Glut1 Deficiency Syndrome and Erythrocyte Glucose Uptake Assay

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Objective: The Glut1 deficiency syndrome (Glut1 DS) phenotype has expanded dramatically since first described in 1991. Hypoglycorrhachia and decreased erythrocyte 3-OMG uptake are confirmatory laboratory biomarkers. The objective is to expand previous observations regarding the diagnostic value of the uptake assay.

Methods: One hundred and nine suspected cases of Glut-1 DS were studied. All cases had a consistent clinical picture and hypoglycorrhachia. The uptake assay was decreased in 74 cases (group 1) and normal in 35 cases (group 2). We identified disease-causing mutations in 70 group 1 patients (95%) and one group 2 patient (3%).

Results: The cut-off for an abnormally low uptake value was increased from 60% to 74% with a corresponding sensitivity of 99% and specificity of 100%. The correlation between the uptake values for the time-curve and the kinetic concentration curve were strongly positive ($R^2 = 0.85$). Significant group differences were found in CSF glucose and lactate values, tone abnormalities, and degree of microcephaly. Group 2 patients were less affected in all domains. We also noted a significant correlation between the mean erythrocyte 3-OMG uptake and clinical severity ($R^2 = 0.94$).

Interpretation: These findings validate the erythrocyte glucose uptake assay as a confirmatory functional test for Glut1 DS and as a surrogate marker for GLUT1 haploinsufficiency.

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D-glucose, the essential fuel for brain metabolism, needs to traverse the luminal and abluminal membranes of the endothelial cell before entering the brain extracellular compartment. Glut1 is the key protein facilitating this entry. It also is abundant in primate erythrocytes and is immunochemically identical to Glut1 in brain microvessels.1,2

Glut1 deficiency syndrome (Glut1 DS, OMIM 606777, #138140) was first described in 1991 by De Vivo and colleagues.3 It is characterized classically by infantile onset seizures, delayed neurological development, disorders of movement, and acquired microcephaly. The laboratory signatures of this disorder are hypoglycorrhachia and decreased uptake of glucose by the patient’s freshly isolated erythrocytes.5 Since the original description, the classical phenotype has expanded to include paroxysmal nonepileptic allelic variants like intermittent ataxia, choreoathetosis, dystonia, and alternating hemiplegia with or without epilepsy.4–8 The spectrum of clinical severity also has expanded. Some patients have minimal clinical findings and appear relatively normal between paroxysmal episodes. Others are severely disabled neurologically with fixed deficits, often never learning to walk or talk.

The distinctive biomarker for Glut1 DS is a low cerebrospinal fluid (CSF) glucose concentration—hypoglycorrhachia. At the beginning, we set the CSF glucose concentration at 40mg/dl (or 2.2mM) as the cutoff value for suspected Glut1 DS cases. However, with the increasing recognition of milder allelic variants, higher CSF glucose values of 41 to 52mg/dl are now being described.6–9 In our experience, the CSF glucose values in 150 cases of Glut1 DS always have been less than 60mg/dl, and the vast majority (>90%) of values have been less than 40mg/dl (De Vivo and colleagues9; unpublished observations). These observations also indicate that the normal range for CSF glucose has never been defined properly.
Furthermore, although necessary, it remains uncertain whether hypoglycorrachia always implies Glut1 DS when present in the appropriate clinical setting.

The molecular basis of GLUT1 haploinsufficiency is heterozygous microdeletions and missense, nonsense, insertion, deletion, and splice site mutations. Recently, we also described probands in 2 unrelated families who transmitted Glut1 DS as an autosomal recessive trait. These observations indicate that the pattern of inheritance is determined by the degree of GLUT1 insufficiency from the allele.

Based on the fact that erythrocytes express the same Glut1 as endothelial cells, we originally reported an erythrocyte 3-O-methyl-D-Glucose (3-OMG) uptake assay to confirm the clinical diagnosis of Glut1 DS. Later, we reported our experience with this diagnostic assay in 22 Glut1 DS patients. In this earlier report, we accepted the clinical picture and hypoglycorrachia as the diagnostic gold standard. The data allowed us to define 60% as the cutoff point between Glut1 DS patients and controls with 86% sensitivity and 97% specificity. Initially, we viewed the assay as a qualitative tool. With more experience, however, we have demonstrated that the assay is semiquantitative and is a surrogate for GLUT1 haploinsufficiency.

We now report further refinements of the 3-OMG uptake assay including a confirmatory kinetic concentration curve assay. Based on the analysis of 109 suspected cases of Glut1 DS, we are able to define a higher cutoff value for the improved uptake assay with increased specificity. Also, we detected a significant correlation between the mean erythrocyte 3-OMG uptake value and the clinical severity score. These results demonstrate that the erythrocyte 3-OMG glucose uptake assay strongly correlates with the clinical diagnosis of Glut1 DS and appears to be the single most important accurate laboratory test to confirm the clinical diagnosis.

Patients and Methods

Human Subjects

The investigatory studies of blood were exempted from full review and approved by the Columbia University Institutional Review Board. Informed consent was obtained from the parents and patients who participated in this study.

Suspected Glut1 DS Patients

Since the original description of the condition in 1991, we have been asked by referring physicians to evaluate patients for the possibility of Glut1 DS. Typically, the clinical findings and the supporting laboratory data are reviewed before performing the erythrocyte glucose uptake assay and searching for a GLUT1 mutation. The following criteria have been used for the selection of clinically suspected Glut1 DS patients; all findings were not necessarily present in each case. All patients had early delays in neurological development and most (90%) had infantile-onset seizures. Their clinical presentation corresponds to the clinical phenotype we described originally, now referred to as the classical phenotype. The classical phenotype remains the common phenotype in our experience. There were no inconsistent clinical or laboratory findings in any case. For example, no patient had optic atrophy, pigmentary retinopathy, hearing loss, normal CSF glucose, or elevated CSF lactate.

2. Laboratory findings: normal blood glucose values (~70–110mg/dl), low CSF glucose values (<40mg/dl), normal or low CSF lactate values (<2.2mM).

Based on these clinical and laboratory criteria, 109 suspected Glut1 DS patients (58 females, 51 males; mean age 6 years (range from 2 months to 26 years) were selected for this study.

Grading of Patient Symptoms

Patient symptoms were given a severity score (0 = none, 1 = mild, 2 = moderate, 3 = severe) for the following subcategories: seizures, microcephaly, and tone abnormalities. Other neurological symptoms were age-dependent, such as language, coordination, and ataxia, and were not graded according to the patient’s age. Monthly seizure episodes were categorized as “mild,” weekly episodes as “moderate,” and daily/several times a week as “severe.” Microcephaly was scored as mild (<1 standard deviation [SD] below the mean), moderate (<2 SD below the mean), and severe (<3 SD below the mean). Tone abnormalities were scored as mild for 3+ tendon reflexes without ankle clonus; moderate for 4+ tendon reflexes with clonus and Babinski signs; or severe if the patient also had difficulty/inability to walk.

Columbia Neurological Score

The Columbia Neurological Score (CNS) is a semiquantitative tool developed previously by our research team to summarize the clinical evaluations of patients with neurological impairment. This tool assesses the following 12 domains: (1) height, weight, and head circumference; (2) general medical exam; (3) fundoscopic exam; (4) cranial nerves; (5) stance and gait; (6) involuntary movements; (7) sensation; (8) cerebellar function; (9) muscle bulk, tone, and strength; (10) tendon reflexes, (11) Babinski signs; and (12) other findings. Results of these domains are scored as normal or abnormal and summarized in the CNS, ranging from 0 to 76, with 76 being perfect. CNS scores of 40 to 49 indicate severe impairment, 50 to 59 are moderate, 60 to 69 are mild, and 70 to 76 are minimal. We have previously shown that the instrument has good interrater reliability and correlates with other measures of disease severity.

Time Curve of the Erythrocyte 3-OMG Uptake Assay

To confirm the diagnosis of Glut1 DS, blood samples from both patients and parents were analyzed using the erythrocyte 3-OMG uptake assay. The blood sample collection, shipment,
storage, reagents, solutions, and time-curve uptake assay were performed as previously described. The time-curve uptake assay was done under zero-trans influx conditions. All assays were performed by the same person (H.Y.). Data in the time-curve were expressed as the natural logarithm of the ratio of intracellular radioactivity at time T and at equilibrium vs time (seconds). The slope of the curve represented 3-OMG uptake. Uptake was expressed as a percentage of the control (where the mean slope value of the controls corresponded to 100%).

**Kinetic Concentration Curve of the Erythrocyte 3-OMG Uptake Assay**
A total of 100μl of prepared erythrocytes was aliquotitted into 1.5ml microcentrifuge tubes from the patient and from the parents, respectively, for the following 3-OMG concentrations: 0.6, 1.0, 1.3, 2.0, 4.0, 6.0, and 10.0mM. Assays were run in triplicate at each concentration. Each tube was mixed with 100μl of 14C-labeled 3-OMG solution for 15 seconds. Uptake was terminated by adding 1.0ml cold stop solution. A background value was obtained by adding stop solution followed by the addition of 14C-labeled 3-OMG solution. Red blood cell (RBC) pellets were then washed and digested, and the data captured as described in the time-curve experiment. The kinetics were calculated by the GraphPad Prism4.

**Creation of New Cutoff Value**
We included 109 suspected patients for the uptake assay, which provided us with sufficient power to reevaluate the previously determined cutoff value of 60% with 22 patients. We plotted the sensitivity (y-axis) against the false positive rate (1 − specificity; x-axis), using the area under the curve (AUC) as a summary measure to facilitate classification. The new cutoff point was determined by maximizing the sum of the sensitivity and specificity of the system. This was visualized graphically in a receiver operating characteristic (ROC) curve. All analyses were performed with the use of SAS software, version 9.2 (SAS Institute, Cary, NC). A 2-sided p value of less than 0.05 was considered to indicate statistical significance.

**Mutation Analysis of the GLUT1 Gene**
Direct sequencing of amplified polymerase chain reaction products from 10 exons, intron-exon boundaries, and the promoter region of the GLUT1 gene was used to screen all 109 patients for mutations as described previously.

**Single Nucleotide Polymorphism Oligonucleotide Microarray Analysis**
Copy number analysis was performed via single nucleotide polymorphism (SNP) oligonucleotide microarray analysis (SOMA) using the Affymetrix Genome Wide Human SNP Array 6.0 (Santa Clara, CA, USA), which includes over 906,600 SNPs and more than 946,000 probes for the detection of copy number variation as previously described.

**Multiplex Ligation-Dependent Probe Amplification**
A multiplex ligation-dependent probe amplification (MLPA) kit (MRC-Holland, Amsterdam, Netherlands) was used to screen 8 patients with decreased 3-OMG uptake without identified sequence based mutations or deletions using SOMA according to the manufacturer’s instructions.

**Statistical Analysis**
CSF and blood glucose, CSF/blood glucose ratio, CSF lactate, and Km (the concentration of substrate that allows the reaction to proceed at half maximal velocity) values were calculated using the Welch Two Sample t test (p < 0.05). Seizure onset and Vmax (maximal velocity of the reaction) analysis utilized the Wilcoxon rank sum test with continuity correction. Seizure severity, tone abnormalities, microcephaly, and mutation type were analyzed using Fisher’s exact test for count data. Statistical analysis was performed with Excel/SPSS, and significance was determined if the corresponding p value was less than 0.05.

Logistic models were used to determine whether the clinical diagnosis could be predicted by the clinical biomarkers, including CSF and blood glucose values, CSF/blood glucose ratio, Vmax, Km, seizure onset or severity, and age at first visit. The model was adjusted for gender, age, and seizure onset.

Certain factors were interrogated using logistic models to determine whether these factors had predictive diagnostic value. The factors included CSF and blood glucose, CSF/blood glucose ratio, Vmax, Km, seizure onset, seizure severity, and age of first visit. The model was adjusted for gender, age, and seizure onset.

**Results**

**Improved 3-OMG Uptake Assay with Higher Specificity**
The clinically suspected 109 Glut1 DS patients and their parents (controls) were stratified according to the 3-OMG uptake time-curve assay. A total of 74 patients were found to have decreased 3-OMG uptake (group 1) and 35 patients had normal 3-OMG uptake (group 2) compared to intraassay controls. The mean uptake for group 1 was 54.2 ± 8.9% (mean ± SD, n = 74) with a median of 55% (range, 37–72%). The mean uptake for group 2 was 107.9 ± 19.1% (mean ± SD, n = 35) with a median of 104% (range, 74–175%). These glucose uptake differences are shown in Figure 1A, B, in which the slope values for suspected Glut1 DS patients are compared to parental control slope values. The difference in uptake assay values between the 2 groups is highly significant (p < 0.001).

We added a kinetic concentration curve for the 3-OMG uptake assay to verify and corroborate the time curve uptake assay (Fig 2A, B). The difference was highly significant (p < 0.005) between the Vmax values for groups 1 and 2 (54.7 ± 9.2% [mean ± SD, n = 74], and 104.2 ± 25.7% [mean ± SD, n = 35], respectively). There were no significant differences between the time-curve uptake and the kinetic concentration curve.
values in group 1 ($p = 0.75$) or group 2 ($p = 0.49$). The correlation between the time-curve uptake values and the $V_{\text{max}}$ values in the 109 patients was strongly positive (correlation coefficient $= 0.85$) (Fig 3). In contrast, we noted no difference between the group 1 mean Km values of $104.3 \pm 28.4\%$ (mean $\pm$ SD, $n = 74$) and the group 2 mean Km values of $102.9 \pm 23.5\%$ (mean $\pm$ SD, $n = 35$).

**ROC Curve Establishes New Cutoff Point**

The ROC curve with the larger patient population established a higher glucose uptake assay cutoff point of 74% (Fig 4) compared to the previously determined cutoff value of 60%. A cutoff at 74% increased the specificity from 97% to 100%. The sensitivity increased from 86% to 99%. Using the new uptake cutoff value of 74%, an additional 17 patients were diagnosed as having Glut1 DS (see Fig 3; Table 1).

**Mutational Analysis of the GLUT1 Gene by Direct Sequencing, SOMA, and MLPA**

We identified a pathogenic heterozygous GLUT1 mutation in 70 of 74 Group 1 patients (95%) including 29 missense, 5 nonsense, 18 oligonucleotide deletion, 5 intragenic large deletion (whole exons or introns), 4 complete gene deletion, 1 contiguous gene deletion, 2 insertion, and 6 splice site (Fig 5). We identified only 1 of 35 Group 2 patients (3%) with a heterozygous missense mutation. Based on the erythrocyte glucose uptake assay and the GLUT1 mutation analysis data, the suspected Glut1 DS patient population could be subdivided into 4 subgroups: decreased uptake with a disease-causing mutation (70 cases); decreased uptake without an identified pathogenic mutation (4 cases); normal uptake with a disease-causing mutation (1 case); and normal uptake without an identified pathogenic mutation (34 cases) (see Fig 5). Of the 34 patients in Group 2 with no mutation, we reanalyzed the
clinical information in an attempt to provide an alternative diagnosis. Ten patients had clinical symptoms suggestive of other disorders, 15 were thought to represent examples of transient hypoglycorrhachia of infancy, and 9 were unknown. We reclassified these 34 patients as “hypoglycorrhachia, not otherwise specified (NOS).”

All parents who served as controls were normal by glucose uptake assay, GLUT1 sequencing and SOMA. Mosaicism could not be totally excluded in the control group as the direct sequencing by polymerase chain reaction will not detect mutant DNA representing less than 10% of total DNA in lymphocytes.

### Comparison of Laboratory Values and Phenotypes Between the Low Uptake (Group 1) and Normal Uptake (Group 2) Groups

The blood glucose values between Groups 1 and 2 were indistinguishable ($p = 0.467$), but the CSF glucose values differed significantly ($p < 0.005$) with the Group 1 values being lower. When comparing the ratio of CSF-to-blood glucose levels, we found no significant difference between the 2 groups ($p = 0.267$) (Table 2). CSF lactate and Vmax also differed significantly ($p < 0.005$

### FIGURE 3: Correlation between the time-curve uptake values and the Vmax values

The unfilled circles represent patients with time-curve uptake values that are <60% of the control value. The filled triangles represent the patients with time-curve uptake values between 62% and 74% who are diagnosed with Glut1 DS based on the new cutoff value of 74%. The unfilled diamonds represent patients with uptake values that are >74%. The correlation between the time-curve uptake values and the Vmax values is significant ($R^2 = 0.85$).

### FIGURE 4: ROC curve to determine new cutoff point for Glut1 DS diagnosis

The optimal cutoff is determined by maximizing the sum of the sensitivity and the specificity. The resulting cutoff is 74%, which yields a sensitivity of 99% and specificity of 100%.

### TABLE 1: 3-O-MG Uptake Cutoff Value, Sensitivity, and Specificity

<table>
<thead>
<tr>
<th>Cutoff Values</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Area</th>
<th>$p$</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>81.3</td>
<td>100</td>
<td>0.907</td>
<td>0.000</td>
<td>0.851–0.962</td>
</tr>
<tr>
<td>66</td>
<td>84.0</td>
<td>100</td>
<td>0.920</td>
<td>0.000</td>
<td>0.868–0.972</td>
</tr>
<tr>
<td>67</td>
<td>88.0</td>
<td>100</td>
<td>0.940</td>
<td>0.000</td>
<td>0.895–0.985</td>
</tr>
<tr>
<td>68</td>
<td>89.3</td>
<td>100</td>
<td>0.947</td>
<td>0.000</td>
<td>0.904–0.989</td>
</tr>
<tr>
<td>70</td>
<td>94.7</td>
<td>100</td>
<td>0.973</td>
<td>0.000</td>
<td>0.936–1.000</td>
</tr>
<tr>
<td>71</td>
<td>96.0</td>
<td>100</td>
<td>0.980</td>
<td>0.000</td>
<td>0.900–1.000</td>
</tr>
<tr>
<td>72</td>
<td>97.3</td>
<td>100</td>
<td>0.987</td>
<td>0.000</td>
<td>0.900–1.000</td>
</tr>
<tr>
<td>74</td>
<td><strong>98.7</strong></td>
<td><strong>100</strong></td>
<td><strong>0.993</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000–1.000</strong></td>
</tr>
<tr>
<td>77</td>
<td>98.7</td>
<td>97</td>
<td>0.989</td>
<td>0.000</td>
<td>0.000–1.000</td>
</tr>
<tr>
<td>80</td>
<td>98.7</td>
<td>97.1</td>
<td>0.979</td>
<td>0.000</td>
<td>0.000–1.000</td>
</tr>
</tbody>
</table>

The bold row indicates the selected cutoff value (74%) which gives the best sensitivity and specificity.

3-O-MG = 3-O-methyl-D-glucose; CI = confidence interval.
and $p < 0.0005$) between the 2 groups, with the Group 1 values being lower. The $K_m$ values did not differ significantly between the 2 groups.

Significant differences between Groups 1 and 2 were found in the clinical domains of tone abnormalities and microcephaly but not in seizure activity (Table 3). Hypotonia was more prevalent in Group 1 (76% vs 41%). Severity also was worse (1.5 ± 1.1 vs 0.77 ± 1.1). Microcephaly was more prevalent (55% vs 25%), and more severe (1.0 ± 1.1 vs 0.38 ± 0.7) in Group 1. Approximately 80% of patients in both groups had seizure activity. While not significant, the trend in seizure severity was less in Group 1. This trend likely was due to a subset of Group 1 patients who were successfully treated with the ketogenic diet at the time of assessment.

When evaluating gender influence in Group 1 patients, females were found to have significantly lower uptake values of 49% ± 8 ($p < 0.001$) and lower $V_{\text{max}}$ values of 52% ± 9 ($p < 0.05$). All other measurements were similar statistically (data not shown). This gender effect also was evident when analyzing the ratio of female to male uptake curves in 88 available control parent sets (60 parents from Group 1, 28 parents from Group 2). The female to male ratio was 95.6% ± 16.8 (95% CI, 92.08–99.12%). The $V_{\text{max}}$ data analysis mirrored these findings supporting the conclusion that glucose transport also is modulated by other factors that are gender-specific.

The time-curve uptake ($p < 0.001$) and $V_{\text{max}}$ value ($p = 0.002$) were the only measures that reliably identified the Glut1 DS patients from the rest of the study population (Table 4). Therefore, these measures permitted a diagnosis of Glut1 DS with great specificity.

**Correlation Significant Between Uptake Assay and Clinical Severity**

Fifty-three patients in Group 1 were stratified clinically according to the CNS: minimal group (CNS 70–76), mild group (CNS 60–69), moderate group (CNS 50–59), and severe group (CNS 40–49).

<table>
<thead>
<tr>
<th>Laboratory Value</th>
<th>Group 1 (mean ± SD)</th>
<th>Group 2 (mean ± SD)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF glucose (mg/dl)</td>
<td>32.31 ± 4.10 (n = 74)</td>
<td>35.53 ± 5.83 (n = 35)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>90.99 ± 18.57 (n = 70)</td>
<td>94.55 ± 17.66 (n = 31)</td>
<td>0.362</td>
</tr>
<tr>
<td>CSF/blood glucose ratio</td>
<td>0.37 ± 0.08 (n = 70)</td>
<td>0.38 ± 0.07 (n = 29)</td>
<td>0.435</td>
</tr>
<tr>
<td>CSF lactate (mM)</td>
<td>0.95 ± 0.20 (n = 54)</td>
<td>1.31 ± 0.71 (n = 25)</td>
<td>0.02</td>
</tr>
<tr>
<td>$K_m$</td>
<td>104.27% ± 28.38% (n = 74)</td>
<td>102.9% ± 23.48% (n = 34)</td>
<td>0.795</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>54.7% ± 9.24% (n = 74)</td>
<td>104.2% ± 25.67% (n = 34)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time-curve uptake</td>
<td>54.2% ± 8.94% (n = 74)</td>
<td>107.9% ± 19.08% (n = 35)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*As percentage of control value.

CSF = cerebrospinal fluid; $K_m$ = the concentration of substrate that allows the reaction to proceed at half maximal velocity; SD = standard deviation; $V_{\text{max}}$ = maximal velocity of the reaction.
50–59), and severe group (CNS 40–49). When comparing the nature of various GLUT1 heterozygous mutations across the 4 groups (Table 5), missense mutations were predominantly found in the mild to moderate clinical categories, whereas insertion, deletion, splice site, nonsense, exon, and unknown mutations were found almost exclusively in the moderate and severe clinical categories. Complete gene deletions were found predominantly in the severe clinical category. There was a significant inverse correlation between the median values of the time-curve uptake and the clinical severity of Glut1 DS ($R^2 = 0.94$; Fig 6). The $R^2$ for $V_{max}$ curve and clinical severity was 0.76 (data not shown).

**Increased Sensitivity and Specificity of the 3-OMG Erythrocyte Uptake Assay**

The sensitivity and specificity for the glucose uptake assay were 99% and 100%, respectively. It was highly specific and proved to be an excellent laboratory test to confirm the clinical diagnosis of Glut-1 DS.

**Discussion**

Glut1 DS was first described in 1991 by De Vivo and colleagues. Since then, about 400 patients have been diagnosed world-wide. However, many patients remain undiagnosed due to lack of awareness of the syndrome or reluctance to perform a diagnostic lumbar puncture. But, hypoglycorrhachia alone is not confirmatory of Glut1 DS. Sixty-nine percent of the 109 cases had a low erythrocyte glucose uptake assay, a disease-causing GLUT1 mutation or both essentially confirming the clinical diagnosis. However, 31% of cases with low CSF glucose had normal uptake assays and negative mutational analysis, essentially ruling out the clinical suspicion of Glut1 DS. We reclassified this group of patients as “hypoglycorrhachia, NOS.” The 15 patients we reclassified as probable cases of transient hypoglycorrhachia of infancy were contacted by telephone to determine their neurological outcome. All, in fact, were

### TABLE 3: Comparison of Seizure Activity, Tone Abnormalities, and Microcephaly Between Group 1 (low uptake) and Group 2 (normal uptake)

<table>
<thead>
<tr>
<th>Category</th>
<th>Patient Group</th>
<th>n</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean (SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tone abnormalities</td>
<td>1</td>
<td>68</td>
<td>16</td>
<td>19</td>
<td>15</td>
<td>18</td>
<td>1.5 ± 1.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22</td>
<td>13</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>0.77 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Degree of microcephaly</td>
<td>1</td>
<td>58</td>
<td>26</td>
<td>14</td>
<td>10</td>
<td>8</td>
<td>1.0 ± 1.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24</td>
<td>18</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0.38 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Seizure activity</td>
<td>1</td>
<td>67</td>
<td>13</td>
<td>19</td>
<td>21</td>
<td>14</td>
<td>1.5 ± 1.0</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>12</td>
<td>2.0 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

Clinical severity ranged from 0 (normal) to 3 (severe). Group 1 patients were more significantly affected in the categories of tone abnormalities and microcephaly. Seizure activity was influenced by the use of the ketogenic diet and the group differences did not achieve significance. SD = standard deviation.

### TABLE 4: Biomarkers that Discriminated Between Group 1 and Group 2 Patients

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF glucose</td>
<td>0.937</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>0.804</td>
</tr>
<tr>
<td>CSF/blood glucose ratio</td>
<td>0.683</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>0.002</td>
</tr>
<tr>
<td>$K_m$</td>
<td>0.108</td>
</tr>
<tr>
<td>Time-curve uptake</td>
<td>0.001</td>
</tr>
<tr>
<td>Seizure onset age</td>
<td>0.287</td>
</tr>
<tr>
<td>Seizure severity</td>
<td>0.243</td>
</tr>
<tr>
<td>Age of visit</td>
<td>0.371</td>
</tr>
</tbody>
</table>

The statistical models were adjusted for age, gender, and age of seizure onset. CSF = cerebrospinal fluid; $K_m$ = the concentration of substrate that allows the reaction to proceed at half maximal velocity; $V_{max}$ = maximal velocity of the reaction.
developing normally as one would expect with this benign diagnosis. The diagnoses in the other 19 cases remain obscure. The uptake assay is performed under zero-trans influx conditions and we have never seen a normal uptake assay in any patient who has a mutation affecting influx of glucose. We have studied only 3 patients (1 reported here and previously18) who have had pathogenic mutations and normal uptake. All 3 mutations were missense detected by GLUT1 gene sequencing and all 3 missense mutations only affected efflux (Wang and colleagues18; unpublished observations). One could argue that these 19 group 2 patients have a clinical condition that is similar to Glut1 DS caused by a different molecular mechanism; and we continue to search for such a possibility.

The laboratory hallmarks of Glut1 DS are normal blood glucose values and low CSF glucose values.17 Our study shows that the CSF glucose values are significantly lower in the Group 1 patients compared to the Group 2 patients (p < 0.005) (see Table 2). Also, the CSF lactate concentrations (0.95 ± 0.2mM) generally fell below the normal mean value of 1.63mM. A low CSF glucose concentration also can be found in other neurological conditions such as infectious meningitis, hypoglycemic states, subarachnoid hemorrhage, and meningeal carcinomatosis.19–23 These conditions can be readily ruled out in Glut1 DS patients. The CSF glucose value also may be lower than normal in mitochondrial diseases. However, the normal-to-low CSF lactate values, in addition to other clinical symptoms, differentiates Glut1 DS from mitochondrial diseases24 and the other clinical conditions listed above.

We have increased the cutoff value for the glucose uptake assay from 60% to 74% (see Fig 4) based on the ROC curve from 109 patients. The new uptake cutoff

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Minimal (70–76)</th>
<th>Mild (60–69)</th>
<th>Moderate (50–59)</th>
<th>Severe (40–49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense (n = 19)</td>
<td>1</td>
<td>9</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Insertion (n = 2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Splice site (n = 3)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Nonsense (n = 4)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Oligonucleotide deletion^a (n = 15)</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Intragenic large deletion^b (n = 3)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Complete gene deletion^c (n = 4)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Contiguous gene deletion^d (n = 1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Unknown (n = 2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

The trend indicates that the milder clinical phenotypes are associated with the less pathogenic mutations.

^aOligonucleotide deletions are ≤4bp.
^bIntragenic large deletions included whole exon or intron deletions.
^cDeletions range from 45 to 82kb.
^dDeletion size is 1.78Mb.
CNS = Columbia Neurological Score.
point of 74% provides a specificity of 100% and a sensitivity of 99%. The larger number of patients (109 patients) in this study compared to our previous report (22 patients) provides more statistical power and increases our confidence that the erythrocyte uptake assay is a valuable diagnostic test and has semiquantitative value as a surrogate measure of haploinsufficiency. A total of 74 patients had decreased 3-OMG uptake (54.2 ± 8.9%) and 35 patients had normal uptake (107.9 ± 19.1%) based on this new cutoff value (see Fig 5). We believe that the clinical and CSF findings and the decreased erythrocyte glucose uptake confirm the diagnosis of Glut1 DS (74 patients) even if a disease-causing mutation cannot be identified. We have been unable to detect any clinical or laboratory differences between the 70 patients with an identified pathogenic GLUT1 mutation and the 4 patients without an identifiable molecular defect. Further studies are continuing to identify pathogenic mutations in these 4 remaining patients. Initially, we found mutations in only 60 cases using gene sequencing techniques. Ten additional mutations were identified by MLPA or SOMA methods, leaving only the 4 cases with decreased uptake values. This complexity of the molecular diagnostic methods required speaks to the value of the functional uptake assay as a sensitive diagnostic screen.

The kinetic concentration assay has confirmed the time-curve uptake value, reinforcing the diagnosis of Glut1 DS. The Vmax value is significantly different between the Group 1 patients (54.7 ± 9.2%, n = 74) and the Group 2 patients (104.2 ± 25.7%, n = 35) (p < 0.0001) (see Figs 2 and 3). Both the time-curve uptake value (54.2% of control values) and Vmax (54.7% of control values) in Glut1 DS patients are significantly different when compared to the suspected cases with normal glucose uptake studies (p < 0.0001).

A total of 70 out of 74 patients with decreased glucose uptake were identified to carry pathogenic GLUT1 mutations, whereas only 1 of 35 patients with normal glucose uptake was found to have a missense mutation (see Fig 5). Of the 70 mutations identified, 60 were detectable by sequence analysis, 5 were partial gene deletions, and 5 were complete gene deletions. The 10 partial or complete gene deletions were detectable by MLPA, but MLPA cannot determine the extent of the deletion beyond the GLUT1 gene, which can include the deletion of other contiguous genes that may be associated with a more severe phenotype. Therefore, we recommend reflexive molecular genetic testing, starting with sequencing and continuing to MLPA for patients meeting clinical diagnostic criteria, and then progressing to high-resolution chromosome microarray for patients with complete gene deletions by MLPA to characterize the size and gene content of the deletion. However, because a small group of Glut1 DS patients have either a normal glucose uptake assay (1 case) or no identifiable pathogenic mutations (4 cases), it is best to continue using both tests when the diagnosis is suspected clinically (see Fig 5). We believe that a normal uptake assay and negative direct sequencing of the gene, ruling out a missense mutation, is a sufficient laboratory interrogation of the suspected patient.

Mutation analysis of the 35 patients with normal erythrocyte glucose uptake revealed a missense mutation (T295M) in only 1 patient. This mutation specifically alters Glut1 conformation and asymmetrically affects glucose flux across the cell membrane. These structure-function studies explain the seemingly paradoxical observation of Glut1 DS with hypoglycorrhachia and "normal" erythrocyte glucose uptake. The erythrocyte uptake assay only measures the zero-trans influx activity. Thus it is possible that other mutations may have similar differential effects on efflux and influx, and these possibilities need to be explored before ruling out Glut1 DS as the clinical diagnosis. The remaining 34 patients with normal glucose uptake and no detectable pathogenic mutation are unlikely to have Glut1 DS as currently defined.

The clinical condition suffered by the 4 Group 1 patients with decreased glucose uptake and no detectable pathogenic mutations in GLUT1 could be caused by an as yet unidentified mutation in GLUT 1 as we only sequenced the promoter region, the 10 coding exons and the intron-exon boundaries. We can not rule out the possibility of pathogenic mutations in the 9 introns, especially the 2 large introns 1 and 2 (~15kb and 12kb), cis acting regulatory sequences or genetic variants affecting mRNA stability, or other trans acting genetic modifiers affecting posttranslational modification, such as glycosylation.

When comparing the prevalence of the distinguishing Glut1 DS clinical features (seizure activity, tone abnormalities, and microcephaly), significant group differences were identified (see Table 3). These differences also argue that the Group 2 patients do not have Glut1 DS. This conclusion also implies that a consistent clinical picture and hypoglycorrhachia are necessary for the diagnosis of Glut1 DS, but not sufficient. A total of 31% of patients with both of these findings did not have Glut1 DS. The expanding clinical phenotypes including the milder intermittent cases also makes the diagnosis even more challenging. A similar analysis will be necessary to extend our conclusions to the milder phenotypic variants.

We noted a trend between the specific type of mutation in the GLUT1 gene and the clinical severity (see Table 5) as Leen and colleagues did recently. Trends between clinical severity and the type of mutation...
could be explained by many factors, including the relative severity of the mutation, the resulting amount of protein produced, mosaicism, and other genetic modifiers.\footnote{10,12} Consistent with these speculations, we demonstrated a significant inverse correlation between the mean time-curve uptake, the Vmax values, and the clinical severity scores of Glut1 DS patients. The time-curve uptake, in fact, had a better correlation efficiency (R$^2$ = 0.94) (see Fig 6) than the Vmax study (R$^2$ = 0.76). The erythrocyte glucose uptake assay is an indication of the functional effect of the mutation as we have argued recently.\footnote{12} These new findings reinforce that argument.

In aggregate, these recent studies allow us to conclude that the erythrocyte glucose uptake assay, when abnormal, essentially confirms the clinical suspicion of Glut1 DS. Furthermore, the degree of uptake abnormality correlates with glucose transport functional activity and has implications regarding clinical severity and prognosis.

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Potential conflicts of interest

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