Prospective Multicenter Validation of a Simple Blood Test for the Diagnosis of Glut1 Deficiency Syndrome

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Abstract

Objective
GLUT1 deficiency syndrome (Glut1DS) is a treatable neurometabolic disease that causes a wide range of neurological symptoms in children and adults. However, its diagnosis relies on an invasive test, i.e., a lumbar puncture (LP) to measure glycorrhachia, and, sometimes complex, molecular analyses of the SLC2A1 gene. This procedure limits the number of patients able to receive the standard of care. We wished to validate the diagnostic performance of METAglut1™, a simple blood test that quantifies GLUT1 at the erythrocyte surface.
Methods
We performed a multicenter validation study in France, involving 33 centers. We studied two patient cohorts: a prospective cohort, consisting of patients with a clinical suspicion of Glut1DS explored through the reference strategy, i.e., LP and analyses of the SLC2A1 gene; a retrospective cohort that included patients previously diagnosed with Glut1DS. All patients were blind-tested with METAglut1™.

Results
We analyzed 428 patients in the prospective cohort, including 15 patients newly diagnosed with Glut1DS, and 67 patients in the retrospective cohort. METAglut1™ was 80% sensitive and >99% specific for the diagnosis of Glut1DS. Concordance analyses showed a substantial agreement between METAglut1™ and glycorrhachia. In the prospective cohort, the positive predictive value of METAglut1™ was slightly higher than that of glycorrhachia. METAglut1™ succeeded to identify patients with Glut1DS with SCL2A1 mosaicism and variants of unknown significance.

Interpretation
METAglut1™ is an easily performed, robust and non-invasive diagnostic test for the diagnosis of Glut1DS, which allows a wide screening of children and adults, including those with atypical forms of this treatable condition.

Classification of Evidence
This study provides class I evidence that a positive METAglut1™ test accurately distinguishes patients with suspected GLUT1 deficiency syndrome from other neurological syndromes as compared to invasive and genetic testing.

Introduction
GLUT1 deficiency syndrome (Glut1DS) is a rare and disabling neurological disease that can be treated. It is therefore of utmost importance to raise awareness about its diagnosis among the medical community. Glut1DS is caused by impaired glucose transport across the blood-brain barrier and into glial cells due to heterozygous, mostly de novo, mutations in the SLC2A1 gene encoding the glucose transporter GLUT1. GLUT1 is a membrane-bound glycoprotein that is particularly abundant in human erythrocytes, and brain endothelial and glial cells. Its dysfunction limits brain glucose availability and leads to brain energy deficiency. Besides the classical severe infantile-onset epileptic encephalopathy, Glut1DS also manifests with a wide range of neurological symptoms in children and adults, including epilepsy, permanent motor disorders, paroxysmal movement disorders, and cognitive impairment, either combined or isolated.1-3 The early detection of Glut1DS is critical 4 as the disease is treatable with ketogenic diets5,6 or novel experimental therapies.7
A recent work estimates the disease incidence in the general population to be higher than 1 in 24,000. This number only takes into account patients presenting with epilepsy. Some patients present only with movement disorders and/or learning difficulties. Given the number of patients currently identified in registries (e.g., about 500-1,000 patients in the USA, 150-200 patients in France, and 60-80 patients in Spain), it is highly likely that a large number of patients with Glut1DS have currently gone undiagnosed. These numbers highlight the importance of tackling underdiagnoses and medical wandering, and urge the medical community to improve both awareness about the disease, and diagnostic strategy. However, this is challenging for physicians as, on the one hand, the clinical spectrum is very protean, encouraging more frequent testing for Glut1DS, but on the other hand, the current diagnostic strategy relies, as a first step, on an invasive and strict procedure—i.e., lumbar puncture (LP) performed in the fasting state with glycemia measured right before LP, followed by genetic analyses (targeted SCL2A1 analysis or gene panels or whole exome sequencing). This diagnostic sequence limits the number of patients able to receive the standard of care.

The easy access to a blood biomarker for the early and fast diagnosis of Glut1DS could be determinant for patient outcome and of major economic impact, as earlier treatment is associated with greater patient’s prognosis. METAglut1™ is a simple test that relies on the quantification of GLUT1 at the erythrocyte surface. It thus provides direct labeling of fresh red blood cells by flow cytometry, similar to routine flow cytometry-based assays in hematology and immunology performed by routine laboratory testing. Only a simple blood draw in an EDTA tube is needed, and it does not require the patient to fast beforehand. A pilot cohort of 30 patients estimated that METAglut1™ was 77% sensitive and very specific for Glut1DS. These encouraging results prompted us to evaluate the diagnostic performance of METAglut1™. To this end, we conducted a multicenter validation study in France involving 33 centers.

**Methods**

**Standard Protocol Approvals, Registrations, and Patient Consents**

The study was registered and approved by ANSM (French Health Authority) and by the ethics committee CPP Ouest V de Rennes (France) under French national identifier ID-RCB 2017-A01473-50. The study was registered on ClinicalTrials.gov under identifier NCT03722212. Written informed consent was obtained from all participants (or guardians of participants) before being enrolled in the study (consent for research).

**Study Design**

To assess whether METAglut1™ has similar diagnostic performances compared to glycorrhachia, we enrolled both a prospective and a retrospective cohort through 33 French participating centers,
involving more than 100 neurologists and neuropediatricians. The recruitment started in September 2018 and ended in March 2021.

The prospective cohort consisted of patients presenting with a clinical suspicion of Glut1DS and blind-tested with METAglut1™ along with the reference strategy, which consists of a LP for glycorrhachia measurement, completed by SLC2A1 molecular analyses. In case of an uncertain diagnosis due to potentially discordant results, we further assessed the patient status with an ex vivo functional glucose uptake assay performed on the patient red blood cells. Glycorrhachia, lactatorrhachia, glycemia and SLC2A1 molecular analyses were determined at each center under current standard of care.

The retrospective cohort consisted of patients already diagnosed with Glut1DS based on the reference strategy (i.e., compatible clinical phenotype associated with pathogenic SLC2A1 variants, or hypoglycorrhachia and SCL2A1 variants of uncertain pathogenicity).

Both cohorts were tested with METAglut1™, blind of the patients’ condition—clinical, biochemical and molecular data were not available to the central lab performing the test.

**Participants**

We enrolled both children and adults. Children were older than age 3 months as early infantile red blood cells are 20% larger than adult cells with higher GLUT1 levels, which is a confounding factor for the interpretation of GLUT1 expression.

For the prospective cohort, we used the following inclusion criteria: (1) patients with classical phenotypes of Glut1DS: i) encephalopathy with drug-resistant epilepsy and microcephaly, or ii) early onset absence epilepsy characterized by EEG, or iii) generalized epilepsy with a personal or family history of paroxysmal exercise-induced dyskinesia; or (2) patients with atypical forms of Glut1DS defined as unexplained forms (i.e., absence of argument for an infectious, inflammatory or tumoral cause) of i) childhood epilepsy occurring after age 4 years characterized by EEG, or severe juvenile epilepsy occurring after age 10 years characterized by EEG, and/or ii) developmental delay or intellectual disability with a history of epilepsy not yet characterized, or a history of drug-resistant epilepsy, and/or iii) paroxysmal movement disorders (pyramidal, ataxic, dyskinetic, ocular), including abnormal movements triggered or aggravated by fasting, exercise, stress or emotion, and/or iv) permanent movement disorders (pyramidal, ataxic, or dyskinetic) with history of epilepsy or learning disorders, and/or v) patient referred to pediatrics for repeated malaise of unknown origin.

For the retrospective cohort, patients already diagnosed with Glut1DS, based on pathogenic SLC2A1 variants, or hypoglycorrhachia and likely pathogenic SCL2A1 variants, were eligible. Patients suspected to have Glut1DS, with compatible phenotype but inconsistent results for the aforementioned parameters (e.g., SCL2A1 variants of uncertain pathogenicity), and so-called possible Glut1DS, were also eligible. For the latter patients, the glucose uptake assay was prescribed to confirm or rule-out the diagnosis.

For all patients, exclusion criteria were: (1) patients with brain imaging suggestive of a cause other than Glut1DS, and 2) situations that could be confounding factors for the interpretation of GLUT1.
expression on erythrocytes: i) patients less than age 3 months, ii) patients with sickle cell anemia, as erythrocytes have higher levels of GLUT1, \(^{13}\) iii) patients having undergone a heterologous bone marrow transplant, or who had a blood transfusion within the last 120 days, as normal erythrocytes can bias the mean GLUT1 expression on the erythrocyte population measured by flow cytometry analysis.

**CSF Analyses**

CSF glucose and lactate were measured at each center under routine care settings, after at least 4 hours of fasting, along with glycaemia, which was measured immediately before the LP.

**SLC2A1 Molecular Analyses**

SLC2A1 molecular analyses were performed either through direct Sanger sequencing and/or multiplex ligation-dependent probe amplification, or through a gene panel tailored for epilepsy or movement disorders or through exome sequencing. Variants were interpreted by geneticists and classified as benign or likely benign (class 1 and 2), variants of unknown significance (VUS) (class 3), probably damaging (class 4), or pathogenic variants (class 5), based on \textit{in silico} predictive algorithms (CADD, AlignGVGD, SIFT, Polyphen2, MutationTaster and Varsome) with Alamut Visual (Interactive Biosoftware, Rouen, France), frequency in international databases (gnomAD, dbSNP) and segregation data, according to ACMG guidelines.\(^{14}\)

**Erythrocyte Analyses**

METAglut1™ (METAFORA biosystems, Paris, France) is a CE marked \textit{in vitro} diagnostic medical device. The innovation stems from seminal work of academic lab, and has been turned into an assay which has received the CE-mark to facilitate implementation in routine testing labs. This test comprises a specially designed assay based on flow cytometry, and a software for automated computation. A soluble ligand that harbors the receptor-binding domain derived from the HTLV2 envelope glycoprotein (H2RBD) is currently the only reagent which recognizes specifically GLUT1 on red blood cells.\(^{15}\) It thus provides direct labeling of fresh red blood cells by flow cytometry, similarly to routine flow cytometry-based assays in hematology and immunology performed by routine testing labs. The sample preparation protocol is minimal, requiring only blood dilution before labeling, followed by washing. Multiple samples can be processed in parallel in a 2-hour run experiment, from sample preparation to results ready to be released. The software includes a series of quality controls of the samples and the run, minimizing time to verify and interpret the data. Altogether the workflow is efficient and ensures a quick turnaround time, which was about 48 hours during the study.

METAglut1™ was performed in a centralized testing laboratory (Laboratoire CERBA, Saint-Ouen l’Aumône, France), and all functional glucose uptake assays were performed at the Institut de Génétique Moléculaire de Montpellier (IGMM, CNRS, France). Both labs were blinded to patient’s clinical diagnosis, or any other biological data, when performing the analyses. In order to avoid bias,
the METAglu1™ result was also blinded to the investigator until the reference strategy was filed in the eCRF, i.e., when the glycorrhachia result was available and molecular analyses prescribed. This procedure ensured proper and timely clinical management of patients with Glut1DS. Blood samples were collected on EDTA tubes, then sent and stored at 4°C until analyses. The METAglu1™ test was performed within 7 days post sampling by the same four trained technicians throughout the study. Results were expressed in terms of differences of GLUT1 detection at the cell surface compared to its mean expression across at least 6 samples. Previous results have demonstrated that coefficients of variation were below 5% both in repeatability and reproducibility experiments, allowing for a deployment in routine testing labs.

When needed, a sensitive functional glucose uptake assay with red blood cells was performed as described.\textsuperscript{16} Once implemented at IGMM, the assay was qualified before the start of the study, and demonstrated a mean coefficient of variation of 6% (<8%). Briefly, blood samples were collected in ACD tubes and shipped and stored at 4°C until analyses. The assay was performed at precisely 7 days post sampling for every patient, in order to minimize variability due to potential red blood cell lesion that would occur during storage. Glucose uptake was expressed as % of the mean of uptakes measured with 2 to 3 healthy blood donors taken as controls and stored for the same amount of time and in the same conditions. Healthy blood donor samples were provided by the French Blood Center (EFS, Saint-Denis, France) under agreement # 16/EFS/007.

\textbf{Glut1DS Diagnosis}

Criteria for Glut1DS followed the most recent recommendations published by an international Glut1DS working group,\textsuperscript{17} and was established by an international scientific committee (FM, RP, AGC, SVB, DCD). Glut1DS was confirmed in patients with a compatible clinical phenotype associated with glycorrhachia below 2.2 mM (40 mg/dl) and a pathogenic or likely pathogenic \textit{SLC2A1} variant. Nonetheless, as glycorrhachia normal range slightly increases with age,\textsuperscript{18} this feature may be more complex to interpret. Therefore, particular attention was given to patients with glycorrhachia comprised between 2.2 (40 mg/dl) and 3 mM (54 mg/dl) and any argument in favor of Glut1DS, such as typical symptoms, or a VUS in the \textit{SLC2A1} gene. In case of an uncertain diagnosis, an abnormal erythrocyte glucose uptake assay confirmed the diagnosis of Glut1DS. Glucose uptake assay positivity threshold was set at 74% of the controls, as previously reported.\textsuperscript{16} Since METAglu1™ is a blood test that can be used as a screening test to improve timely diagnosis, its positive and negative predictive values are critical. Thus, we decided to compare the diagnostic performances of glycorrhachia and METAglu1™ using thresholds with high specificity. For CSF, hypoglycorrhachia threshold positivity was set at 2.2 mM (40 mg/dl), as specificity diminishes drastically above this value,\textsuperscript{19, 20} along with a CSF/blood glucose ratio below 0.45, and lactatorrhachia below 1 mM (9 mg/dl). For METAglu1™, the initial pilot study set a threshold of positivity at a 20% decrease of GLUT1 expression on erythrocytes as diagnostic for Glut1DS.\textsuperscript{11} In other words, if a sample showed more than a 20% decrease in GLUT1 expression, it was specific of Glut1DS. This
interpretation threshold was chosen *a priori* for METAglut1™ diagnostic performances. *Post hoc* analyses showed that the assay was even more specific at the -24% cut-off. Performance assessment for METAglut1™ was thus obtained at the latter refined interpretation threshold.

**Data Collection and Security**

Data related to patient’ symptoms were collected within the eCRF upon patient’s enrollment, allowing investigators to provide some more details following the specification of the summarized clinical presentation (inclusion criteria). Last patients were recruited in March 2021 and data collection ended in July 2021. A thorough data management plan was implemented with on-site monitoring, automated controls of eCRF and recoding after queries and data reviewing.

**Statistical Analyses**

Statistical analyses were performed on all patients selected in the study and whose data were declared usable during a data review, and for whom the masking procedure was fulfilled. Statistical analyses were univariate descriptive or based on cross-tabulations. Data management and statistical analyses were performed using SAS® V9-4 software (North Carolina, USA) by CEMKA (Bourg-la-Reine, France). The 95% confidence intervals were calculated for the main diagnostic performance criteria and when their estimation was considered necessary. The Cohen’s kappa coefficient was used for concordance analyses, with a null hypothesis kappa = 0 with one-side test. Based on an estimated prevalence of 2% among eligible patients for this study, a number of 115 patients would lead to a power of 0.9 with a significant level of 0.05.

**Data Availability**

The data that support the findings of this study are available from the corresponding authors on reasonable request. Permissions are required to gain access to the data resources, subject to successful registration and application process.

**Results**

**Patient Groups**

We enrolled 549 patients in the prospective cohort: 28% presented with classical phenotypes, and 72% had atypical clinical presentations. Sex ratio (determined from pre-existing medical records) was close to 1/1, with 46% of females; 14% were infants with ages between 3 months and 2 years, 69% were children between 2 and 18 years, and 17% were adults. These demographic data were in line with the literature, with no bias towards sex, and a diagnosis that was made mostly in children with a mean age of 7 years. In the retrospective cohort, we enrolled 87 previously diagnosed patients with Glut1DS. Ethnicity data were not available. Table 1 summarizes the clinical characteristics of all included participants. After data collection, review and analysis by the scientific committee, the 636 enrolled patients were classified as patients either with Glut1DS or not.
During the course of the study, a total of 15 index cases (8 with classical phenotypes and 7 with atypical phenotypes), and overall, 20 patients with Glut1DS (i.e., 3 families of 2 to 3 patients) were newly diagnosed among the 428 prospective participants for whom glycorrhachia and/or molecular analyses could be obtained (Figure 1). Among these 428 patients, 205 index patients for whom molecular analyses were available were used to compute the sensitivity and specificity of METAglut1™, as well as its positive and negative predictive values (Figure 1). Concordance analyses between METAglut1™ and glycorrhachia were performed on a larger group of 350 prospective patients for whom both glycorrhachia and METAglut1™ were available (Figure 1). Only index cases were used to compute diagnostic performances of the diagnostic tests parameters as Glut1DS mostly occurs de novo and familial cases would have introduced bias due to the enrichment of certain SLC2A1 variants.

Within the retrospective cohort of 87 patients, demographic data were comparable to those of the prospective cohort – sex ratio of 1/1, 4% of infants with ages between 3 months and 2 years, 64% of children between 2 and 18 years, and 32% of adults. From the 77 patients with Glut1DS that were retained for analyses, 60 index patients were used for sensitivity analysis, and 54 index patients for concordance analyses between METAglut1™ and glycorrhachia (Figure 1).

**Diagnostic Performance of METAglut1™**

When using a threshold of 76% GLUT1 detection on erythrocytes, METAglut1™ sensitivity was found to be 85% (95% CI 76 – 94) in the retrospective cohort of 60 index patients with Glut1DS. Sensitivity in the prospective group of 205 patients, including 15 index patients with Glut1DS, was 60% (35 – 85). Specificity confirmed to reach 99% (98 – 100), and positive and negative predictive values were 90% (71 – 100) and 97% (95 – 99), respectively. A drop in GLUT1 detection greater than 28%, which was the case for most patients, was associated with a positive predictive value of 100%. Overall, METAglut1™ was 80% sensitive and >99% specific for the diagnosis of Glut1DS. Notably, METAglut1™ allowed us to detect a SCL2A1 mosaicism in a 12-year-old patient presenting with a moderate phenotype (Figure 2).

Fifteen out of 75 (20%) index patients with Glut1DS were negative for METAglut1™. All these patients presented with mild to moderate phenotypes (Table 2). The glucose uptake assay could be performed for 12 of these 15 false negative patients, and 8 had a negative glucose uptake assay (Figure 3). Of note, 3 of these patients had glucose uptake kinetics that were remarkably higher than controls (>160%), while remaining in the normal range for METAglut1™ (Table 2). All pathogenic variants associated with METAglut1™ false negative results were missense mutations (Table 2), while nonsense mutations, deletions and premature stop codons were only found in the METAglut1™ positive patients (data not shown).

**Comparison of METAglut1™ Diagnostic Performance to Glycorrhachia**
In our study, the 2.2 mM (40 mg/dl) glycorrhachia threshold matched the low-end value of glycorrhachia in the non-Glut1DS population (Figure 4). When performing calculation on patients with both tests available, the diagnostic performances of METAglut1™ were highly comparable to those of glycorrhachia with a sensitivity of 92% (84 - 99) for glycorrhachia versus 85% (75 – 95) for METAglut1™ in the retrospective cohort; an equal sensitivity of 67% (40 – 93) in the prospective cohort; an equal negative predictive values of 99% (98 – 100); and positive predictive values of 73% (46 – 99) for glycorrhachia versus 89% (68 – 100) for METAglut1™. The diagnostic performance of the CSF/blood glucose ratio was similar to that of glycorrhachia. Furthermore, we observed a mean lactatorrhachia of 1 mM (9 mg/dl) (range: 0.7 to 1.4 mM – 6 to 12 mg/dl) for 90% of patients with Glut1DS, compared to a mean of 1.4 mM (12 mg/dl) (range: 0.8 to 1.9 mM – 7 to 17 mg/dl) for 90% of patients without Glut1DS, supporting that CSF lactate is low-normal to low in Glut1DS. 18 The small number of prospective patients, which is inherent to a rare disorder, did not allow us to perform statistical comparisons between the sensitivity of the two tests. Nevertheless, we applied a concordance analysis to further match the two diagnostic tests. METAglut1™ and glycorrhachia were compared on the subset of 350 prospective patients for which both tests were available. The two tests were in agreement, with a Cohen’s kappa coefficient of 0.59 (0.33 – 0.85; significantly better than 0 with p = 0.004) (Figure 4). Glut1DS being a rare disease, the large number of double negative cases (336) imbalances the matrix (6 double positives, and 5 versus 3 single positives), explaining the only moderate to strong agreement of 0.59 along with a wide 95% confidence interval in the prospective cohort. Since both the prospective and retrospective cohorts have been analyzed by the same central lab unbeknownst of their status, we also performed concordance analyses in the cumulated cohort. The Cohen’s kappa coefficient then reached 0.78 (0.69 – 0.87; significantly better than 0 with p < 0.001), which is considered as a substantial agreement (341 double negative, 43 double positives, and 13 versus 7 single positives). The mean value falls close to the 0.8 threshold of Cohen’s kappa coefficient to state an almost perfect agreement. The low range of the 95% confidence interval matched 0.7 that is classically considered as a good concordance, and its upper range encompassed values that are considered as an almost perfect agreement.

Discussion

We demonstrated that the METAglut1™ blood test can expedite the diagnosis of Glut1DS with 80% sensitivity and > 99% specificity. Those values are comparable to the 77% sensitivity found in the pilot cohort. 11 Moreover, this multicenter prospective cohort allowed to estimate the positive and negative predictive values of METAglut1™, which reached 90% (71 – 100) and 97% (95 – 100), respectively. The number of 20 patients with Glut1DS found in the prospective cohort is in line with previous reports. 22 Indeed, the occurrence of Glut1DS within the so-called classical clinical forms has been estimated between 5% to 10%, whereas 1% to 2 % of patients with Glut1DS are expected among the so-called atypical clinical forms. 23 Our study enrolled 156 patients with a classical phenotype,
which would translate into an expected 7 to 16 patients among this population, and 393 patients with an atypical phenotype, corresponding to an expected 4 to 8 patients with Glut1DS. Thus, a total of 11 to 24 patients was expected in the prospective cohort, which compares very favorably with the 20 patients with Glut1DS whom we diagnosed in our study. Accordingly, the cohort used in the present study can be validated as representative for Glut1DS.

Since hypoglycorrhachia is a hallmark of Glut1DS and that LP is used as a first test in the current diagnostic strategy, we wished to compare glycorrhachia levels to METAglut1™. In the retrospective and prospective cohorts, the diagnostic performances of both tests were highly similar in terms of sensitivity, specificity, and positive and negative predicted values. They also demonstrated good concordance at the patient level. A slightly higher sensitivity was found for glycorrhachia in the retrospective cohort compared to METAglut1™ (92% versus 85%, respectively). Such a bias was expected since most patients in this cohort were previously diagnosed based on hypoglycorrhachia. Moreover, our study allowed us to determine the positive predictive value of glycorrhachia, which appeared (even at the 2.2 mM / 40 mg/dl threshold) to be lower than that of METAglut1™ (73% versus 89%, respectively).

Our study confirmed that one Glut1DS patient out of 5 is negative for METAglut1™. Among these patients, we found that two-thirds (8/12) were also negative for the glucose uptake assay, a very sensitive functional assay, which is considered the gold standard for assessing Glut1DS. Noticeably, the best cutoffs values are equivalent for both erythrocyte tests, with approximately 74-76% of the controls, and is likely due to similar biological parameters measured by the two assays. These findings highlight important diagnostic challenges in some patients with Glut1DS. A possible explanation for METAglut1™ false negative results is that mutations are likely to bear different consequences on GLUT1 function in erythrocytes than they do on the blood brain barrier, in part likely due to tissue-specific GLUT1 partners that can distinctively modulate the impact of the mutation at the erythrocyte surface. Since METAglut1™ relies on the quantification of GLUT1 presence at the erythrocyte surface by the H2RBD specific ligand, mutations that do not prevent either its binding, or the transporter to be properly expressed at the cell surface, whether this stems from a problem of translation or folding or trafficking, are likely to remain undetected. This observation suggests that METAglut1™ false negatives reflect rather an altered glucose uptake kinetics than an impact on the actual presence of the protein at the cell surface. Accordingly, it appeared that all false negative patients harbored missense mutations and presented with milder phenotypes. Although it is not a definitive conclusion, these mutations may only partially affect glucose transport, triggering moderate phenotypes in patients.

To our knowledge, our study is the first to prospectively and thoroughly validate a circulating biomarker against the reference diagnostic strategy that includes glycorrhachia. The very good
diagnostic performance of METAglut1™ in our multicenter cohort, which is the largest reported for this disease, reinforces the clinical relevance of this test, which can be easily and rapidly used by prescribers. Unfortunately, this study did not allow us to formally establish the diagnostic time gained with METAglut1™ since most investigators opted to run both tests in parallel during in-patient clinics for practical reasons. With a typical turnaround of 48 hours, the test is robust with few retests necessary, thus warranting an easy adoption in routine clinical settings, notably in outpatient clinics. METAglut1™ can be proposed as a first-line investigation to test for Glut1DS without the constraints of a spinal tap, fasting or expertise to interpret metabolic changes related to age. Incidentally, but quite illustrative, this study led to diagnose a few patients with Glut1DS who, many years prior, had a LP displaying hypoglycorrhachia but that went unnoticed, therefore leading to major delays in diagnosis and treatment. The specificity of METAglut1™ for Glut1DS is also of great added value, unlike hypoglycorrhachia that can be also caused by hypoglycemia, meningitis, subarachnoid hemorrhage, or ventriculoperitoneal shunt systems. The high positive predictive value of METAglut1™ supports its use as a screening test for Glut1DS on patients, since a positive result is actionable by triggering early treatment and therefore likely to greatly improve prognosis. Furthermore, while next generation sequencing (NGS) methodologies keep expanding worldwide, only a few countries can offer NGS as first-line investigations for the diagnosis of rare diseases, mainly in pediatrics. At best, turnaround time is of several weeks to several months – as illustrated in our study with a large number of unavailable molecular analyses –, which is not desirable for epileptic children with Glut1DS. Moreover, the interpretation of SCL2A1 variants can be quite challenging, with frequent missense variants and a great proportion of VUS. In our study, METAglut1™ turned positive for 3 patients with SLC2A1 VUS. Likewise, METAglut1™ can provide critical information with regard to puzzling molecular analyses such as VUS or genetic mosaicism.

Glut1DS is an urgent diagnosis for patients, not to miss critical time where early treatments can be initiated to support brain development and function. To this end, we suggest to perform METAglut1™ in any patient, after age 3 months, who presents with intellectual disability or mixed specific neurodevelopmental disorder, and/or epilepsy (especially drug resistant or ketogenic diet responsive), and/or deceleration of head growth, and/or permanent movement disorders (cerebellar ataxia, dystonia, or spasticity), and/or paroxysmal movement disorders (Figure 5). If performed at an early symptomatic stage, this simple test will identify right away 80% of patients with Glut1DS among those with developmental and epileptic encephalopathy, intellectual disabilities or movement disorders. The high positive predictive value (90%) of METAglut1™ is of paramount importance for the diagnosis of Glut1DS and the initiation of dedicated treatments as soon as possible. Moreover, the good negative predictive value of the test (>95%) can be sufficient to rule out Glut1DS in most cases. However, in case of a negative result but a strong clinical suspicion, measurement of glycorrhachia and/or SLC2A1 molecular analyses are warranted to further explore the possibility of Glut1DS (Figure
5). Given the current estimated prevalence of Glut1DS, the availability of a simple blood test is a major milestone for patients with Glut1DS as their diagnosis and treatment will occur much faster.

References


epilepsy and absence epilepsy, and the estimated frequency of GLUT1 deficiency syndrome. *Epilepsia* 2015; 56(12): e203–208. DOI: 10.1111/epi.13222


**Figure legends**

**Figure 1. Patient groups used for the calculation of diagnostic performance**

We enrolled 549 patients in the prospective cohort and 87 patients in the retrospective cohort. Within the prospective cohort, 121 participants had to be excluded from the analyses, mainly because METAglut1™ was the only available test for these patients (n = 116), with neither available glycorrhachia (lumbar puncture refused by the patients or their caregivers), nor molecular analyses, or because the final diagnosis remained uncertain (n = 5). After excluding mainly patients for whom molecular analyses were not available (n = 218), as well as a few familial cases (n = 5), 205 index patients were used to compute the sensitivity, specificity, positive and negative predictive values of METAglut1™. These performance criteria were also computed on 350 prospective patients with available glycorrhachia, after excluding 5 familial cases and 10 patients for whom blinding was not maintained – e.g., patients for whom glycorrhachia was performed as a confirmatory test following METAglut1™ or molecular analyses. Concordance analyses between METAglut1™ and glycorrhachia were performed on this same subgroup of patients.

Within the retrospective cohort, 10 patients were excluded due to previous enrollment in the initial cohort (n = 5), inconclusive or missing data (n = 1), or uncertain Glut1DS diagnosis (n = 4). From the 77 remaining patients, 60 index patients with Glut1DS were used for sensitivity analysis, after excluding 7 familial cases and revised Glut1DS diagnosis (n = 10). The 54 index patients for whom glycorrhachia was available from the retrospective cohort were used for concordance analyses between METAglut1™ and glycorrhachia, after merging them with the prospective cohort of 350 index patients.

PPV: positive predictive value, NPV: negative predictive value.
Figure 2. Identification of a case of genetic mosaicism with METAglut1™

A 12-year-old child presented with a moderate phenotype (myoclonic epilepsy, attention and executive function deficit), and a glycorrhachia of 2.6 mM (47 mg/dl). His red blood cells analyzed with METAglut1™ showed an unusual feature with 2 distinct red blood cell populations. One population representing 36% of the patient red blood cells had a normal expression of GLUT1, while the other representing 64% of red blood cells had a distinct lower level of GLUT1 (53% of the controls). Parents of the child were healthy, with normal GLUT1 levels on red blood cells. Upon deeper sequencing analysis, it was confirmed that 36% of SLC2A1 copies in the patient’s blood DNA and 22% in his oral swab DNA harbored a de novo premature stop codon in the SCL2A1 gene (pArg330†).
Figure 3. Glucose uptake assay

Glucose uptake assay by red blood cells was performed on METAglut1™ false negative patients. The glucose uptake assay interpretation threshold was set at 74% of controls. In 12 index patients with Glut1DS, 4 turned out to have an abnormally low glucose uptake, whereas 5 displayed a normal glucose uptake and 3 an abnormally high glucose uptake.

Figure 4. Concordance analysis between glycorrhachia and METAglut1™

All patients with both glycorrhachia and METAglut1™ available at the completion of the study were used to draw the comparative distribution of the two biomarkers. The recommended interpretation thresholds are represented with dashed lines at 2.2 mM (40 mg/dl) for glycorrhachia, and 76% of normal expression for METAglut1™. The Cohen’s kappa coefficient was computed on the prospective cohort (n = 350 patients), as well as the whole cohort – 350 prospective patients and 48 retrospective patients –, for whom both glycorrhachia and METAglut1™ were available.
Figure 5. Proposed new diagnostic algorithm for Glut1DS diagnosis in standard of care

Positive and negative predictive values of METAglut™ are those obtained from the prospective cohort, with all patients with confirmed Glut1DS, whether they had glycorrhachia available or not.

Glut1DS: Glut1 deficiency syndrome; DEE: developmental and epileptic encephalopathy; Se: sensitivity; Spe: Specificity; PPV: positive predictive value; NPV: negative predictive value; CSF: cerebrospinal fluid.
Table 1: Clinical characteristics of all included study participants

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>Prospective cohort</th>
<th>Retrospective cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 549</td>
<td>n = 87</td>
</tr>
<tr>
<td>Sex, Female, n (%)</td>
<td>251 (46%)</td>
<td>43 (49%)</td>
</tr>
<tr>
<td>Age at inclusion in years, mean (SD)</td>
<td>11.6 (13.1)</td>
<td>13.2 (13.4)</td>
</tr>
<tr>
<td>Age at inclusion in classes, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3-24] months</td>
<td>76 (14%)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>[2-18] years</td>
<td>382 (69%)</td>
<td>56 (64%)</td>
</tr>
<tr>
<td>&gt;= 18 years</td>
<td>91 (17%)</td>
<td>28 (32%)</td>
</tr>
<tr>
<td>Inclusion criteria (prospective cohort)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classical form, n (%)</td>
<td>156 (28%)</td>
<td>-</td>
</tr>
<tr>
<td>Encephalopathy with drug-resistant epilepsy and microcephaly</td>
<td>18 (12%)</td>
<td>-</td>
</tr>
<tr>
<td>Early Onset Absence Epilepsy (EOAE) characterized by EEG</td>
<td>136 (87%)</td>
<td>-</td>
</tr>
<tr>
<td>Generalized epilepsy with a personal or family history of PED</td>
<td>2 (1%)</td>
<td>-</td>
</tr>
<tr>
<td>Atypical form, n (%)</td>
<td>393 (72%)</td>
<td>-</td>
</tr>
<tr>
<td>Epilepsy (CAE, JAE, Intellectual disability with history of epilepsy drug-resistant or not)</td>
<td>129 (33%)</td>
<td>-</td>
</tr>
<tr>
<td>Abnormal movement (permanent or paroxysmal)</td>
<td>129 (33%)</td>
<td>-</td>
</tr>
<tr>
<td>Epilepsy associated with abnormal movement</td>
<td>11 (3%)</td>
<td>-</td>
</tr>
<tr>
<td>Other associated atypical forms</td>
<td>124 (31%)</td>
<td>-</td>
</tr>
<tr>
<td>Inclusion criteria (retrospective cohort)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with confirmed Glut1DS diagnosis at inclusion</td>
<td>-</td>
<td>74 (85%)</td>
</tr>
<tr>
<td>Patients with pending diagnosis at inclusion (inconsistent biological or genetic data)</td>
<td>-</td>
<td>13 (15%)</td>
</tr>
<tr>
<td>Neurological symptoms at inclusion (prospective) or at diagnosis (retrospective)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epilepsy, n (%)</td>
<td>352 (64%)</td>
<td>29 (33%)</td>
</tr>
<tr>
<td>Atypical absences</td>
<td>124 (23%)</td>
<td>7 (8%)</td>
</tr>
<tr>
<td>Partial epilepsy (focal)</td>
<td>26 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>Myoclonic epilepsy</td>
<td>23 (4%)</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>Tonic / generalized clonic epilepsy</td>
<td>46 (8%)</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>Febrile epilepsy</td>
<td>8 (1%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Atonic epilepsy</td>
<td>4 (1%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>Several types of associated epilepsy</td>
<td>120 (22%)</td>
<td>12 (14%)</td>
</tr>
<tr>
<td>Not reported</td>
<td>1 (&lt;1%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Paroxysmal movements, n (%)</td>
<td>83 (15%)</td>
<td>17 (20%)</td>
</tr>
<tr>
<td>Dyskinesia</td>
<td>42 (7%)</td>
<td>8 (9%)</td>
</tr>
<tr>
<td>Episodic ataxia</td>
<td>21 (4%)</td>
<td>7 (8%)</td>
</tr>
<tr>
<td>Abnormal eye movements</td>
<td>16 (3%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>Not reported</td>
<td>4 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>Epilepsy with paroxysmal movements, n (%)</td>
<td>47 (9%)</td>
<td>28 (32%)</td>
</tr>
<tr>
<td>Not reported</td>
<td>67 (12%)</td>
<td>13 (15%)</td>
</tr>
</tbody>
</table>
N= number; SD= standard deviation; EOAE= Early Onset Absence Epilepsy; PED= paroxysmal exercise-induced dyskinesia; CAE= Childhood Absence Epilepsy; JAE= Juvenile Absence Epilepsy.
### Table 2: Clinical and biological description of Glut1DS patients non detected by METAglut1™

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Cohort</th>
<th>Age at inclusion (y)</th>
<th>Sex</th>
<th>CSF glucose mM*</th>
<th>Glut1 expression %</th>
<th>Glucose uptake %</th>
<th>SLC2A1 variant# Class</th>
<th>Age at onset and 1st symptom</th>
<th>Epilepsy</th>
<th>Permanent motor disorder</th>
<th>Paroxysmal movement disorder</th>
<th>Cognitive impairment</th>
</tr>
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<tbody>
<tr>
<td>01</td>
<td>R</td>
<td>10.8</td>
<td>F</td>
<td>1.8 (32)</td>
<td>91</td>
<td>164</td>
<td>c.94G&gt;C; p.(Val32Leu)</td>
<td>0.5 y Epilepsy</td>
<td>Generalized tonic-clonic epilepsy</td>
<td>Ataxia</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>02 Family A index case</td>
<td>P</td>
<td>18.2</td>
<td>F</td>
<td>NA</td>
<td>99</td>
<td>67</td>
<td>c.101A&gt;G; p.(Asn34Ser)</td>
<td>3 y Epilepsy</td>
<td>Generalized tonic-clonic epilepsy</td>
<td>No</td>
<td>No</td>
<td>Moderate ID</td>
</tr>
<tr>
<td>03 Family A</td>
<td>P</td>
<td>2.7</td>
<td>M</td>
<td>NA</td>
<td>98</td>
<td>66</td>
<td>c.101A&gt;G; p.(Asn34Ser)</td>
<td>1 y Epilepsy</td>
<td>EAOE, generalized tonic-clonic epilepsy</td>
<td>No</td>
<td>No</td>
<td>Mild ID</td>
</tr>
<tr>
<td>04 Family A</td>
<td>P</td>
<td>6.9</td>
<td>M</td>
<td>NA</td>
<td>101</td>
<td>70</td>
<td>c.101A&gt;G; p.(Asn34Ser)</td>
<td>5 y Epilepsy</td>
<td>Atomic seizures</td>
<td>No</td>
<td>No</td>
<td>Mild ID</td>
</tr>
<tr>
<td>05</td>
<td>R</td>
<td>7.6</td>
<td>M</td>
<td>1.6 (29)</td>
<td>NA</td>
<td>96</td>
<td>c.102T&gt;G; p.(Asn34Lys)</td>
<td>0.3 y Epilepsy</td>
<td>EAOE, focal epilepsy</td>
<td>No</td>
<td>No</td>
<td>Coordination and attention deficit</td>
</tr>
<tr>
<td>06</td>
<td>P</td>
<td>10.4</td>
<td>M</td>
<td>1.9 (34)</td>
<td>92</td>
<td>105</td>
<td>c.1300T&gt;G; p.(Phe434Val)</td>
<td>2 y Epilepsy</td>
<td>EAOE</td>
<td>No</td>
<td>No</td>
<td>Mild ID</td>
</tr>
<tr>
<td>07</td>
<td>R</td>
<td>16.7</td>
<td>M</td>
<td>1.9 (34)</td>
<td>113</td>
<td>112</td>
<td>c.193T&gt;C; p.(Trp65Arg)</td>
<td>0.5 y Ocular movement</td>
<td>No</td>
<td>Dystonia</td>
<td>Yes</td>
<td>Mild ID</td>
</tr>
<tr>
<td>08</td>
<td>P</td>
<td>56.1</td>
<td>F</td>
<td>2.1 (38)</td>
<td>107</td>
<td>100</td>
<td>c.377G&gt;A; p.(Arg126His)</td>
<td>4 y Psychomotor delay</td>
<td>No</td>
<td>Dystonia, spasticity</td>
<td>No</td>
<td>Mild ID</td>
</tr>
<tr>
<td>09</td>
<td>R</td>
<td>21.2</td>
<td>M</td>
<td>1.8 (32)</td>
<td>91</td>
<td>52</td>
<td>c.376C&gt;T; p.(Arg126Cys)</td>
<td>2 y Epilepsy</td>
<td>EAOE</td>
<td>Spasticity</td>
<td>Yes</td>
<td>Coordination, attention and executive function deficit</td>
</tr>
<tr>
<td>10</td>
<td>R</td>
<td>17.6</td>
<td>F</td>
<td>2.1 (38)</td>
<td>89</td>
<td>77</td>
<td>c.376C&gt;T; p.(Arg126Cys)</td>
<td>0.5 y Ocular movement</td>
<td>Focal epilepsy</td>
<td>Spasticity (mild)</td>
<td>Yes</td>
<td>Coordination, attention and executive function deficit</td>
</tr>
<tr>
<td>11</td>
<td>R</td>
<td>39.4</td>
<td>M</td>
<td>NA</td>
<td>99</td>
<td>65</td>
<td>c.499G&gt;A; p.(Gly167Ser)</td>
<td>5 y Malaises</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Attention and executive function deficit</td>
</tr>
</tbody>
</table>

*CSF: cerebrospinal fluid. Glut1 expression and Glucose uptake are measured in percentage. SLC2A1 variant is a coding genetic variant associated with Glut1DS. Class 4 and 5 are different levels of severity. Coordination, attention and executive function deficit,永 idiocy, No Dystonia, mild spasticity. EAOE: epilepsy of unknown etiology.
<table>
<thead>
<tr>
<th></th>
<th>P#</th>
<th>R</th>
<th>2.5</th>
<th>M</th>
<th>Class</th>
<th>5 y</th>
<th>Atypic absences</th>
<th>Attention and executive function deficit</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>R</td>
<td>12.3</td>
<td>M</td>
<td>NA</td>
<td>79</td>
<td>c.493G&gt;A; p.(Val165Ile)</td>
<td>2.5 y Paroxysmal movement disorder</td>
<td>No</td>
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<td>Family B</td>
<td>P</td>
<td>39.9</td>
<td>F</td>
<td>NA</td>
<td>99</td>
<td>c.539T&gt;A; p.(Met180Lys)</td>
<td>No</td>
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<tr>
<td>14</td>
<td>Family B index case</td>
<td>P</td>
<td>32.8</td>
<td>F</td>
<td>2.4 (43)</td>
<td>99</td>
<td>c.539T&gt;A; p.(Met180Lys)</td>
<td>No</td>
</tr>
<tr>
<td>15</td>
<td>Family B</td>
<td>P</td>
<td>67.8</td>
<td>M</td>
<td>NA</td>
<td>106</td>
<td>c.539T&gt;A; p.(Met180Lys)</td>
<td>2 y</td>
</tr>
<tr>
<td>16</td>
<td>P</td>
<td>4.8</td>
<td>M</td>
<td>1.9 (34)</td>
<td>90</td>
<td>c.884C&gt;T ;p.(Thr295Met)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>Family C index case</td>
<td>R</td>
<td>17.3</td>
<td>M</td>
<td>NA</td>
<td>110</td>
<td>c.940G&gt;A; p.(Gly314Ser)</td>
<td>6 y</td>
</tr>
<tr>
<td>18</td>
<td>Family C</td>
<td>R</td>
<td>38.6</td>
<td>M</td>
<td>NA</td>
<td>121</td>
<td>c.940G&gt;A; p.(Gly314Ser)</td>
<td>4 y</td>
</tr>
<tr>
<td>19</td>
<td>P</td>
<td>11.9</td>
<td>F</td>
<td>2.3 (41)</td>
<td>91</td>
<td>c.955G&gt;C; p.(Ala319Pro)</td>
<td>3 y</td>
<td>Malaise</td>
</tr>
<tr>
<td>20</td>
<td>R</td>
<td>3.7</td>
<td>F</td>
<td>1.3 (23)</td>
<td>117</td>
<td>c.929C&gt;T; p.(Thr310Ile)</td>
<td>3 y</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>21</td>
<td>R</td>
<td>3.4</td>
<td>M</td>
<td>1.9 (34)</td>
<td>87</td>
<td>c.376C&gt;T; p.(Arg126Cys)</td>
<td>0.5 y</td>
<td>Malaise</td>
</tr>
</tbody>
</table>

P= prospective; R= retrospective; F= female; M= male; y= year; EAOE= early absence onset epilepsy; ID= intellectual disability; NA= non available; in bold, abnormal values; in italics, abnormally high glucose uptake. * In (), values of CSF glucose in mg/dl. # Previously reported variants: 1–8


Prospective Multicenter Validation of a Simple Blood Test for the Diagnosis of Glut1 Deficiency Syndrome

Fanny Mochel, Domitille Gras, Marie-Pierre Luton, et al.

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