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Endogenous glucose production in patients with glycogen storage disease type Ia estimated by oral D-[6,6-2H2]-glucose

Alessandro Rossi a,b,*, Maaike H. Oosterveer c,d,*, Theo H. van Dijk d, Aycha Bleeker c, Martijn Koehorst d, David A. Weinstein e, Barbara M. Bakker c,*, Terry G. J. Derks a,‡

1 Section of Metabolic Diseases, Beatrix Children’s Hospital, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands
2 Department of Translational Medicine, Section of Pediatrics, University of Naples “Federico II”, Via Sergio Pansini 5, 80131, Naples, Italy
3 Laboratory of Pediatrics, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.
4 Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.
5 University of Connecticut School of Medicine (Farmington, CT), Connecticut Children’s Medical Center, 282 Washington St, CT 06106, Hartford, Connecticut, USA.

* shared first authors; # shared last authors

‡Corresponding author:
Terry G. J. Derks, MD, PhD, University of Groningen, University Medical Center Groningen, Beatrix Children’s Hospital, Section of Metabolic Diseases, P.O. Box 30.001, 9700 RB Groningen, The Netherlands.
Mail: t.g.j.derks@umcg.nl Tel: +31-50-3611036. Fax: +31-50-3614235
ORCID ID: 0000-0002-7259-1095

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ABBREVIATIONS

CBG Capillary blood glucose
CGM Continuous glucose monitoring
CNGDF Continuous nocturnal gastric drip feeding
DBS Dried blood spot
EGP Endogenous glucose production
GSDIa Glycogen storage disease type Ia
HV Healthy volunteers
$R_a$ Rate of appearance
UCCS Uncooked corn starch
ABSTRACT

Context. Glycogen storage disease type Ia (GSDIa) is an inborn metabolic disorder characterized by impaired endogenous glucose production (EGP). Monitoring of GSDIa patients is prioritized, because of ongoing treatment developments. Stable isotope tracers may enable reliable EGP monitoring.

Objective. The aim of this study was to prospectively assess the rate of appearance of endogenous glucose into the bloodstream (\(R_a\)) in GSDIa patients after a single oral D-[6,6-\(^2\)H_2]-glucose dose.

Design. Ten adult GSDIa patients and ten age-, sex-, BMI-matched healthy volunteers (HV) were enrolled. For each participant, three oral glucose tracer tests were performed: 1) preprandial/fasted, 2) postprandial, and 3) randomly fed states. Dried blood spots were collected before D-[6,6-\(^2\)H_2]-glucose administration and 10, 20, 30, 40, 50, 60, 75, 90, 120 minutes thereafter.

Results. Glucose \(R_a\) in fasted HV was consistent with previously reported data. The time-averaged glucose \(R_a\) was significantly higher in i) preprandial/fasted GSDIa patients than HV and ii) postprandial HV compared to fasted HV (\(p<0.05\)). A progressive decrease in glucose \(R_a\) was observed in preprandial/fasted GSDIa patients; the change in glucose \(R_a\) time-course was directly correlated with the change in capillary glucose (\(p<0.05\)).

Conclusions. This is the first study to quantify glucose \(R_a\) in GSDIa patients using oral D-[6,6-\(^2\)H_2] glucose. The test can reliably estimate EGP under conditions in which fasting tolerance is unaffected but does not discriminate between relative contributions of EGP (e.g., liver, kidney) and exogenous sources (e.g., dietary cornstarch). Future application is warranted for longitudinal monitoring after novel genome-based treatments in GSDIa patients in whom nocturnal dietary management can be discontinued.
INTRODUCTION

Glycogen storage disease type Ia (GSDIa) is an inborn disorder of carbohydrate metabolism characterized by severe fasting hypoglycemia due to impaired endogenous glucose production (EGP). GSDIa is caused by glucose 6-phosphatase-α (G6Pase-α) deficiency due to biallelic G6PC1 gene variants, resulting in defective glycogenolysis and gluconeogenesis, which in turn impairs EGP. Strict medically prescribed nutrition therapy is the cornerstone of the treatment. Although controversies still exist, the medically prescribed dietary regimens include frequent daytime feedings which are restricted in fructose, sucrose and (ga)lactose, as a form of substrate reduction. In addition, uncooked cornstarch (UCCS), extended-release cornstarch (Glycosade®) or continuous nocturnal gastric drip-feeding (CNGDF) serve as product supplementation. The general aims of these strict diets is to achieve normoglycemia, to normalize secondary metabolic perturbations, and to prevent long-term, chronic complications as much as possible. Liver transplantation is an increasingly recognized alternative treatment option; its advantages and disadvantages need to be weighed individually. Novel treatment approaches for GSDIa are currently being investigated, including adeno-associated virus (AAV) serotype 8 (AAV8)-mediated gene therapy (NCT05139316) and mRNA-mediated therapy (NCT05095727). In vivo genome editing is in clinical development following promising results in GSDIa mice. Ideally, because of these novel treatments, EGP would be restored, allowing GSDIa patients no longer being dependent on strict medically prescribed diets and safely sleep throughout the night, without hypoglycemia risks.

The recent multi-stakeholder international priority setting partnership for liver GSDs has emphasized the need for new, low burdensome methods for monitoring metabolic control. Appropriate monitoring of GSDIa patients is crucial to reliably assess the acute and long-term efficacy of any treatment. Amongst the primary outcome parameters used in the aforementioned clinical trials are changes in i) the percentage time-in-range as measured by continuous glucose
monitoring (CGM), and ii) time to hypoglycemia assessed by in-hospital controlled fasting challenges.

In theory, stable isotope methods have the potential to monitor EGP in GSDIa patients in a reliable, longitudinal, minimally invasive, safe fashion. At least two major methodological aspects challenge the use of stable isotopes in GSDIa patients. First, most approaches require intravenous or nasogastric tube administration of stable isotope tracers using a primed continuous infusion and repeated venous blood sampling during the test. Second, GSDIa patients depend on dietary treatment to maintain euglycemia and cannot be studied safely under fasted conditions. Consequently, any stable isotope approach is likely to result in EGP overestimation due to the contribution of unlabeled exogenous dietary glucose, which increases the total glucose rate of appearance ($R_a$; i.e. the rate of endogenous glucose production by liver, kidney and intestine, plus any glucose originating from dietary sources).

In recent years, intraperitoneal or oral stable isotope-labelled glucose administration followed by serial dried blood spot (DBS) sampling has proven a valid approach to assess glucose $R_a$ and EGP in small laboratory animals. Since these animals were fasted, glucose $R_a$ was equal to EGP. These studies suggest that oral administration of glucose tracers may provide a minimally invasive approach to estimate glucose turnover in humans. The aim of the current study was to overcome the limitations of traditional stable-isotope approaches by developing a minimally invasive method enabling $R_a$ quantification in GSDIa patients which can be translated in regular care and clinical studies. Here we report our prospective, investigator-initiated human pilot study in ten adult GSDIa patients and ten age-, sex- and BMI-matched healthy volunteers receiving a single oral D-[6,6-2H2]-glucose dose. For each participant glucose $R_a$ was estimated under three conditions: overnight fasted before breakfast, postprandially after lunch, and at a random time.
SUBJECTS AND METHODS

Study approval

The Medical Ethical Committee of the University Medical Center Groningen (UMCG), the Netherlands approved the study protocol (ref. no. METc 2020/342). The study was conducted according to the principles of the Helsinki Declaration of 1975 as revised in 2013. All participants provided written informed consent prior to inclusion in the study.

Study design

This was a prospective, investigator-initiated human pilot study (ENGLUPRO GSDIa, NCT04311307). Study days 1 and 2 were scheduled at the UMCG after written informed consent was signed by the participants. Information on participants’ demographic, genotype (for GSDIa patients) and diet was collected. The procedures on study days 1 and 2 were performed under the supervision of a research nurse and a physician. The procedures on study day 3 were performed at home (or in hotel, in case of participants’ preferences) by the participants without supervision, after participants were provided with detailed instructions and written information during study days 1 and 2. Participants stayed at the hotel (or at home, in case of participants’ preferences) during the night between day 1 and 2. All participants were asked to return the study material to the study site after completion of all study procedures.

Study participants

The trial was conducted at the UMCG between October 2020 and July 2021. Patients with GSDIa and an equal number of age-, gender- and BMI-matched healthy volunteers (HV) were recruited at the UMCG. Inclusion criteria were (a) age > 16 years, (b) stable medical condition before the start of the test procedures and for patients with GSDIa (c) confirmation of GSDIa with enzyme assay and/or G6PC1 variation analysis. Exclusion criteria included (a) pregnancy, (b) recent (< 1 month) history of hospitalization due to hypoglycaemia, (c) intercurrent illness (defined as (a combination...
of) decreased dietary intake, vomiting, diarrhoea and fever (>38.5 °C) in the week prior to the study visit, and for HV also (d) confirmed diagnosis or history suggestive of diabetes mellitus, (e) first grade family member with a confirmed diagnosis associated with fasting intolerance, (f) symptoms or signs by suggestive of fasting intolerance, metabolic instability, fever or gastrointestinal complaints.

Based on their G6PC1 genotype (or clinical features in case the genotype was not available), patients with GSDIa were classified as “severe” (2 non-sense or active site variants or clinical ascertainment before the age of two years) or “attenuated” (2 missense variants not in the active site or 1 non-sense or active site variant and 1 missense variant not in the active site or clinical ascertainment after the age of two years). Variants were reported according to ClinVar, or based on published literature in case a mutation was not published/deposited on ClinVar. Each participant was assigned a code which was used to label the study materials (i.e., data collection forms, biological material, cloud data).

Study procedures

The study protocol is presented in Figure 1. For each participant, three glucose stable isotopes blood load tests (referred to as ‘glucose-SIB tests’) were performed. The first two glucose-SIB tests were performed at the study site under supervised conditions, during which 1.25 grams of D-[6,6-²H₂]-glucose dissolved in water (referred to as the ‘study drink’) were either orally administered before breakfast (glucose-SIB test 1) or after lunch (glucose-SIB test 2). The glucose-SIB test 3 was performed outpatient (at home or hotel) without supervision at a random time, after careful instruction of the study participant.

For each glucose-SIB test, data were collected at multiple time points, i.e., at baseline just before taking the study drink, and subsequently at 10, 20, 30, 40, 50, 60, 75, 90, 120 (and 180 if available)
minutes after taking the study drink. For each time point the following data were collected: 2 dried blood spot (DBS) samples, capillary blood glucose (CBG) concentrations (capillary blood was obtained by fingerprick), continuous glucose monitoring (CGM) values, information on any symptoms or signs. The glucose-SIB test was stopped in case 1) the CBG dropped to <3.6 mmol/L and the participant showed other signs or symptoms of hypoglycaemia or 2) the participant requested to discontinue the experiment.

On day 1, a CGM device was placed (Dexcom G6®) either the upper arm or the abdomen. Instructions on how to appropriately use the CGM device were provided by an experienced research nurse. Additionally, all participants (patients and volunteers) were given the same type of CBG device (Freestyle freedom Lite®) to be used for the entire study. Participants were also instructed on how to fill a food diary during the entire study.

On day 2, glucose-SIB test 1 was started between 05:00-06:00 a.m. and before breakfast, according to each participant’s feeding pattern. For GSDIa patients the timing of the last meal prior to glucose-SIB test 1 was accommodated to their usual fasting tolerance. Healthy volunteers were asked to fast for at least 8 hours. For 3 GSDIa patients proper fasting was not possible due to CNGDF. In these patients, glucose-SIB test 1 was performed during the last 2 hours of CNGDF. Glucose-SIB test 2 was started between 12:00-02:00 p.m. on day 2, after taking lunch according to each participant’s regular (prescribed) nutrition regimen. For glucose-SIB test 1 and 2, detailed information on the last (or concurrent) meal were recorded for each participant. On day 2 all participants were provided with instructions for glucose-SIB test 3, CGM and shipping of the study material back to the site.

In order to minimize the risk of CGM malfunction and/or disconnection during glucose-SIB test 3, participants were asked to perform this test between day 3 and 7 and to send the study material back to the site on study day 10.
Non-investigational medical product: the ‘study drink’

The D-[6,6-2H2]-glucose was purchased from Cambridge Isotope Laboratories, Inc.©, Massachusetts, United States. The study drink was prepared by Apotheek A15, and purity was confirmed to be 97%. The study drink was stored at the UMCG Pharmacy at the study site according to good manufacturing practice (GMP). For each participant, three vials (i.e., 1 vial for each glucose-SIB test) were distributed to the investigators one day before study day 1. Each study vial contained 1.25 grams of D-[6,6-2H2]-glucose powder (each glucose molecule carried two hydrogen atoms as deuterium with a molar mass of 182.17) and was labelled with the participant’s study code. This dose was based on pilot studies previously performed in healthy subjects (van Dijk TH, unpublished observations). The D-[6,6-2H2]-glucose powder was dissolved in water in two steps (total volume of 200 mL) by the investigators for glucose-SIB tests 1 and 2, and by the participants for glucose-SIB test 3 after instruction.

Outcome measures

The primary study endpoint was assessing glucose $R_a$. Calculated glucose $R_a$ was compared between: 1) GSDIa patients versus matched healthy volunteers; 2) severe and attenuated GSDIa patients; 3) the pre-prandial and the fed state (i.e. glucose-SIB test 1 and glucose-SIB test 2) in GSDIa subjects; 4) the pre-prandial and fed state (i.e. glucose-SIB test 1 and glucose-SIB test 2) in healthy volunteers; 5) the controlled hospital setting and at home setting (i.e. glucose- SIB test 1 and glucose-SIB test 3) in GSDIa patients; 6) the controlled hospital setting versus at home setting (i.e. glucose-SIB test 1 and glucose-SIB test 3) in healthy volunteers. CBG and CGM data were collected and stored as exploratory endpoints and these results have been published previously.14

Analytical procedures

Glucose derivatization and GC-MS measurements
Collected DBS were air dried horizontally for at least 3 hours at room temperature avoiding direct sunlight. Sample preparation for gas chromatography mass spectrometry (GCMS) analysis to determine the fractional distribution of glucose isotopomers was performed according to Van Dijk et al. 2003. Briefly, a 6.5 mm disk was punched out from each DBS, wetted with 50 µl water for 15 minutes followed by the addition of 500 µl ethanol and incubated overnight at room temperature. After centrifuging, the 400 µl of the supernatant was transferred to a Teflon-capped reaction vial and dried at 60°C under a stream of N₂. Glucose was subsequently converted to its pentaacetate derivative by adding 300 µl pyridine/acetic anhydride (1:2) to the residue and incubating for 30 min at 60°C (or overnight at room temperature). After drying at 60°C under a stream of N₂, the residue was dissolved in 200 µL ethylacetate and transferred into an injection vial for GCMS analysis (GC: Agilent 7890A, MS: Agilent 5975C inert MSD; Agilent Technologies, Amstelveen, The Netherlands). Derivatives were separated on a Zebron ZB-1701 30 m x 0.25 mm ID (0.25 µM film thickness) capillary column (Phenomenex, Utrecht, The Netherlands). Mass spectrometric analyses were performed by positive chemical ionization with ammonia. Out of the ions monitored, i.e., m/z 408–412 (mo-m₄), the fractional contribution of D-[6,6-²H₂]-glucose in blood glucose is represented by M₂, which was used for calculations.

Correction for naturally occurring isotopes and assessment of isotopomer data quality

The isotopologue distributions obtained by GCMS analysis were corrected for naturally occurring isotopes as previously described. The correction was based on the measured average isotopologue distribution in the baseline samples of all glucose-SIB test (i.e., samples collected just before the study drink was taken). In most baseline samples the measured M₂ fraction deviated less than 0.3 % from the theoretical prediction by ChemCalc (https://www.chemcalc.org/). Only baseline samples from participant 004_glucose-SIB test 3, participant 008_glucose-SIB test 2, and participant 018_glucose-SIB test 2 contained 0.45, 0.96 and 0.39 % excess M₂ as compared to the theoretical prediction, respectively. To account for the possibility that certain foods consumed by these...
participants affected the basal isotopomer abundances, the data from these three tests were corrected for the participant’s own baseline sample of the respective glucose-SIB test.

Model description

To derive the $R_a$ from the tracer data, we used the two-compartment model and parameter fitting procedure recently described by Vieira Lara et al. The difference with the latter study design was that our subjects received only $^2$H$_2$-labelled glucose in the study drink, whereas they supplied a mixture of labelled and unlabelled glucose. This was taken into account in the modelling of the data.

In brief, we considered two compartments, the external (stomach) compartment (compartment 1) and the blood plasma (compartment 2). The pool sizes ($q$) and concentrations ($c$) of labelled glucose were denoted by $q_1$ and $q_2$, $c_1$, and $c_2$ respectively, and those of unlabeled glucose in plasma analogously by $Q_2$ and $C_2$. Pool sizes $Q$ and $q$ were expressed in µmol kg$^{-1}$ (referring to kg body weight), and concentrations $C$ and $c$ in mmol/L.

The tracer model consisted of three rates $v$ (Figure 2): import of glucose into the blood plasma compartment ($v_1$), elimination from the plasma into the tissues ($v_2$); and metabolism or storage of glucose before it enters into the blood ($v_L$, with subscript $L$ denoting loss). All rates $v$ were expressed in µmol kg$^{-1}$ min$^{-1}$ and described by first-order kinetics, with rate constants $k$ in min$^{-1}$ with the following rate equations for the tracer:

$$v_1(q_1) = k_1 \cdot q_1$$

$$v_L(q_1) = k_L \cdot q_1$$

$$v_2(c_2) = k_2 \cdot c_2 \cdot Vol$$
The conversion factor $Vol$ in $\text{mL kg}^{-1}$ was the distribution volume of the plasma compartment and served as a conversion factor between concentration and pool size. Analogously, the elimination rate of the unlabeled glucose was defined by:

$$v_2(C_2) = k_2 \cdot C_2 \cdot Vol$$

In contrast to the model of Vieira Lara, current model lacked the equations for uptake and loss of unlabelled glucose since the bolus did not contain unlabelled glucose in the present study. Finally, the rate of appearance of unlabeled glucose was quantified as the $R_a^*$ in $\text{mM min}^{-1}$ or $R_a$ in $\text{µmol kg}^{-1} \text{min}^{-1}$.

This then led to the following set of ODEs:

$$\frac{dq_1}{dt} = -(k_1 + k_L) \cdot q_1$$

$$\frac{dc_2}{dt} = \frac{k_1 \cdot q_1}{Vol} - k_2 \cdot c_2$$

$$\frac{dC_2}{dt} = -k_2 \cdot C_2 + R_a^*(t)$$

By implication time $t$ was expressed in min.

*Calculation of $R_a$ from the tracer time courses*

The analytical solution of the above-described model as derived before reads:

$$c_2(t) = C \cdot (e^{-k_2t} - e^{-k_at})$$

with:

$$k_a = k_1 + k_L$$

The $c_2$ time courses were obtained by multiplication of the measured total glucose concentrations and the measured $M_2$ enrichment at each time point. Based on the data of all subjects and tests in
this study, and following the procedure outlined by Vieira Lara,\textsuperscript{13} $C$ was estimated from the data at 0.34 mM and $k_a$ was constrained between 0.028 and 0.33 min$^{-1}$. Subsequently, $k_a$ and $k_2$ were fitted to each individual tracer time course, yielding their values for each individual test subject and test. It can be derived\textsuperscript{13} that:

$$C = \frac{q_1(0) \cdot F}{Vol} \cdot \frac{k_a}{k_a - k_2}$$

in which $q_1(0)$ is the amount of tracer administered and $F$ (dimensionless) the bioavailability of the tracer, \textit{i.e.} the fraction of the tracer that reaches the sampled plasma pool:

$$F = \frac{k_1}{k_1 - k_L}$$

We assumed a constant bioavailability $F$ (equal for all subjects) of 0.89 based on triple-tracer tests with 88 healthy subjects.\textsuperscript{18} This then allowed to compute the distribution volume $Vol$:

$$Vol = \frac{q_1(0) \cdot F}{C} \cdot \frac{k_a}{k_a - k_2}$$

We note that an independent assessment of $Vol$ and $F$ would have required an intravenous administration of the label.

First, the time-averaged $R_a$ was computed, based on the fitted $k_2$. To this end, the time-averaged tracee concentration ($C_{2,avg}$) was computed by integrating the concentration of unlabeled glucose over time (area under the curve) and dividing by the duration of the test ($t_{end} - t_0$). A steady state was assumed for the tracee concentration. At steady state the rate of glucose elimination from the plasma pool equals the rate of appearance, and thus:

$$R_{a,average}^* = k_2 \cdot C_{2,avg}$$

$$R_{a,average} = k_2 \cdot C_{2,avg} \cdot Vol$$
Second, only for glucose-SIB test 1 (i.e., preprandial/fasted conditions) the $R_a$ was computed as a function of time. The time course of unlabeled glucose was fitted to a linear function:

$$C_2(t) = a + b \cdot t$$

Hence:

$$\frac{dC_2(t)}{dt} = b$$

At the same time (above):

$$\frac{dC_2(t)}{dt} = -k_2 \cdot C_2(t) + R_a^*$$

Setting the two expressions equal to each other gives:

$$R_a^*(t) = b + k_2 \cdot C_2(t)$$

To convert $R_a^*$ in mM to $R_a$ in µmol/kg/min, $R_a^*$ was multiplied by the distribution volume $Vol$. Note that the estimated $R_a$ in µmol/kg/min is based on an assumed bioavailability $F$ of 0.89\(^{18}\) whereas the estimated $R_a^*$ in mM is independent of $F$.

In three tests, $k_2$ could not be calculated, since there was a less than 25% decrease between the peak concentration of D-[6,6-\(^2\)H\(_2\)]-glucose and the concentration at the last time point. This concerned participant 008 (HV) glucose-SIB test 1 (25% decrease), participant 019 (HV) glucose-SIB test 2 (2% decrease), and participant 020 (GSDIa attenuated) glucose-SIB test 2 (6% decrease). These tests were excluded from further computational analysis (Supplemental file 1).\(^{19}\)

**Software and statistical analysis**

Correction for natural abundance of isotopes was performed in Excel 2019. All other calculations on glucose kinetics were done in Python, using Jupyter Notebook 6.1.4. Statistical analysis was performed using IBM SPSS Statistics 23. The comparisons between numerical variables were performed by Student’s t-test corrected for Fisher’s exact test. The normality of the distribution was
checked by the Shapiro–Wilk test. Comparison between CBG curves generated in GSDIa patients and healthy volunteers both during glucose-SIB test 1 and glucose-SIB test 2 was performed by two-way ANOVA analysis with the Geisser-Greenhouse correction. CBG and CGM values collected during glucose-SIB test 3 were excluded from the ANOVA analysis in order to minimize possible bias due to data collected under non-supervised conditions. Correlation study was performed by Spearman’s rank correlation. Statistical significance was set at $p < 0.05$. For results on 1) CBG values, 2) comparison between CBG and CGM and 3) correlation between change in glucose $R_a$ and change in CBG GSDIa patients were analysed as a group ($n=10$). For results on 1) glucose $R_a$ (both time-averaged and time courses) and, 2) $M_1$ fraction GSDIa patients with an attenuated ($n=6$) and a severe ($n=4$) phenotype were analysed separately, unless stated otherwise.
RESULTS

Study participants

Ten GSD Ia patients (5 females, 5 males) with a median age of 22.2 years (range: 17.8-53.1) and a median BMI of 26.1 kg/m² (range 22.4-29.8) were enrolled. Ten age-, gender-, and BMI-matched healthy volunteers (HV) were also enrolled. Table 1 presents the clinical and genetic characterization of the GSDIa patients, whereas the details of their nutrition diary are summarized in Table 2.

Safety

No serious adverse events were recorded during the glucose-SIB tests. Three GSDIa patients showed asymptomatic CBG < 3.6 mmol/L between 90 and 120 minutes during glucose-SIB test 1. These events were deemed unrelated to the study drink and most likely related to GSDIa.

Glucose concentrations

CBG data collected under supervised conditions (i.e. glucose-SIB tests 1 and 2) are presented in Figure 3 and Supplemental file 2. During glucose-SIB test 1 (i.e., supervised preprandial/fasted condition), no major increases in glucose concentrations were observed after the ingestion of the study drink in any of the participants. As expected, based on the metabolic defect, a significant progressive decrease in glucose concentrations during glucose-SIB test 1 was observed in GSDIa patients as compared to HV (p< 0.01) (Figure 3A-B). A major increase in glucose concentrations was observed during glucose-SIB test 2 (supervised postprandial condition) in both GSDIa patients and HV. No difference between the groups was observed (p>0.05) (Figure 3C-D). A comparison of the CBG and CGM measurements was previously reported elsewhere.¹⁴

Glucose Rₘ
Fifty-nine $M_2$ glucose curves [glucose-SIB test 1, $n=20$ (10 GSDIa and 10 HV); glucose-SIB test 2, $n=20$ (10 GSDIa and 10 HV); glucose-SIB test 3, $n=19$ (10 GSDIa and 9 HV)] were generated. For participant 012, CBG were not collected during glucose-SIB test 3 and therefore an $M_2$ curve could not be generated. Data from the fifty-six approved tests were subsequently used for computational modelling. Kinetic constants and other model parameters are presented in Supplemental file 3.

Time-averaged glucose $R_a$ and glucose $R_a$ time courses are presented in Table 3 and Figure 4. The time-averaged glucose $R_a$ was significantly higher in GSDIa patients than in HV ($p<0.05$) (for both $R_a$ in $\mu$mol/kg/min and mM/min) in glucose-SIB test 1 (i.e., supervised preprandial/fasted condition) (Figure 4A). No significant differences in the time-averaged glucose $R_a$ between GSDIa patients and HV were observed in glucose-SIB test 2 (i.e., supervised postprandial condition) and glucose-SIB test 3 (i.e., unsupervised random fed condition) for both glucose $R_a$ in $\mu$mol/kg/min and mM/min (Figure 4B-C). Mean glucose $R_a$ in HV was significantly higher in glucose-SIB test 2 compared to glucose-SIB test 1 ($p<0.05$) for both $R_a$ in $\mu$mol/kg/min and mM/min but did not differ significantly between glucose-SIB tests 1 and 2 in GSDIa patients (Figure 4A-B). No significant correlation between the time-averaged glucose $R_a$ and 1) fasting time before glucose-SIB test 1, 2) CH intake before glucose-SIB test 2, 3) daily UCCS/Glycosade intake or 4) overnight UCCS/Glycosade intake was found in GSDIa patients. Time courses of glucose $R_a$ during glucose-SIB test 1 showed a stable trend in HV and a progressive decrease in GSDIa patients (Figure 4D). The median decrease in glucose $R_a$ during glucose-SIB test 1 was 28.5% in GSDIa patients with an attenuated phenotype and 14% in GSDIa patients with a severe phenotype. No significant correlation between glucose $R_a$ calculated either at baseline or at the end (+120 minutes) of glucose-SIB test 1 and fasting time prior to glucose-SIB test 1 was found in GSDIa patients. Among GSDIa patients, the change in glucose $R_a$ (i.e. the difference between the glucose $R_a$ at the end of the test and the glucose $R_a$ at baseline) was directly correlated with the change in CBG (i.e. the difference between the CBG concentration at the end of the test and the CBG at baseline) during glucose-SIB test 1 (Figure 5).
DISCUSSION

This is the first study in GSDIa patients aiming to quantify glucose $R_a$ using a single oral $d-[6,6-^{2}H_{2}]$-glucose dose. We demonstrated that oral administration of $d-[6,6-^{2}H_{2}]$-glucose combined with DBS sampling allows estimation of glucose $R_a$ both in GSDIa patients and healthy volunteers. Moreover, we showed that calculated glucose $R_a$ is influenced by the subject feeding status (e.g., frequent feedings and UCSS/Glycosade). As such, glucose $R_a$ likely exceeded EGP in GSDIa patients and fed healthy volunteers due to the contribution of unlabelled dietary glucose.

Assessing EGP in GSDIa patients has been a key research challenge for decades. In theory, EGP represents an ideal biomarker for monitoring GSDIa patients. Firstly, it is directly influenced by defective G6Pase-$\alpha$ activity. Secondly, it is significantly lower in patients with GSDIa compared to the healthy population. Thirdly, it can be monitored over time. EGP can be estimated by (stable isotope labelled) tracer studies. Hence, proper background knowledge on physiology and stable isotope technique is essential to adequately interpret the data. EGP represents the amount of glucose which is physiologically produced by the body to avoid hypoglycemia. The liver ensures up to $\sim 80\%$ of EGP, with the remainder largely accounted for by the kidneys. Biochemically, two main processes contribute to EGP, namely gluconeogenesis and glycogenolysis. When stable isotope tracers are employed, the glucose $R_a$ refers to the sum of unlabelled glucose produced endogenously by the organs (i.e. EGP), plus that from exogenous sources (e.g. diet, i.v. glucose) appearing per unit of time in the bloodstream.

Intriguingly, previous stable isotope studies have shown that GSDIa patients display considerable residual EGP despite (often almost complete) G6Pase-$\alpha$ deficiency (Table 4). In those studies, EGP was derived from the glucose $R_a$. Since many adult GSDIa patients require frequent meals and UCCS every 4-6 hours (or alternatively i.v. glucose infusion or carbohydrates administration via N.G. tube) to maintain euglycaemia, the EGP was likely overestimated. This limitation applies to any approach aiming at quantifying EGP in GSDIa patients. Importantly, different tracers,
administration routes and feeding conditions were used in previous studies, potentially contributing to the variability observed among GSDIa patients. Based on previous encouraging results obtained in non-GSD Ia mice and taking into account the above-mentioned considerations, we tested the feasibility of glucose $R_a$ and EGP quantification in adult GSDIa patients after a single oral D-[6,6-$^2$H$_2$]-glucose dose. Collected data showed that this method allows to quantify glucose $R_a$ in adult patients with GSDIa and healthy volunteers. It is unlikely, however, that the estimated $R_a$ exclusively represents EGP in GSDIa patients. The contribution of UCCS may explain why the glucose $R_a$ in test 1 was higher in patients than in HV, since the HV had fasted for at least 8 hours.

Time-averaged glucose $R_a$ in preprandial patients was 7.7-27.0 µmol/kg/min (1.4-4.9 mg/kg/min). Our results are in line with previous studies (Table 4), confirming the reported variability among GSDIa patients. In this study that included a larger number of GSDIa patients, glucose $R_a$ was significantly higher in preprandial GSDIa patients compared to healthy volunteers while no major differences were observed between patients and healthy volunteers in (random) fed states. Although no correlation between glucose $R_a$ and fasting time prior to glucose-SIB test 1 and CH intake prior to glucose-SIB test 2 or daily UCCS intake was found in GSDIa patients, results support the role of exogenous sources (i.e., diet) to the estimated glucose $R_a$ in patients. The slowly digested UCCS likely represented a major contributor to the estimated glucose $R_a$. Cornstarch is relatively highly enriched in $^{13}$C. Yet, the baseline $M_f$ fraction in blood glucose observed in GSDIa patients (0 - 0.2%) was found to be negligible, suggesting that the UCCS used by the patients in this study was not overenriched in $^{13}$C compared to the diet of HV.

Insulin resistance is observed in GSDIa and UCCS requirements decrease in ageing GSDIa patients. Although none of the GSDIa patients included in this study displayed increased HbA1c levels during regular care visits, these potential contributing factors were not formally assessed in the present study and may have affected glucose $R_a$ in a post-prandial state.
Time-averaged glucose $R_a$ was 7.5-13.9 µmol/kg/min (1.4-2.5 mg/kg/min) in fasted healthy volunteers. After a physiological overnight fast, glucose metabolism is in steady state, meaning that the rate of glucose entering the bloodstream ($R_a$) equals the flow rate of glucose leaving the bloodstream (Rate of disappearance; $R_d$). In this situation there is no contribution of exogenous sources to the glucose $R_a$, such as it occurs in healthy who do not require UCCS. Therefore, it can be assumed that the estimated glucose $R_a$ equals the EGP in fasted healthy volunteers. Indeed, estimated glucose $R_a$ in fasted healthy volunteers is in line with data on EGP previously published upon continuous glucose tracer infusion, supporting the reliability of our method.\(^{20}\)

The time course analysis performed on the preprandial/ fasted tests revealed a stable glucose $R_a$ in healthy volunteers while a progressive decline was observed in GSDIa patients. The degree of glucose $R_a$ decline directly correlated with the decline in CBG. Moreover, the decline in glucose $R_a$ was sharper in patients with an attenuated phenotype compared to those with a severe phenotype. Again, the progressive depletion of the UCCS may have contributed to this finding. The different interval between subsequent UCCS doses (likely broader in GSDIa patients with an attenuated phenotype than in severely affected patients) may contribute to the difference observed between the two patient subgroups. Indeed, mean fasting time prior to glucose-SIB test 1 was shorter in patients with a severe versus attenuated GSDIa phenotypes (2 hours and 24 minutes vs 2 hours and 54 minutes). Whether this difference was statistically significant could not be ascertained in this relatively small study.

For data analysis, conversion of the $R_a$ from mM/min to µmol/kg/min required an estimation of the distribution volume of the tracer. To this end it was assumed that the bioavailability of the tracer was equal for all study participants. This did not affect the differences observed between the study groups (either when expressing $R_a$ in mM/min or µmol/kg/min). However a potential contribution of residual intestinal and/or hepatic G6Pase activity to the labelled glucose bioavailability cannot be ruled out. Inter-patient differences in residual G6Pase activities may affect intestinal uptake and/or
hepatic retention, resulting in differences in glucose tracer bioavailability, hence contributing to the observed variability among GSDIa patients.\textsuperscript{38,39} The relatively low number of patients included in the present study reflects the challenge of patient recruitment when studying rare disorders. It is unknown whether the current sample size adequately reflects the large clinical and biochemical heterogeneity observed in GSDIa.\textsuperscript{40}

Despite such limitations in GSDIa patients, our test can be used to adequately estimate EGP in fasted healthy volunteers and presumably under conditions in which fasting tolerance is not impaired, such as diabetes and chronic kidney disease. Theoretically, it may develop into a baseline assessment method in patients in whom glycogenolysis or gluconeogenesis is decreased (e.g., ketogenic GSD subtypes, fructose-1,6-bisphosphatase deficiency) and as a confirmation tool to clarify the impact of genetic variants of unknown significance. Importantly, as this approach does not require intravenous infusion or collection of multiple venous blood samples it can be performed in outpatient settings. As such, this method overcomes organizational, financial and psychological burden related to hospital admission, frequent venous sampling and specific sample storing and processing.

Interestingly, in several of the 15 currently registered clinical trials for GSDI patients, outcome parameters for efficacy have been selected that are counterintuitive or even dangerous, such as controlled fasting challenges. Novel techniques are urgently needed to capture alterations in metabolic flux homeostasis, that may better reflect therapeutic changes in pharmacodynamics and pharmacokinetics.\textsuperscript{41} such as CGM\textsuperscript{7,14} and stable isotope methods.\textsuperscript{9,42} As glucose $R_a$ is directly linked to the underlying enzyme defect in GSDIa, it has the potential to develop into a Primary Disease Activity Biomarkers (PDAB) as opposed to current biomarkers.\textsuperscript{43} The case-controlled application of the oral D-[6,6-$^2$H$_2$]-glucose method may be develop into a tool to longitudinally monitor GSDIa patients who discontinued nocturnal dietary management after a so-called single shot innovative treatment (such as gene transfer, or gene editing approach). In these individuals the contribution of
the diet to the estimated glucose $R_d$ is expected to decrease because of less intense dietary management. Because current mRNA treatments require repeated in-hospital intravenous administrations, a modified approach by making use of short-term multi tracer i.v. solution of [U-$^{13}$C]-glucose, [2-$^{13}$C]-glycerol, [1-$^2$H]-galactose and acetaminophen may allow to reliably estimate EGP and separately quantify contributions of gluconeogenesis and glycogenolysis.$^{44,45}$

Acknowledgements

The authors would like to thank all the patients and healthy volunteers for their participation in the study. We are grateful to Giancarlo Parenti, Rebecca Riba-Wolman and Folkert Kuipers for fruitful discussion on the study protocol and manuscript. We appreciate the assistance of Emmalie A. Jager and Candelas Gross Valle. We thank Margreet Steinfort and Petra Haarsma for practical support and precious help with the study procedures. All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest.

Data Availability

Data analyzed during the current study are available from the corresponding author on reasonable request.
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29. Rother KI, Schwenk WF. Glucose production in glycogen storage disease I is not associated with increased cycling through hepatic glycogen. Am J Physiol 1995;269(4 Pt 1):774


<table>
<thead>
<tr>
<th>Participant</th>
<th>Age (years)</th>
<th>Gender</th>
<th>BMI (kg/m²)</th>
<th>Genotype (G6PC1 variants)</th>
<th>Clinical ascertainment (years)</th>
<th>Phenotype</th>
<th>Dietary management</th>
<th>Nocturnal carbohydrate intake (g/kg/hour)</th>
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<td>M</td>
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Table 1. Clinical and molecular characteristics of the study participants.

UCCS: uncooked cornstarch; CNGDF: continuous nocturnal gastric drip-feeding; N.A.: not available

1median and range are shown
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<th>GSDIa patient</th>
<th>Fasting interval before glucose-SIB test 1 (hours)</th>
<th>CH intake before glucose-SIB test 2 (g/kg)</th>
<th>Feeding status during glucose-SIB test 3</th>
<th>Matched healthy volunteer</th>
<th>Fasting interval before glucose-SIB test 1 (hours)</th>
<th>CH intake before glucose-SIB test 2 (g/kg)</th>
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Table 2. Participants’ nutritional details during each of the three glucose-SIB tests.

CH: carbohydrates, glucose-SIB test: glucose stable isotopes blood load test performed before breakfast (test 1), after lunch (test 2) or at a random time (test 3).

¹continuous nocturnal gastric drip-feeding
### Table 3. Time-averaged Glucose $R_a$ in GSDIa patients and healthy volunteers.

For each glucose-SIB test the range of measured glucose $R_a$ is expressed in µmol/kg/min, mg/kg/min and mM/min, parameters which are commonly used in preclinical and clinical settings, respectively.

<table>
<thead>
<tr>
<th>Glucose $R_a$</th>
<th>Glucose-SIB test 1 (preprandial/fasted state)</th>
<th>Glucose-SIB test 2 (fed state)</th>
<th>Glucose-SIB test 3 (random fed state)</th>
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<td></td>
<td>µmol/kg/min</td>
<td>mg/kg/min</td>
<td>mM/min</td>
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<td>GSDIa attenuated</td>
<td>7.6-24.1</td>
<td>1.4-4.3</td>
<td>0.06-0.11</td>
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<td>GSDIa severe</td>
<td>7.6-27.3</td>
<td>1.4-4.9</td>
<td>0.04-0.17</td>
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<td>Healthy volunteers</td>
<td>7.5-13.9</td>
<td>1.4-2.5</td>
<td>0.03-0.08</td>
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Table 4. Previous studies assessing endogenous glucose production (EGP) in GSDI patients using stable isotope tracers. Due to the various feeding conditions in the different studies, results are presented as glucose rate of appearance ($R_a$). For each study $R_a$ is presented both as µmol/kg/min and mg/kg/min, parameters which are commonly used in preclinical and clinical settings, respectively.

*Unlabelled glucose i.v. infusion at variable rate (3-12 mg/kg/min)
# Some subjects were assessed in a fasted (5-12 h) state and some subjects during unlabelled glucose i.v. infusion at variable rate (1.8-9.9 mg/kg/min)
§ Unlabelled glucose NG tube infusion at variable rate (5.8-8.7 mg/kg/min)
^Only cumulative data available
† Subjects were assessed during unlabelled glucose iv infusion (1.4 mg/kg/min) before and after glucagon challenge
¶ Depending on the fasting time
IV: intravenous; MRS: magnetic resonance spectroscopy; NG: nasogastric

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<th>Glucose $R_a$µmol/kg/min</th>
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<td>Taslikian</td>
<td>1984</td>
<td>5 (1 GSDIa, 4 GSDIb)</td>
<td>$6,6-^2$H$_2$-glucose IV</td>
<td>Fasted (0.75 h after i.v. glucose infusion discontinuation)</td>
<td>16.7-26.7</td>
<td>3.0-4.8</td>
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<td>Schwenk</td>
<td>1986</td>
<td>6 (2 GSDIa, 4 GSDIb)</td>
<td>$6,6-^2$H$_2$-glucose via NG tube</td>
<td>I.v. glucose infusion*</td>
<td>0.0-16.1</td>
<td>0.0-2.9</td>
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<td>Kalderon</td>
<td>1988</td>
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<td>10.0-19.4</td>
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<td>5 (4 GSDIa, 1 GSDIb)</td>
<td>$13^C$-glucose via NG tube</td>
<td>I.v. glucose infusion*</td>
<td>6.1-19.4</td>
<td>1.1-3.5</td>
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<tr>
<td>Collins</td>
<td>1990</td>
<td>6 (5 GSDIa, 1 GSDIb)</td>
<td>$3-^3$H-glucose/$6,6-^2$H$_2$-glucose IV</td>
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<td>4.4-30.5</td>
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<tr>
<td>Rother</td>
<td>1995</td>
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<td>Weghuber</td>
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<td>$6,6-^2$H$_2$-glucose + MRS</td>
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<td>Huidekoper</td>
<td>2010</td>
<td>1 (GSDIa)</td>
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<td>Fasted (2.0-2.6h after drip-feeding discontinuation)</td>
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<td>0.7-0.9</td>
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FIGURE LEGENDS

Figure 1. Study protocol.

T1: glucose-SIB test 1 (before breakfast); T2: glucose-SIB test 2 (after lunch); T3: glucose-SIB test 3 (random time).
- *: supervised; #: unsupervised; ‘one additional samples at +180 was collected in a subset of participants.

Figure 2. Two-compartment model of tracer kinetics. The aim is to compute the rate of appearance (Ra) of unlabeled glucose. Ra represents the sum of endogenous glucose production (mainly by the liver) and other sources of unlabeled glucose (e.g. in the non-fasted tests including intestinal glucose uptake). Q2 is the pool of unlabeled glucose in the plasma compartment (the tracee), q1 the pool of labelled glucose (tracer) administered orally, and q2 the pool of labelled glucose tracer observed in the plasma. Reaction rates v depend on rate constants k which are assumed to be identical for tracer and tracee, since these are biochemically indistinguishable.

Figure 3. Capillary and CGM glucose concentrations during glucose-SIB test 1 and glucose-SIB test 2. Capillary glucose concentrations (CBG) during glucose-SIB test 1 and 2 in GSDIa patients [n=100 (i.e. 10 time points × 10 participants) per each glucose-SIB test] and healthy volunteers [HV, n=100 (i.e. 10 time points × 10 participants) per each glucose-SIB test]. Results are calculated as compared to median baseline values calculated in each subgroup (i.e., GSDIa and HV, respectively) for each glucose-SIB test (100%=median of baseline values in each subgroup) and presented as median with range (grey circles show single participants’ values). 20%= 1 mmol/L glucose.
Figure 4. Glucose $R_a$ in the study participants. (A-C): Time-averaged glucose $R_a$ calculated with fixed $F$ and constrained $C$ and $K_a$. Mean and standard deviation are shown. (D) Glucose $R_a$ time course calculated with fixed $F$ and constrained $C$ and $K_a$. A line connecting the mean value calculated at each time point (thick line) and standard deviation (shaded area) are shown (green: GSDIa attenuated, blue: GSDIa severe, red: healthy volunteers). *$p<0.05$.

Figure 5. (A) Correlation between the change in glucose $R_a$ and capillary blood glucose concentrations (CBG) during glucose-SIB test 1 in GSDIa patients ($r=0.79, **p<0.01$). $\Delta$ was calculated as $|\frac{\text{value end test } 1 - \text{value start test } 1}{\text{value start test } 1}| \times 100$. The baseline values were considered as value start test; values at $+120$ (or $+180$) were considered as value end test. (B-D) Relationship between calculated glucose $R_a$ and CBG during glucose-SIB test 1 in GSDI patients with severe (B) and attenuated (C-D) phenotypes who developed hypoglycemia at the end of glucose-SIB test 1.
Figure 1
272x145 mm (x DPI)
Figure 2
64x70 mm (x DPI)
Figure 3

Figure 3

Glucose-SIB test 1 (preprandial/fasted state)

A  GSD la

B  HV

Capillary glucose (% of baseline)

Time after study drink (min)

Glucose-SIB test 2 (postprandial state)

C  GSD la

D  HV

Capillary glucose (% of baseline)

Time after study drink (min)
Figure 4
Glucone-SIB test 1 (preprandial/fasted state)
Glucone-SIB test 2 (fed state)
Glucone-SIB test 3 (random state)

D Glucose-SIB test 1 (preprandial/fasted state)

Time (min)

Figure 4
233x156 mm (x DPI)