary study parameters/endpoints

Identification of phenotypic presentation/ disease characteristics to facilitate recognition of disorders in other undiagnosed patients

8.1.3 Other study parameters

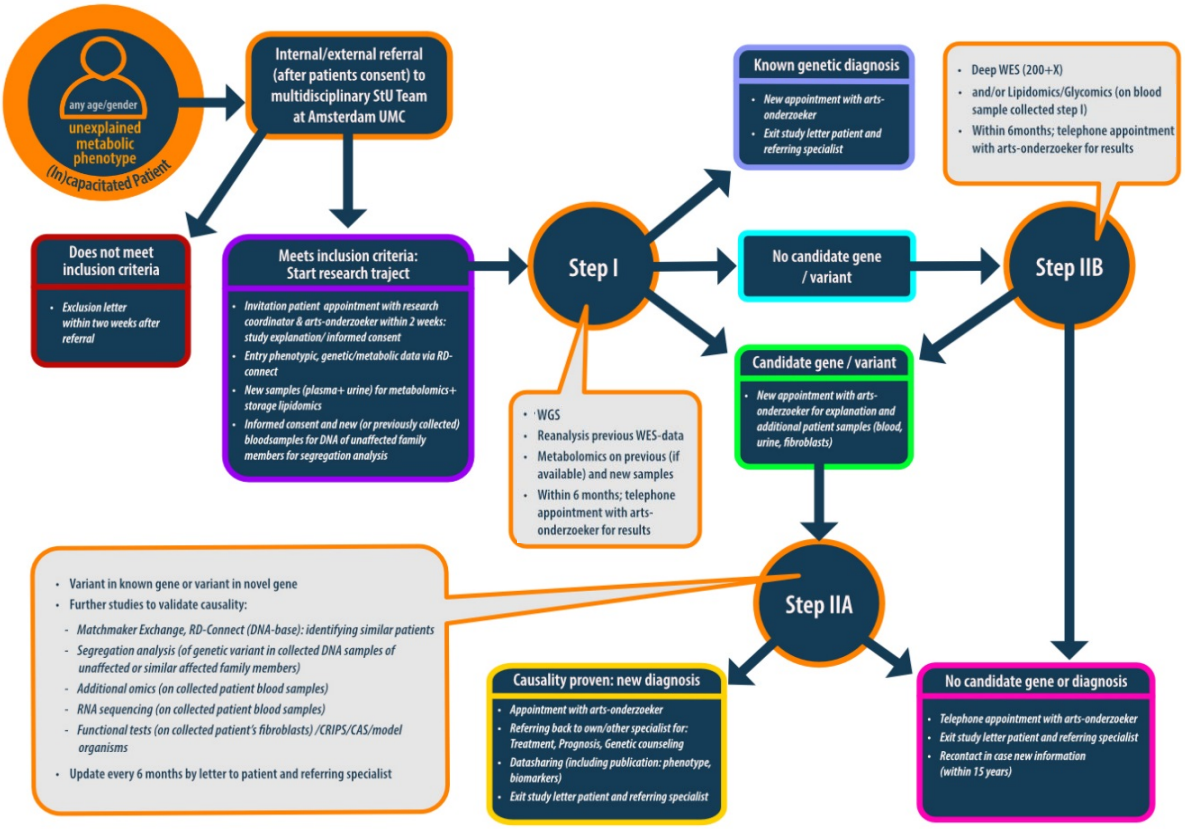
Evaluation of the yield of the diagnostic approach used in the study. Yield of integrated analysis of the –omics datasets and whole exome/whole genome sequencing dataset for validation of causality of the identified variants.

8.2 Randomisation, blinding and treatment allocation

Not applicable

8.3 Study procedures

See Figure 1, pag24



pigmentosa (RP)), endocrine (hypoparathyroidism, hypoglycemia)
Intoxication: neurological (encephalopathy, regression, movement disorder, psychiatric symptoms), ophthalmological (lens luxation), organic (liver and kidney function abnormalities)
Storage: neurological (regression, psychiatric symptoms), ophthalmological (cataract/corneal clouding), organic (hepatosplenomegaly, cardiac hypertrophy, skeletal abnormalities, short stature, coarse facial features, umbilical/inguinal hernia)
[2] **abnormal**: metabolites in body fluids (CSF, urine, blood)/ Functional studies at a biochemical/ cellular level/radiological (neuro)imaging **suggesting a deficient metabolic pathway or process** and/or a VUS (variant of unknown significance) in a gene involved in metabolism

* adult/pediatric metabolic physician, neurologist, psychiatrist, clinical geneticist

Patient referral

Patients with an unexplained metabolic phenotype are eligible for inclusion in the study (see inclusion criteria 4.2, pages 19-20) *only* once all 'standard of care' clinical diagnostic tests have proved inconclusive (see inclusion criteria 4.2, pages 19-20). Thus, no clinical diagnostic procedures will be postponed because of participating in the study.
The treating physician will ask the patient for consent to share his/her data with the multidisciplinary clinical meeting of the ZOEMBA team. After patient consent, the referring specialist will make a patient resume, using a standard format, which will be used for discussion at the multidisciplinary patient meeting the "ZOEMBA-team triage meeting" (1x per 2-4 weeks).

The ZOEMBA team for this meeting exists of:

- Study coordinators: Dr. C.D. van Karnebeek (Study PI, Pediatrician in Inborn errors of metabolism & Biochemical Geneticist– Amsterdam UMC), Dr M. Langeveld (internal medicine specialized in IEM), Prof N.M. Verhoeven-Duif (Study CoPI, head of the Laboratory of Metabolic Diagnostics, UMC Utrecht), Dr. S.N. van der Crabben (coordinating postdoctoral fellow, clinical geneticist specialized in IEM, Amsterdam UMC)
- A clinical and laboratory representative per participating UMC (when not already represented by the study coordinators):
    - Amsterdam UMC: Prof. dr. H. Waterham (PI at the laboratory of GMD), Dr F. Vaz (Clinical Chemist IEM, Laboratory GMD, specialized in lipidomics)
    - UMCU: Dr. P.M. van Hasselt (Pediatrician in IEM) and Dr. J.J. Jans (laboratory specialist, Laboratory of Metabolic Diagnostics, specialized in metabolomics)
    - UMCG: Prof. dr. F.J. van Spronsen (Pediatrician in IEM), Dr. M.R. Heiner-Fokkema (Clinical Chemist IEM, Laboratory of Metabolic Diseases, specialized in IEM)
    - MUMC: Prof. dr. M.E. Rubio-Gozalbo (Pediatrician in IEM), Dr. J. Bierau (Clinical Chemist IEM, Laboratory for GMD)
    - Radboudumc: Dr. M. Janssen (Internist in IEM), Dr. K.M. Coene (Clinical Chemist IEM, Translational Metabolic Laboratory)
    - Erasmus UMC: Dr. H.H. Huidekoper (Pediatrician in IEM), Dr. G.J.G. Ruijter, Clinical Chemist, Laboratory for Inherited and Metabolic Diseases)
- The referring medical specialist

Patient journey: exclusion or inclusion

In case of exclusion (see page 21) the patient and referring specialist will receive a letter of exclusion within 2 weeks after ZOEMBA-team triage meeting.

When meeting inclusion criteria, patients will receive an invitation for an appointment with the MD-PhD student at the outpatient clinic. During the visit the patient will receive information about the study. When patients have understood the procedure, informed consent (1) will be asked for within 24 hours.

During the whole of the study, appointments will be combined with regular hospital visits if possible.

Informed consent

Retrieving data from the patient's medical records

Medical data including medical and social history (developmental milestones, level of education, neuropsychological testing), family history, and physical and neurologic examination

Diagnostic/laboratory data including cerebral MRI-scan, skeletal X-rays, ECG.

In case of specific dysmorphic features the patient has an option to consent for the use of medical photographs for teaching goals and/or for publication. Specific dysmorphic features can be essential for establishing a quick clinical diagnosis and can lead to significant shortening the diagnostic trajectory.

All relevant patient data are documented pseudo-anonymized in standardized data collection forms (synonym: Case Report Form, CRF).

Safeguarding previous (stored) samples

Neonatal dried blood spot card (to be requested from RIVM)

Plasma samples

Urine samples

CSF

Skin fibroblasts, muscle biopsy, chorionic villi

Amniotic fluid.

Data sharing (pseudo-anonymized) through RD-Connect

1) For identification of additional patients with a VUS in the same gene

2) To share knowledge according to FAIR principles. Data will be shared pseudo-anonymized through this international database

Sampling blood and urine

Of all patients, blood and urine will be collected

Blood:

Adults: 50 ml in heparinized tubes for metabolomics and 0,5 ml in EDTA tube for lipidomics (total of 50,5 ml)

Children: 10 ml in heparinized tubes for metabolomics and 0.5 ml in EDTA tube for lipidomics (total of 10,5 ml)

Children <2 years of age: assessed on a case-by-case basis (prioritization of investigation)

From the heparinized blood, 3 dried blood spots (DBS) will be made. Samples will be treated and stored as described in a standardized protocol.

Urine: of all ages a minimum from 4- 10 ml will be collected.

Sampling from family members
Blood samples of unaffected parents and/or siblings will be asked for to store DNA. Sample collection for DNA isolation consists of:
In adults: 10 ml blood in EDTA tubes.
In children: 2 ml blood in EDTA tubes.

Of unaffected family members (parents and/or siblings) informed consent will be asked to use previously collected DNA samples. In case no (not enough) DNA is available consent is asked for collection of new blood samples for storage of DNA. For the reanalysis of WES-data and WGS, DNA of these family members will be used as reference. In case a candidate variant is identified, DNA of unaffected family members will be used to test *inheritance pattern of his variant only* (in **Step I**). No other tests will be run on the DNA from the unaffected family members.
In case of similarly affected family member, this patient can be included, after informed consent. He/she will be treated similar to the index patient.

Diagnostic Step I

- Reanalysis previous WES-data
The bioinformatic pipeline will be optimized to detect pathogenic variants. The bioinformatician will generate a variant list with potential pathogenicity determined by comparison to existing databases such as ClinVar, Exac or HGMD. After use of *in silico* predictors and consideration of conservation and minor allele frequencies, potentially pathogenic variants will be reported for discussion within the StU-team.

- Samples
The blood samples will be processed within 1 hour after sampling. DBS will be made, after which the samples are centrifuged, aliquoted and stored at the Amsterdam UMC at -80 °C. Urine samples will be stored at -80 °C.

- WGS
WGS will be performed with a minimum coverage of 45x for the patient and 30x for the parents. Raw data will be provided as fastq files and variant calls will be provided as BAM files and vcf files. The analysis will be run, like WES, as trios whenever possible. In case the parents of a patient are not available for research, DNA of other preferably affected siblings

or unaffected family members will be used instead, only after consent. DNA will be directly transferred to the Radboudumc in Nijmegen for the distribution of the material to an external party. WGS data will be generated by BGI Genomics, a commercial international genome sequencing facility located in Shenzhen, China. BGI Genomics is the world's leading provider of genomic sequencing services and proteomic services, now serving customers in more than 66 countries. They provide academic institutions, pharmaceutical companies, health care providers and other organizations with integrated genomic sequencing and proteomic services and solutions across a broad range of applications spanning. They have almost 20 years of genomics experience helping customers achieve their research goals by delivering rapid, high quality results using a broad array of cost-effective, cutting-edge technologies, including innovative DNBseq™ sequencing technology.The generated WGS data is thereafter transferred to our ZOEMBA bioinformatics specialist in the Radboudumc. Here the WGS data is analyzed and interpreted in order to find a diagnosis. No data or (surplus) material will be stored by BGI genomics and will be destroyed shortly after data transferal.

The Radboudumc has signed a contract with this third party, regarding material ownership, confidentiality and destruction of sample after use. We highlight the following clauses, which address the aforementioned topics:

1.1 BGI Genomics shall deliver data to Radboudumc no later than 60 days following the completion of services related to the Data delivered under the agreement. Data will be shipped to Radboudumc as generated

1.2 Data delivered to Radboudumc shall be deleted by BGI Genomics no sooner than 30 days after data delivery of the data to Radboudumc as provided in 1.1 unless otherwise specified in writing by the parties, and will be destroyed in accordance with all applicable laws.

1.3 Samples from Radboudumc will be sequenced for the studies by BGI Genomics may provide additional samples in the context of these clinical utility studies with the prior written consent hereto from Radboudumc. Data analysis will be performed by Radboudumc scientists, but if desired by Radboudumc, collaboration with BGI Genomics scientist can take place and will be made possible by BGI Genomics.

1.4 Upon delivery, Radboudumc shall own all rights to the Data. Under no circumstances shall BGI Genomics have any ownership interest in the Data.

- *Metabolomics*

The coded and anonymized DBS will be transported to the section Metabolic Diagnostics of the UMC Utrecht and plasma samples (1ml) will be shipped to Radboudumc for metabolomics in DBS and plasma respectively. Packing, shipping, and tracking of samples will be coordinated by the research coordinator.

Patients will have a telephone appointment with the arts-onderzoeker within 6 months to discuss the first results of the reanalysis of the WES-data and of the metabolomics. The arts-onderzoeker will explain further diagnostic trajectory and expected timeline.

Potential outcomes of Diagnostic step 1 (see Figure 1)

1. Known genetic diagnosis

Patients will be invited for an appointment with the arts-onderzoeker and one of the MD's from the StU team at the outpatient clinic to discuss the findings. Hereafter, patients and their referring physicians will receive a letter explaining the diagnosis and confirming the exit of the study. Follow up/treatment of the patient will be organized by the referring physician.

2. No genetic diagnosis

The following additional tests will be performed (**Step IIB**, **see Figure 1**)

- *Deep WES (200+X) (performed on previously collected DNA)*

For deep WES the bioinformatic pipeline will be optimized to detect pathogenic variants (as has been described under Diagnostic step 1). Deep WES data will be generated by the involved UMC's.

- *(and/or) Lipidomics/Glycomics on previously collected blood samples in Step I.*

Lipidomics will be performed in EDTA plasma (50 µl) at the Amsterdam UMC, location AMC. Analysis of plasma protein glycosylation will be performed in heparinized plasma (<50 µl) at the Radboudumc Nijmegen.

Patients will have a telephone appointment with the arts-onderzoeker within 6 months to discuss the results of these tests.
In case a candidate genetic variant is found, patients will go to **3 (Step IIA), see below.**
In case the cause for the patient's phenotype still remains unclear, possibility of a future diagnosis and recontact still remains possible if other patients have been identified via data-sharing (see **underneath 3 (Step IIA)**).

3. Candidate genetic variant (**Step IIA, see Figure 1**)

When either direct after diagnostic Step 1 or via Step IIB, a candidate gene has been identified, patients will be invited for an appointment with the arts-onderzoeker at the outpatient clinic for explanation of the finding and further plans.
Additional samples will be collected and additional tests will be performed: repeat metabolomics (blood and urine; similar to **Step I**), RNA-analysis and skin biopsy for fibroblasts (in case no previous sample is available)). These tests are needed to further study causality of the identified genetic variant.

Sampling blood and urine
Of all patients, blood and urine will be collected
Blood
Adults: 50 ml in heparinized tube for repeat and additional metabolomics
and 20 ml in PAX tubes for transcriptomics (a total of 70 ml of blood will be drawn)
Children: 10 ml in heparinized tubes for repeat and additional metabolomics and 3 ml blood in PAX tubes for transcriptomics (total of 13 ml)
Children <2 years of age: assessed on a case-by-case basis (prioritization of investigation)

From the heparinized blood in the tube, 3 dried blood spots (DBS) will be made. Samples will be treated and stored as described in a standardized protocol.

Urine: of all ages a 4-10 ml will be collected.

Skin biopsy
When no previous fibroblasts are available, additional consent will be asked to perform a skin biopsy. Skin biopsies will be taken according to standard clinical procedures.
In case the patient will receive anesthesia for a planned procedure (not study-related), a skin biopsy will be combined with this procedure if possible.

After collecting the additional samples, the following will be pursued to further validate causality in **Step IIA**:
- *Datasharing* via RD-Connect (for MatchMaker Exchange)
- *DNA segregation analysis* of the identified candidate genetic variant in unaffected family members will be performed to establish correlation between variant and phenotype.
- *Lipidomics* (in case lipid metabolism is suspected to be affected) is performed in EDTA plasma (50 µl) at the Amsterdam UMC, location AMC.
- *RNA sequencing* will be performed in the collected blood samples and/or fibroblasts.
- For *functional studies*, fibroblast cell cultures, derived from the patient's skin biopsy, will be used. These studies will be performed by the associated research groups of prof. dr. Hans Waterham, dr. Riekelt Houtkooper, dr. André van Kuilenburg (Amsterdam UMC, location AMC), dr. Gijs van Haaften, dr. Judith Jans and prof. Nanda Verhoeven (UMC Utrecht) and dr. Richard Rodenburg (Radboudumc).

Depending on the type and number of additional tests, the generation and interpretation of these final results may take from 3 months up to 1 year. Therefore, patients and the referring

specialists will receive an update every 6 months by letter to inform them on the latest results.

When results correlate with the patient's phenotype and a diagnosis can be made, the patient will follow point 1 and will be invited for an appointment with the 'arts-onderzoeker' and the MD involved at the outpatient clinic to discuss the findings.

When, with additional testing, the causality between the genetic variant and the patient's phenotype still remains unclear, unraveling the genetic cause still remains possible by identifying another (phenotypical/metabolic/genetic) similar patient though the database of RD-connect. In case new information becomes available leading to a possible diagnosis during, patients will be recontacted by the arts-onderzoeker.

Research meetings on patient data

To discuss the results of data collected from Step I, Step IIA and Step IIB and to establish a validation plan, a biweekly research meeting, called the ZOEMBA diagnostic meeting will be organized. The referring medical specialist and involved lab scientists will be invited to attend the meeting. The meeting will be conducted by the participators of the ZOEMBA team below and will be organized via teleconference.

The ZOEMBA team for this meeting exists of:

- Study coordinators: Dr C.D. van Karnebeek (Study PI, biochemical geneticist and pediatrician specialized in IEM, Amsterdam UMC), Prof. dr. N.M. Verhoeven-Duif (Study CoPI, head of the laboratory of GMD, UMC Utrecht), Dr. S.N. van der Crabben (coordinating postdoctoral fellow, clinical geneticist specialized in IEM, Amsterdam UMC)
- Omics datasets: Dr F. Vaz (lipidomics, Amsterdam UMC), Dr K. Coene (metabolomics Radboudumc), Dr J. Jans (metabolomics, UMC Utrecht), Prof D. Lefeber (glycomics, Radboud UMC)
- Functional metabolic assays: Prof. Dr. H. Waterham and dr. A. van Kuilenburg (Amsterdam UMC), Dr R. Rodenburg (Radboudumc)
- Model organism studies: Dr G. van Haaften (UMC Utrecht)
- Clinical molecular geneticist: Dr. M. Alders (Amsterdam UMC)
- Clinical geneticists: Dr. J.D. van de Kamp, Dr. P. Zwijnenburg (Amsterdam UMC)
- Internal Medicine, specialized in IEM, Dr M. Langeveld

8.4 Withdrawal of individual subjects

Capacitated patients can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a patient from the study for urgent medical reasons.
In case of incapacitated subjects (children or patients with an ID) any sign or behaviour (verbal or non-verbal) that can be interpreted as resistance will be taken into account and discussed with the caregivers and family. When consistent and seen as resistance this will lead to immediate withdrawal of the study (gedragscode verzet minderjarige en gedragscode verzet verstandelijk gehandicapten, NVK).
In case of withdrawal all data will be removed from the study file and biological samples of the patients will be destroyed. Data uploaded at RD-Connect will either stay there or be removed depending on the patients or the legal guardians wishes .

8.4.1 Specific criteria for withdrawal (if applicable)

Not applicable

8.5 Replacement of individual subjects after withdrawal

Because this study is about RD and single patient diagnosis, we will as such not replace individuals after withdrawal.

8.6 Follow-up of subjects withdrawn from treatment

Patients who withdraw from the study will follow their regular care (no specific study follow-up).

8.7 Premature termination of the study

Premature termination of our study will follow in the highly unlikely case that no patients will be included.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground to assume that continuation of the study will jeopardise patient's health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.
The only invasive procedures undertaken in this study are vena puncture and skin biopsy. We do not expect any adverse events from this procedure, but in case of occurrence they will be reported to the accredited METC.

9.2.2. Serious adverse events (SAEs)

Not applicable since non-interventional study and study procedures (vena puncture/skin biopsy) cannot lead to a serious adverse event.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable

9.3 Annual safety report

Not applicable

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

SAEs need to be reported till end of study within the Netherlands, as defined in the protocol

9.5 Data Safety Monitoring Board (DSMB)

Our study does not require the installation of a DSMB. Nonetheless, we plan to perform a mid-term evaluation after one year to evaluate the inclusion progress: in case the rate is lower than expected we will try to identify causal factors. The mid-term analysis will be performed by the ZOEMBA team.

10. STATISTICAL ANALYSIS

The only statistics will be a descriptive analysis on the number of patients included. We will also report the number of patients with a confirmed diagnosis and the average time to diagnosis.

10.1

Not applicable

10.2 Primary study parameter(s)

Not applicable

10.3 Secondary study parameter(s)

Not applicable

10.4

Not applicable

10.5 Other study parameters

Not applicable

10.6 Interim analysis (if applicable)

Not applicable

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (version 8, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

11.2 Recruitment and consent

Patients

The treating physician of a patient with an unexplained metabolic phenotype (in whom all standard of care clinical diagnostic tests yielded inconclusive results), will ask the patient and/or her/his parents or legal representative for oral consent to share his/her data with the multidisciplinary clinical meeting of the ZOEMBA team.

When meeting inclusion criteria, patients and/or her/his parents or legal representative will receive an invitation for an appointment with the arts-onderzoeker at the outpatient clinic. During this visit the patient will receive information about the study. When patients and/or her/his parents or legal representative have understood the procedure informed consent will be asked for and new blood samples and urine will be collected. Informed consent of unaffected family members (parents or siblings in case parents are deceased/unavailable) will be asked for use of stored DNA and selected analysis. In case no DNA (not enough DNA) is available informed consent is asked to draw new samples for storage of DNA. In case of a similar affected family member, this patient can be included, after informed consent and will be treated similarly to the index patient.

11.3 Objection by minors or incapacitated subjects

In case of enrolment of children or incapacitated adults in this study, any sign or behaviour (verbal or non-verbal) that can be interpreted as resistance will be taken into account and discussed with the caregivers and family. When consistent and seen (by caregivers) as resistance this will lead to immediate withdrawal of the study (gedragscode verzet minderjarigen en gedragscode verzet verstandelijk gehandicpaten, NVK).

11.4 Benefits and risks assessment, group relatedness

Children and adults enrolled in this study will have an unexplained metabolic phenotype. Without diagnosis, in general, these patients will undergo continuing invasive diagnostics

(e.g. blood withdrawal, muscle biopsies, cerebral MRI-scans under sedation) to identify the cause of their disease. Moreover, a diagnosis could open therapeutic options and thereby ameliorate future by preventing worsening of (neurodegenerative) symptoms.

Possible benefits for participants of this study:

- Identification of the genetic cause of the affected individuals phenotype, leading to an accurate diagnosis and potential therapeutic, management and counselling options.
- Identification of the gene and the functional analysis will lead to improved understanding of the pathophysiology; providing options for further study and potential therapeutic investigation.

Possible risks/discomfort for participants of this study:

- The risk of drawing blood (2 times in this study) may include some dizziness, discomfort around the bruise and a very low risk of infection.
  We will make every effort to coordinate the drawing of blood with other medical procedures where blood is taken during routine medical care.
  Skin biopsy (taken once if not previously available) is usually a safe procedure. Complications, such as infection, bleeding and scarring may very rarely occur. Participants may, very rarely, experience discomfort with topical anesthetic administration. In order to minimize the possibility of discomfort and complications, skin biopsies will be performed by trained medical staff experienced with this procedure. Whenever possible skin biopsies will be taken when a patient will receive anesthesia for a planned procedure (not study related).
- With the reanalysis of WES, deep sequencing and/or WGS there is a new, very low, possibility of incidental findings.
  Incidental findings (also known as secondary or additional findings) can be identified through use of WES or WGS. As all patients have given informed consent for WES in diagnostics setting, this issue has already been discussed in a diagnostic setting. There will not be an intentional analysis for incidental findings (Boycott et al., 2015). However, incidental findings are likely to be found in some study participants. It is therefore important to ensure that parents (and children where appropriate) are aware of the possibility of incidental findings should they choose reanalysis of WES and/or WGS.
  Non-paternity, if found, will be neither discussed nor disclosed.
  Incidental findings will be reported after discussion within the team of incidental findings of the UMC involved, when information on the incidental finding is deemed important for the health of the patient and/or his her parents/relatives.

The health care professional members of the study team, in conjunction with any additional expert geneticists needed for consultation, will determine whether or not an incidental finding is likely to be actionable.
Dr. CD van Karnebeek (PI) or Dr. S.N. van der Crabben, will be responsible for initial reporting any incidental findings to the family and for ensuring that any appropriate follow-up is preformed, including genetic counselling.

- Psychological toll from potential disappointment or frustration at either the lack of any helpful result or the length of time it takes to arrive at a result.
  We will be careful to set expectations appropriately at the time of enrollment and will carefully monitor patients and/or caregivers for any signs of the above. In case of worries, we will give support where-ever possible.

11.5 Incentives (if applicable)

Because all invasive procedures (i.e. vena puncture and skin biopsy) will take place in the Amsterdam UMC, location AMC, all participants are covered by the WMO-proefpersonenverzekering of the AMC. For the other participating UMC's separate appointments with local insurance policies will be made.

12 ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Data can only be collected after written permission of the patient or parent/guardian is obtained. Patients or parents/guardians must agree that their data may be used for scientific investigation. When no consent is given, their data may not be used.
After inclusion in the study, patients or parents/guardians consent for collecting of their phenotypical, genotypical (previous exome data) and metabolic data. Data collection and handling will be performed following the hereby described procedures:

Coding data

In each participating UMC, patients will be assigned a unique, non-identifiable alphanumeric code consisting of a sub code for the participating UMC. The sub code of the UMC allows the (virtual) subdivision of the data of each contributing UMC. The key is stored in a secure repository and is only available to the arts-onderzoeker and the participating clinical principial investigator of each contributing UMC; these are either certified medical and / or lab specialists (VKGN, NVK, VKGL etc).

Collecting of data in Case Report Forms

The pseudo-anonymised patient data will be collected and documented in standardized data collection forms, Case Report Forms (CRF). These CRF's will be stored in REDcap, a secure web application for building and managing online surveys and databases. Only the arts-onderzoeker, the bioinformatician, the participating clinical principial investigator and the involved clinical chemists IEM of each UMC will get accounts to log in and have access to the CRF's. In order to make a proper interpretation of the metabolic and genetic data it is necessary for the bioinformatician and laboratory specialists to have access to the pseudo-anonymised data (e.g. age, gender, use of medication). Patient data will be entered in the CRF with use of Pheno Tips © in order to use standardized phenotyping based on the Human Phenotype Ontology (HPO) (Girdea et al., 2013).

Collecting and storing (old and new) metabolic and genetic data and samples

The (old and new) metabolic data is uploaded to the Radboudumc "Digital Research Environment (DRE)" by the specialized laboratory specialist of the diagnostic laboratory of IEM from one of the participating UMCs.
The diagnostic genetic data, derived from the WES-data of the participating patient, will also be uploaded to the DRE by the molecular geneticist of the diagnostic DNA laboratory of the

participating UMC. Additional new genetic data derived from the study (e.g. WGS, RNA-analysis) will be uploaded by the laboratory/research specialist involved per UMC. Both the metabolic and genetic data will be stored, processed and (re)analyzed in the DRE.

The DRE is a recently developed and operational cloud-based computing and storage ICT infrastructure. It is located within Microsoft Azure cloud and designed by Radboudumc and Rapid Circle. The source code is entirely owned by Radboudumc. The DRE is designed as an enclosed, secured, research environment with subdivided, login-restricted, project based working space. Each workingspace has its own, login based, subnet. Only the arts-onderzoeker, bioinformatician (data-steward), clinical principial investigators of each participating UMC and the involved laboratory specialist of the participating UMCs will get login authorization codes.
Even in the unlikely event of unauthorized access to these systems, the research data and phenotypic records are not considered sufficiently unique to allow identification of a research participant, thus minimizing the risk to the participant. Genomic data is considered unique, but does not identify a participant on its own without combination with other data.

Metabolic samples (old and new) that have been send for analysis to a specialized laboratory of a participating UMC, not being the UMC where the patient is treated, will be send back for storage of the remaining sample. DNA of (un)affected family members of a patient as well as the patient's and his/hers fibroblast cell lines, will remain in the participating UMC treating the patient. Storage of DNA and tissue samples will take place at the originating institution and its associated biobank, maintaining the link to the alphanumeric patient code. The biobank catalogue will be regularly backed up and will comply with ontology standards for biobanking set out by BBMRI, RD-Connect and EuroBioBank. Storage will be for 20 years. It is expected that within these years, with the use of (gen)omics, new diagnoses will be established in our study group. Also over time, using novel or improved technologies, new biomarkers/profiles may be identified, which may serve as diagnostic and/or therapeutic efficacy parameters.

<u>Sharing data via RD-Connect</u>
Sharing data in RDs, such as an IEM, is mandatory for two reasons:

1) To identify similar patients via Match Maker Exchange
   In case patients have a VUS or no diagnosis, identifying a patient with a similar VUS, or identifying a phenotypical similar patient, opens possibilities for further study or reanalysis of the genetic data. In case phenotype and genotype are similar in both (most often not related) patients, chances of a (possible) diagnosis increase.
2) Knowledge sharing for rare disease

> Because of paucity of data in patients with a RD, such as an IEM, it is essential to share data to increase diagnostic yield and for development of (new) diagnostic methods and future therapeutics. To ensure datasharing of new IEM's/rare diseases, within and beyond this project, data should be stored based according to the FAIR (Findable, Accessible, Interoperable, Reusable) principles.

To make data Findable (according to FAIR principles), data will be shared internationally with other authorized researchers through the existing, well-managed, secure, large-scale, controlled-access web-based, repository of the RD-Connect platform (https://platform.rd-connect.eu/). The arts-onderzoeker will upload pseudo-anonymised, abstracted portions of the patient data (limited fields derived from Pheno Tips within the CRF's). The bioinformatician will upload the patient's (pseudo-anonymised) genetic metadata from the DRE. In addition all other project assets (methods, software, results, samples, researchers) will be uploaded.

RD-Connect stores thousands of other similar samples and uses best practices for data security. The RD-Connect platform has received ethics committee approval from the Parc de Salut MAR - Clinical Research Ethics Committee under reference number: 2015/6456/I dated October 27th 2015. All researchers and clinicians requesting access to RD-Connect are required to provide information to certify their identity. They are required to sign an Adherence agreement, for authorized access to data and biospecimens in the RD-Connect platform to be used. In addition they need to sign for the Code of practice for integrated user access to RD-Connect platform for health-related information and human biological samples. The RD-Connect platform is hosted at the CNAG-CRG (Parc Científic de Barcelona, Baldiri Reixac 4, 08028 Barcelona, Spain). It is included in IT security. Data protection audits are performed on a regular base, contracted by the CRG to external companies.

Raw sequencing data in .fastq or .bam format plus collection of basic experimental metadata, including experiment description (type of experiment, library information, kit used, sequencer and read-length) and QC metrics (mean or median coverage) will be submitted. This mandatory metadata is required by the European Genome-phenome Archive (EGA), where the raw data will be submitted for long-term controlled-access storage. Genomic data submitted to RD-Connect is stored during analysis on high performance servers at the CNAG-CRG disconnected from the general Internet and with appropriate user permissions. Processed genomic data is indexed in a non-relational database within a server with limited internet access. Processed data is accessed through an API accessible only to authorized users logging in to a Central Authentication System. Genomics data, phenotypic data and

metadata is backed up regularly. Although RD-Connect acts as a submission broker to the EGA, the decision power for sharing the raw data hosted by the EGA remains with Data Access Committees (DAC, usually the original data submitter).

12.2 Monitoring and Quality Assurance

During the study inclusion will be monitored bi-monthly by an independent research monitor. He/she will register if all informed consents are signed correctly.

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments (including covering letter with reasons for the amendment, extract of the modified documents showing previous and new wording and new version of the modified documents), will be notified to the METC that gave a favourable opinion.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

12.4 Annual progress report

The investigator will submit a summary of the progress of the study to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included, adverse events, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the lapse of the study period of the establishment of a causal diagnosis in all enrolled patients (derived from Step IIA or IIB (see Figure 1, pag. 24).

The investigator will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within two years after the end of the study, the investigator will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

Research data will be submitted as articles to professional journals (vaktijdschriften) and peer-reviewed (international) journals. Also, an abstract of our research data reporting our findings will be submitted for presentations on scientific, public and/or patient organisation meetings for oral presentation(s). A summary will be published on the UMC public websites. All investigators named in this project and who actively participated in this project will be (co-)author in publication of the research data according to international guidelines for publication (i.e. rules of the Vancouver Convention drawn up by the International Committee of Medical Journal Editors). Author line-up will be discussed amongst the PIs/ investigator(s) of the study, and will be based on activities performed for the manuscript/study.

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14 Amendments

Amendment 1, 23-10-2019:

Addition of a research expert to the Amsterdam UMC team. Addition of the name of the arts-onderzoeker to the PIFs.

Amendment 2, 25-03-2020:

Adaptation of study protocol. “Whole genome sequencing” (WGS) is now offered to **every** participant in the study. Additionally the diagnostic tool is applied at an earlier timepoint in the protocol during STEP 1, instead of during STEP 2B. WGS data is generated by an external commercial facility (BGI Genomics, located in Shenzhen, China).