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The challenge of understanding and predicting phenotypic diversity in urea cycle disorders

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Abstract

The Urea Cycle Disorders Consortium (UCDC) and the European registry and network for Intoxication type Metabolic Diseases (E-IMD) are the worldwide largest databases for individuals with urea cycle disorders (UCDs) comprising longitudinal data from more than 1,100 individuals with an overall long-term follow-up of approximately 25 years. However, heterogeneity of the clinical phenotype as well as different diagnostic and therapeutic strategies hamper our understanding on the predictors of phenotypic diversity and the impact of disease-immanent and interventional variables (e.g. diagnostic and therapeutic interventions) on the long-term outcome. A new strategy using combined and comparative data analysis helped overcome this challenge. This review presents the mechanisms and relevant principles that are necessary for the identification of meaningful clinical associations by combining data from different data sources, and serves as blueprint for future analyses of rare disease registries.

Keywords

UCDC; E-IMD; urea cycle disorders; rare disease registries; prediction modelling

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Informed Consent and Animal Rights

This article does not contain any studies with human or animal subjects performed by the any of the authors.

Strategies for combined and comparative data analyses in rare diseases

In North America, the Urea Cycle Disorders Consortium (UCDC) was founded by the National Institutes of Health in 2003 as part of the Rare Diseases Clinical Research Network (https://www1.rarediseasesnetwork.org/cms/UCDC), among which the Longitudinal Study has been following the objective to improve knowledge on the natural history, treatment and clinical outcome of individuals with urea cycle disorders (UCDs) [1]. In analogy to this initiative, the European registry and network for Intoxication type Metabolic Diseases (E-IMD; https://www.e-imd.org/) was founded in 2011 within the framework of the EU Health Program 2008-2013 [2]. Since then both consortia pursued the mutual aim to improve health of individuals with UCDs through improved understanding on natural history, elucidation of key pathomechanisms, early prediction of clinical severity, evaluation of safety and efficacy of dietary treatment, pharmacotherapy and liver transplantation, development of evidence-based consensus care recommendations, and empowerment of patients and their families.

Both consortia, largest of their kind in North America and Europe, agreed on a strategic alliance for collaborative and comparative data analysis to foster the achievement of the mutually set goals. This alliance has built the basis for the establishment and longitudinal follow-up of the worldwide largest UCD cohort with more than 1,100 individuals and for the success in addressing some of the most relevant research and care questions, such as understanding the impact of non-interventional (e.g. disease-immanent severity) and interventional (e.g. conservative therapy, liver transplantation) parameters as well as diagnostic strategies (e.g. newborn screening) on long-term health outcomes of individuals with UCDs. To answer these questions and to improve the evidence level of consensus care guidelines [3], a transatlantic combined and comparative data model has been established. The data model consisted of more than 570 variables from UCDC and 490 variables from E-IMD, with an overlapping set of 250 interoperable variables that were used for subsequent data analyses (**Figure 1**).

Before combined and comparative data analysis could be started, however, various technical and strategical aspects needed to be evaluated carefully. For instance, both registries started long before the implementation of important advances in the field of rare disease registries, such as the common data elements (https://eu-rd-platform.jrc.ec.europa.eu/set-of-common-data-elements_en), the European Rare Disease Registry Infrastructure (ERDRI; https://eu-rd-platform.jrc.ec.europa.eu/erdri_en) and the FAIR principles for scientific data management and stewardship (https://www.go-fair.org/fair-principles/). To overcome this shortcoming, a strategy was established on how to deal with the partially overlapping and partially different coding systems, terminologies, and nosologies of the UCDC and E-IMD registries, as previously described [4].

From previous clinical studies on UCDs and other rare diseases we have learned important lessons which have been utilized for the subsequent data analysis: Patient cohorts followed by patient registries do not necessarily match the case mix found in a distinct population, particularly since fatal disease courses and individuals with severe phenotypes are likely to be under-represented in registries due to a recruitment bias [1,5,6], and the frequency of individuals with an attenuated phenotype might be under-estimated in pediatric cohorts due to 'atypical' disease courses and late onset of symptoms [7,8].

The importance of developing severity-adjusted statistical analyses plans to fairly evaluate the impact of diagnostic and therapeutic interventions on health outcomes is also confirmed by the finding that the initial presentation is an important predictor of survival and neurological outcome in individuals with UCDs. Particularly, the predictive impact of age at disease onset, initial peak plasma ammonium concentration, duration

of the initial hyperammonemic encephalopathy, and coma on initial admission have been demonstrated in various cohorts in the US, Europe, and Japan [5,6,9-15].

A further important finding from previous observational studies is that registries often contain many variables that highly correlate, e.g. variables describing the motor phenotype leading to different conclusions [16,17]. Either the number of variables used for the analysis could be reduced, or alternatively, all correlating variables aiming to describe a certain aspect, such as the motor phenotype, could be used to build a superordinate variable. This is a powerful strategy to reduce the negative consequences of missing values, another frequent challenge of patient registries.

Another example for the correlation of single variables is cognitive function. It has been shown in the combined UCDC and E-IMD cohorts that the five cognitive domains, i.e., intelligence, executive functions, memory, visuomotor integration, and visual perception, were not independent and that intelligence was the only domain to correlate with all other domains. Therefore, global IQ was considered an appropriate single parameter to represent cognitive functions, reducing the complexity of subsequent analyses [18,19]. Another challenge highlighted by previous studies is the apparent direct link of some variables, leading to wrong assumptions. For instance, individuals with UCDs were found to have a higher frequency of behavioral abnormalities compared to the general population, but did not present with a disease group-specific behavioral pattern. The best explanation for this result was the high frequency of intellectual disability in the UCD cohort and the fact that intellectual disability and behavioral problems are causally linked, similar to other diseases [20]. With these and other lessons learned in mind both consortia have established a robust strategy for combined and comparative data analysis which is based on three major pillars: (1) *Interoperability* was achieved by the definition of core data sets with a decent

degree of data aggregation while minimizing loss of relevant information. (2) *Representativeness* was achieved by providing a balanced case mix in both registries. (3) *Severity-adjustment* was achieved by identification of and stratification by important disease modifiers. Definitions and examples of the major analysis principles are depicted in **Supplementary Table 1**. Based on this research strategy, the impact of diagnostic and therapeutic interventions on anthropometrical, cognitive and metabolic endpoints was successfully evaluated (**Figure 2**).

Impact of diagnostic and therapeutic interventions on health outcomes in UCDs

In general, conservative management of individuals with UCDs with low protein diet and sodium or glycerol phenylbutyrate (PBA), both reducing the plasma concentrations of essential amino acids, particularly branched-chain amino acids (BCAA), involve the risk of iatrogenic growth failure. Despite this theoretical risk, the overall growth and weight development of longitudinally followed individuals of the UCDC and E-IMD sample showed normal intrauterine and postnatal linear growth if patients remained asymptomatic until preschool age [21]. In contrast, symptomatic individuals were at risk of progressive growth retardation independent from the underlying UCD type and the extent of natural protein restriction. Instead, growth impairment was associated with disease severity and with reduced or borderline plasma BCAA concentrations. This is substantiated by the fact that liver transplantation, and thus the absence of recurrent hyperammonemia and use of nitrogen scavenging drugs, was associated with normalization of plasma BCAA concentrations and improvement or normalization of growth parameters [21]. A recent longitudinal study from Japan confirmed the higher proportion of short stature and poor weight gain with higher disease severity, since it was more frequently found in the neonatal onset group compared to the late-onset

group [22]. A thorough analysis of the dietary management in the E-IMD sample revealed that supplementation of essential amino acid mixtures helped to obtain normal plasma BCAA concentrations [23].

Importantly, the manifestation of hyperammonemic encephalopathy is largely explained by hyperammonemia-induced disruption of the glutamate/glutamine cycle, which is required for the recycling of the energy-rich carbon backbone of glutamate, the major excitatory neurotransmitter, for the maintenance of ammonium transfer between astrocytes and neurons as well as between the brain compartment and blood circulation, the inactivation of synaptic glutamate and the maintenance, and, finally, the preservation of neurotransmitter homeostasis [24-28]. A disruption of this core pathway results in synergistically acting brain energy impairment with over-activation of glutamatergic signaling and thus excitotoxicity, as well as glutamine-induced brain edema. This mechanism is further aggravated by the negative impact of hyperargininemia on the autoregulation of cerebral perfusion and creatine biosynthesis (except for arginase 1 deficiency), as well as by neuronal nitrosative stress and argininosuccinate-induced neurotoxicity in argininosuccinic aciduria (ASA) [29-31]. This explains why the brain is centrally involved in hyperammonemia-induced pathomechanisms and cognitive dysfunction is a prevalent finding in individuals with UCDs. We wondered which disease-immanent variables best predicted cognitive outcome and which diagnostic and therapeutic interventions had a positive impact on cognitive functions of individuals with UCDs. For this purpose, we evaluated more than 500 individuals in the combined UCDC and E-IMD sample. Intellectual disability was associated with the respective UCD subtype and onset type, and was lower in asymptomatic compared to symptomatic individuals. Furthermore, cognitive impairment inversely correlated with the initial peak plasma ammonium concentration during the first hyperammonemic episode (HAE), particularly for individuals with

mitochondrial (proximal) rather than those with cytosolic (distal) UCD subtypes [19], which points towards additional disease-modifying mechanisms others than hyperammonemia as implied by other studies [32]. Early identification by newborn screening and subsequent early treatment appeared to be neuroprotective for individuals with citrullinemia type 1 (CTLN1) and ASA until preschool age.

The impact of conservative management and liver transplantation on long-term health outcomes has been subject to ongoing debates; however, research on this topic has been hampered so far by small sample sizes, the huge naturally occurring variation in prescribed dietary treatment protocols and heterogeneous implementation of long-term medications [15,33]. Apart from studies analyzing the safety and efficacy of nitrogen scavengers [34-37], systematic and comparative data investigating the impact of long-term scavenger therapy on biochemical endpoints, metabolic stability and cognitive outcome are scarce [38-40]. The combined UCDC and E-IMD analyses allowed to address this research question under real-world conditions, unravelling that long-term alternative pathway therapy with PBA was not associated with better cognitive outcome as compared to treatment with sodium benzoate (BZA) in individuals with ornithine transcarbamylase deficiency (OTC-D), the most prevalent UCD. Combined scavenger therapy (PBA and BZA) did not appear to ameliorate cognitive outcome but rather reflected a more severe disease status [19]. Preliminary analyses suggested that in contrast to nitrogen scavenger therapy, liver transplantation, which becomes relevant if intensified conservative management has failed to protect individuals from potentially life-threatening recurrent HAE, appeared to be beneficial for the preservation of cognitive functions [19,21]. This was supported by a recent analysis from the United Network for Organs Sharing database, suggesting that early liver transplantation should be considered in individuals with UCDs to prevent progressive neurological injury [41], and moreover by data from Japan investigating neonatal-onset

and high-risk individuals with UCDs [42,43]. However, for individuals with a severe phenotype, the life-long impact of the first HAE on cognitive function cannot be reversed by liver transplantation [44], while it is effective for preventing individuals from recurrent hyperammonemic attacks and improving long-term survival [44,45]. Future severity-adjusted analyses are indispensable to evaluate the impact of liver transplantation on health-related outcome parameters in comparison to conservative medical management. Given the phenotypic and outcome diversity of each UCD subtype, future analyses characterizing phenotypic parameters and outcome determinants should optimally focus on separate evaluations for each of the UCD subtypes on an individual level for subsequent comparative analysis to assess which parameters or modifiers are weighed more in one UCD subtype compared to another. Moreover, implementation of machine learning approaches as well as artificial intelligence tools into combined and comparative studies might further improve scientific algorithms to get novel insights into determinants of clinical outcome as already proven beneficial for other inborn errors of metabolism (such as newborn screening for isovaleric aciduria) [46,47].

From bench to bedside: Early prediction of individual disease severity

The integration of additional data sources represents a powerful strategy to expand the analytical capability in UCD research. This strategy proved successful for residual enzymatic activity as determined by a novel (biallelic) mammalian expression system for prediction modelling in the three most prevalent UCDs; i.e. male OTC-D (mOTC-D), CTLN1 and ASA [48-50]. Given the broad phenotypic spectrum in UCDs, which ranges from life-threatening hyperammonemic encephalopathy within the first days of life to an attenuated phenotype with protein aversion, headache and

neuropsychiatric symptoms with an onset any time after the newborn period, reliably and precisely predicting the individual disease course and neurocognitive outcome can be challenging, which bares the risk of an eventual over- or undertreatment of affected individuals. Especially the effect of the genetic background on protein function and, subsequently, on the metabolic disease course remained only partially understood thus far. These challenges could be successfully overcome by the integration of *in vitro* residual enzymatic activity as novel variable, which enabled correlation analysis with biochemical and clinical real-world data of included probands giving raise to the establishment of a reliable disease prediction model. The research strategy relies on the introduction of patient-specific pathogenic variants as reported in the UCDC and E-IMD registries into mammalian expression vectors applying standard site-directed mutagenesis techniques, which are subsequently co-transfected in mammalian cells (e.g. COS-7) in an equimolar manner for both alleles, thereby mimicking the endogenous homozygous or compound-heterozygous expression of mutated proteins in affected individuals. The cumulative residual enzymatic activities are finally determined applying directly-coupled enzymatic assays, which take advantage of redox-reactions for the spectrophotometric real-time quantification of enzymatic activity. This modelling approach enabled to reliably quantify the degree of enzymatic dysfunction of homozygous and most importantly compound heterozygous pathogenic variants. Theoretically, residual enzymatic activities could also be quantified using liver biopsy samples taken from affected individuals, which has already been performed to confirm suspected diagnosis of an underlying UCD [51-53]. However, systematic investigations on liver biopsy samples to establish a reliable disease prediction model are expected to be associated with a high degree of data variability given the non-standardized location of sample collection sites, which are likely thought to result in heterogeneous sample constitution due to the periportal expression of UCD enzymes.

Subsequently, numeric values of residual enzymatic activity determined by the *in vitro* expression system are correlated with major biochemical and clinical variables of UCDs as reported in the UCDC and E-IMD registries such as but not limited to peak plasma ammonium concentration at initial decompensation, number of HAEs during the individual observation period, cognitive standard deviation score (SDS) at last follow-up visit and presence or absence of hepatocellular injury, impaired kidney function or mortality (**Figure 3**) [48-50].

In vitro residual enzymatic activity as determined by the depicted modelling approach not only correlated with the actual severity of the initial HAE as reflected by the peak plasma ammonium concentration at disease onset, but was also associated with the number of subsequent HAEs during the individual disease course as well as the cognitive SDS at last follow-up visit in CTLN1 and ASA. Recursive partitioning enabled the identification of a robust threshold value of residual enzymatic OTC, ASS1 and ASL activity that reliably differentiates between individuals with a severe (≤5% of OTC activity for mOTC-D, ≤8% of ASS1 activity for CTLN1 and ≤9% of ASL activity for ASA) or attenuated phenotype (>5% of OTC activity for mOTC-D, >8% of ASS1 activity for CTLN1 and >9% of ASL activity for ASA) [48-50]. Importantly, *in vitro* residual enzymatic OTC, ASS1 and ASL activity as determined by the biallelic expression system qualify as predictive biomarkers, as they reflect disease severity and reliably predicts the clinical outcome in mOTC-D, CTLN1 and ASA according to prerequisites for biomarkers as defined by the US Food and Drug Administration [54,55]. The discrimination of individuals with a predicted severe or attenuated phenotype of cytosolic UCDs is of importance for (1) the counseling of affected individuals and their families with regard to the expected disease course and long-term health outcome at a very early stage of the disease (e.g. when genetic test results are obtained), (2) the establishment of individualized and severity-adjusted care concepts, and (3) the design

and interpretation of clinical trials that aim at evaluating the impact of diagnostic or therapeutic interventions such as future enzyme replacement or gene therapies.

However, residual enzymatic OTC, ASS1 and ASL activities do not allow to predict the *exact* peak plasma ammonium concentration at disease manifestation or the *exact* number of subsequent HAEs, since additional precipitating factors such as the severity of the catabolic state leading to metabolic decompensation, delay between the onset of symptoms and initiation of treatment as well as adherence to long-term treatment also determine the severity of a specific metabolic decompensation. As enzymatic activities are determined using supraphysiological substrate concentrations, the dysfunctional effect of pathogenic variants associated with altered kinetic properties ("leaky mutations", *Km* variants) due to decreased substrate binding of the ASS1 or ASL enzyme could be under-estimated by this modelling approach [56], which needs to be addressed in future research. Moreover, only exonic variants have been investigated by the presented modelling approach due to the applied technique of cloning the respective coding sequence into the expression vectors, which does not allow to assess the effect of intronic variants associated with defective splicing. Systematically integrating genotype-specific *in vitro* residual enzymatic activities with the results of widely used *in silico* algorithm tools (such as aRgus [57]) for multilevel visualization of single nucleotide variants and associated pathogenicity score modeling for vulnerability assessment with regard to protein structure, stability and protein function might bare the potential of significantly improving risk stratification and phenotypic prediction in the future.

To this end, stratification of individuals according to their predicted severe or attenuated phenotype enabled the severity-adjusted evaluation of the impact of an early diagnosis by newborn screening (NBS) on the metabolic disease course in

CTLN1 and ASA [58]. NBS programs for cytosolic UCDs identify a higher proportion of individuals with an attenuated phenotype in comparison to pre-NBS cohorts. This phenotypic shift to more attenuated phenotypes after the start of NBS has been describes for other inherited metabolic diseases, such as mild isovaleric aciduria [59]. However, early identification by NBS and subsequent early therapeutic interventions reduce the severity of the initial HAE as reflected by the reported peak plasma ammonium concentration at disease manifestation for both individuals with a severe and attenuated phenotype. This reduction of peak plasma ammonium concentration might improve the prognosis from 50% mortality during the initial episode to lower mortality rates [11,15,60,61], and might be associated with a better neurocognitive long-term outcome in both severely and mildly affected individuals surviving the initial HAE [15,60,62,63]. Of note, peak plasma ammonium concentration in the group of an attenuated phenotype lie within the normal range after identification by newborn screening [58]. These are promising but preliminary results and thus require careful evaluation of the benefits and harms of NBS programs in an even larger and prospectively followed cohort of individuals with UCDs.

Conclusion and Outlook

Combined and comparative data analyses from the UCDC and E-IMD consortia study group serve as a blueprint and useful strategy for identification of important associations that need to be confirmed in a longitudinal manner for providing evidence-based recommendations that are translated into patient care. We propose that any future clinical trial for UCDs and other rare diseases would benefit from evaluation against meaningful endpoints by adhering to the three principles *Interoperability*, *Representativeness*, and *Severity-adjustment*. Implementation of numerical variables, reflecting the intrinsic disease severity, is indispensable for precise in-depth efficacy evaluation of diagnostic and therapeutic interventions in the field of rare diseases as well as for reliable prediction of individual disease severity, risk stratification and indication to treat, paving the way to personalized medicine.

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Data availability statement

Supporting data can be found in the UCDC database, registered at the US National Library of Medicine (https://clinicaltrials.gov), and in the E-IMD registry, recorded on the German Clinical Trials Register (https://www.drks.de). Datasets reviewed and generated for this work are not publicly available due to existing data protection laws. Furthermore, data ownership is retained by the members of the UCDC and E-IMD consortia that make data available for specific research purposes. Upon request data availability is subject to the consent of both consortia.

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Figure Legends

Figure 1: The principle of traffic lights to overcome the technical challenge when analyzing data from UCDC and E-IMD. Approximately 190 variables were equivalent between both registries, while 60 variables were harmonisable building an overlapping overall set of approximately 250 variables. Exemplarily, variables from the *"Physical and Neurological Examination Form"* are shown. Green color symbolizes equivalent variables, yellow color depicts harmonisable variables, and red color shows non-harmonisable variables.

Figure 2: Overview of the UCDC and E-IMD analysis strategy. Based on the three principles (I) Interoperability, (II) Representativeness, and (III) Severity-adjustment, a combined and comparative data analysis strategy could be performed evaluating diagnostic and therapeutic interventions on various long-term outcome parameters, e.g. cognitive, metabolic, and anthropometrical outcome. For detailed information on the various projects, please see ***Suppl. Table 2***.

Figure 3. Methodological approach for the establishment of disease prediction models based on *in vitro* residual enzymatic activity. (A) The coding sequence of the respective gene of interest is cloned into mammalian expression vectors harboring a FLAG- or MYC-tag to distinguish both alleles. Subsequently, patient-derived pathogenic variants are introduced applying standard site-directed mutagenesis. (B) Transfection of a suitable mammalian cell line (e.g. COS-7) applying lipofection protocols. (C) Cells are lysed after an appropriate incubation time allowing sufficient translation of the (dysfunctional) proteins and subjected to either mRNA expression analysis (using qRT-PCR), protein expression analysis (using standard Western Blot technique) or quantification of residual enzymatic activity (using a directly-coupled enzymatic assays with spectrophotometric read-out). (D) Numeric values of residual enzymatic activity are correlated to biochemical and clinical outcome parameters of the respective patients as reported in disease registries (e.g. UCDC and E-IMD). For detailed information see the respective publications as listed in ***Suppl. Table 2***. Subfigures A and B are partially adapted from [64,65].

The technical challenge in combining and comparing data from UCDC and E-IMD	
	Approximative number of variables
Equivalent variables	190
Harmonisable variables	60
Non-harmonisable variables	320 (UCDC), 240 (E-IMD)

Variables from UCDC and E-IMD (Physical and neurological examination Forms)	
UCDC	E-IMD
ight [cm]	Height [cm]
eight [kg]	Weight [kg]
Head circumference [cm]	Head circumference [cm]
Head, nose and throat [Findings in free text / SNOMED]	Specification of non-metabolic diagnoses (Findings in ICD-10 codes]
ead, nose and throat [Status as tick boxes]	Not available in E-IMD
s and ears [Findings in free text / SNOMED]	Specification of non-metabolic diagnoses (Findings in ICD-10 codes]
d ears [Status as tick boxes]	Not available in E-IMD
Lungs and chest [Findings in free text / SNOMED]	Specification of non-metabolic diagnoses (Findings in ICD-10 codes]
Lungs and chest [Status as tick boxes]	Lungs and chest [Status as tick boxes]
diovascular system [Findings in free text / SNOMED]	Specification of non-metabolic diagnoses (Findings in ICD-10 codes
Cardiovascular system [Status as tick boxes]	Cardiovascular system [Status as tick boxes]

Figure 1.tif

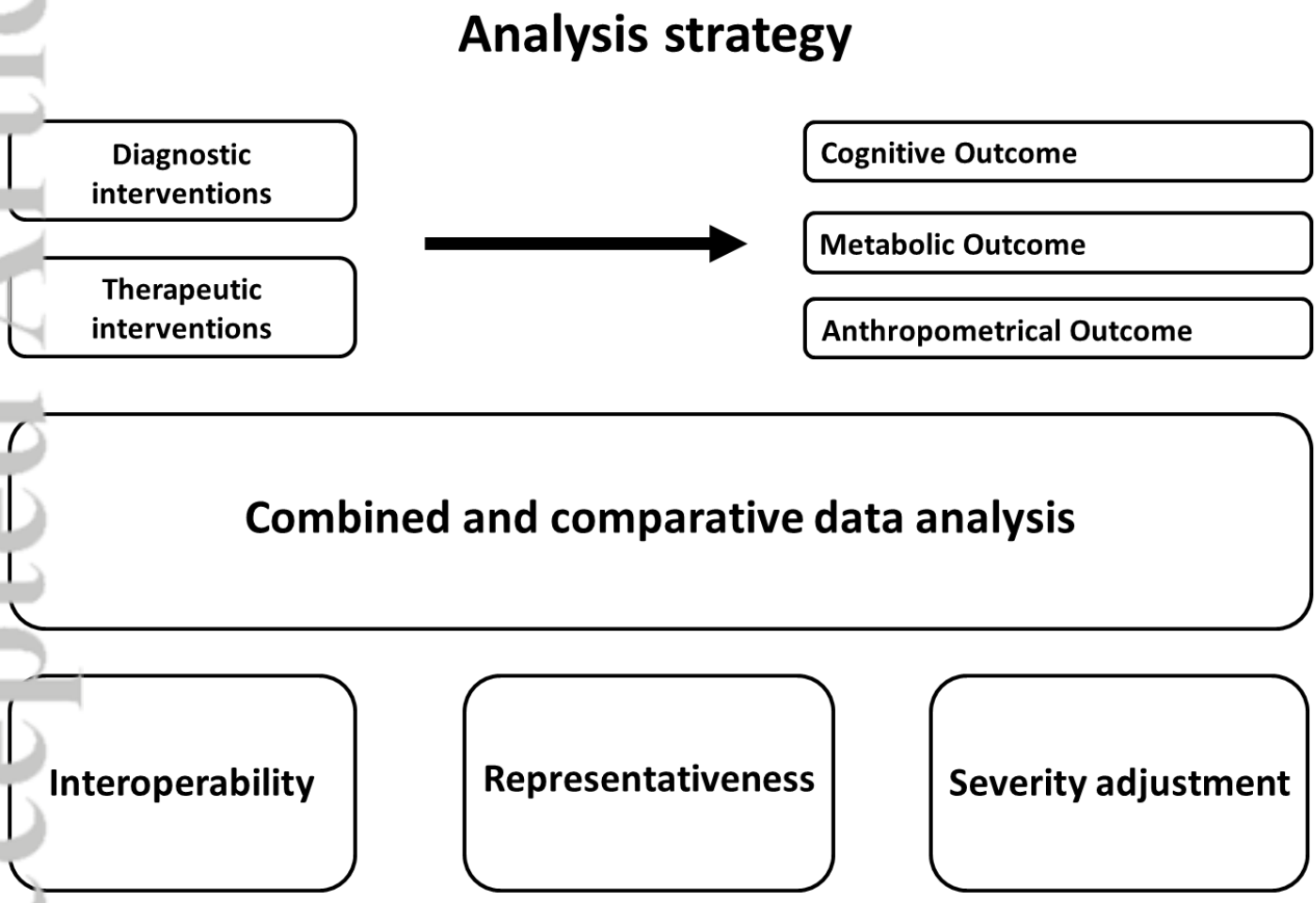

Figure 2.tif

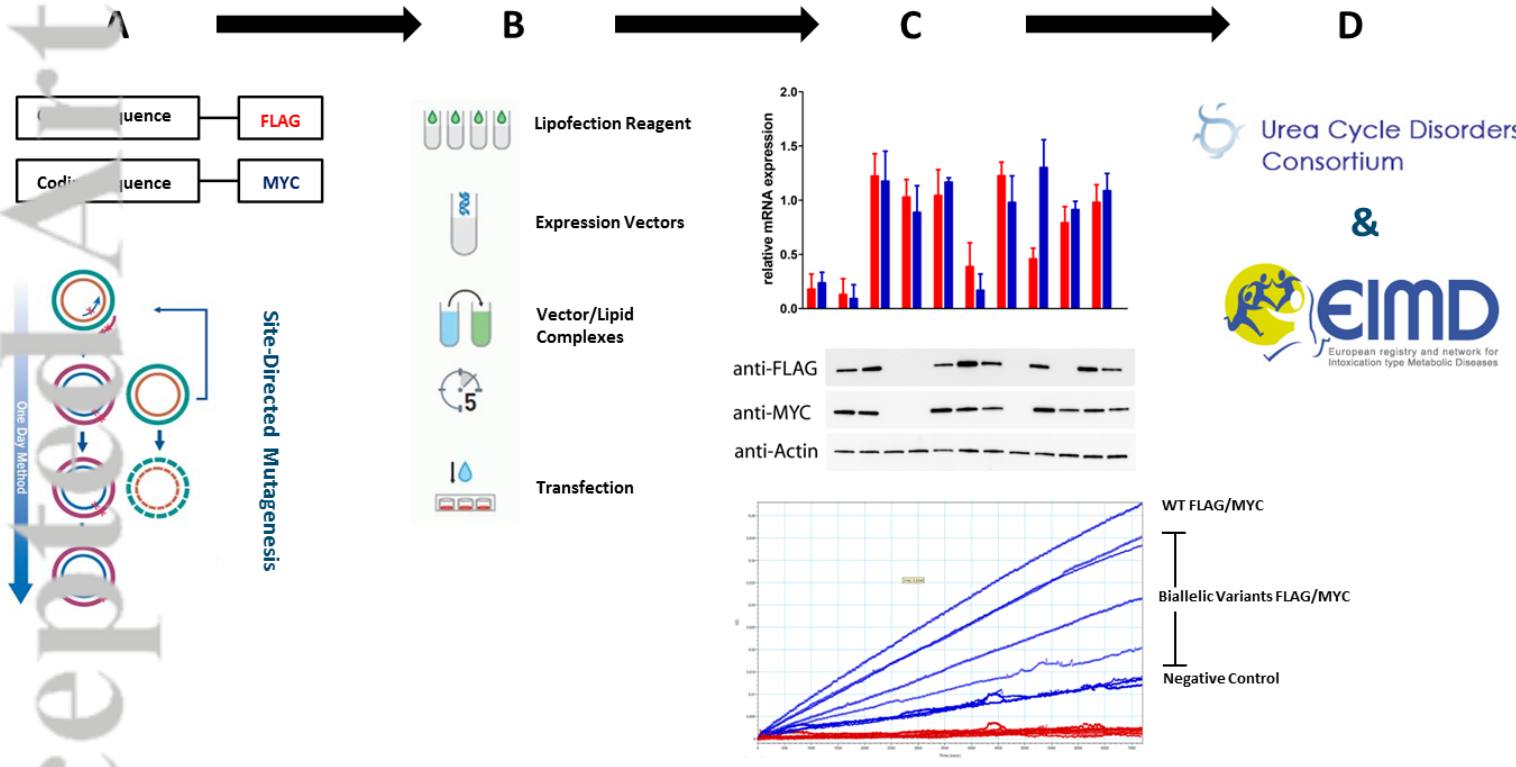

Figure 3.tif