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Development of minimally invasive ^{13}C-glucose breath test to examine different exogenous carbohydrate sources in patients with glycogen storage disease type Ia

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ABSTRACT

Background: Glycogen storage disease type Ia (GSD Ia) is an autosomal recessive disorder caused by deficiency of glucose-6-phosphatase (G6Pase), resulting in fasting hypoglycemia. Dietary treatment with provision of uncooked cornstarch (UCCS) or a novel modified cornstarch (*Glycosade*®) is available to treat hypoglycemia, yet choice of carbohydrate to achieve a desirable glycemic control is debated. ^{13}C-glucose breath test (^{13}C-GBT) can be used to examine glucose metabolism from different carbohydrate sources via $^{13}CO_2$ in breath.

Objectives: Our objectives were: 1) establishing the use of a minimally invasive ^{13}C-GBT to examine in vivo glucose metabolism in healthy adults, and 2) using ^{13}C-GBT to measure utilization of the standard UCCS vs. *Glycosade*® in GSD Ia and healthy controls.

Design: **Experiment 1-** Ten healthy adults (6F: 4 M, 22-33y) underwent ^{13}C-GBT protocol twice as a proof-of-principle, once with oral isotope dose (glucose 75 g + [U-$^{13}C_6$] D-glucose 75 mg) and once without isotope (only glucose 75 g) to test sensitivity of natural ^{13}C-enrichment. Breath samples were collected at baseline and every 20 min for 240 min. Rate of CO_2 production was measured at 120 min using indirect calorimetry. Finger-prick blood glucose was measured using a glucometer hourly to test hypoglycemia (glucose <4 mmol/L). **Experiment 2-** Three GSD Ia (12y, 13y, and 28y) and six healthy controls (2F: 4 M, 10-32y) underwent ^{13}C-GBT protocol twice: with UCCS or *Glycosade*® (based on their current prescribed dose 42-100 g) after ~4 h fast based on our GSD Ia patients with fasting tolerance.

Results: **Findings 1-** Maximum ^{13}C-enrichments occurred at 200 min without and with [U-$^{13}C_6$] D-glucose in all healthy adults, suggesting natural enrichment is sensitive for the ^{13}C-GBT. **Findings 2-** *Glycosade*® utilization was lower than UCCS utilization in 12y and 13y GSD Ia, but was similar in the 28y GSD Ia.

Conclusions: ^{13}C-GBT is a novel minimally invasive functional test to examine glucose metabolism in GSD Ia, and test new products like *Glycosade*®, which has the potential to improve nutritional management and individualized carbohydrate supply in GSD.

Abbreviations: ^{13}C-GBT, ^{13}C-glucose breath test; APE, atom percent excess; AUC, area under the curve; BIA, bioelectrical impedance analysis; BMI, body mass index; C_{max}, maximum peak enrichment in $^{13}CO_2$ oxidation; CGM, continuous glucose monitor; CREU, clinical research and evaluation unit; $F^{13}CO_2$, rate of glucose oxidation; FCO_2, CO_2 production rate using indirect calorimetry; FFM, fat free mass; FM, fat mass; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; GSD I, glycogen storage disease type I; GSD Ia, glycogen storage disease type Ia; HSCT, hematopoietic stem cell transplantation; OGTT, oral glucose tolerance test; PKU, phenylketonuria; REE, resting energy expenditure; t_{max}, time to reach maximum $^{13}CO_2$ oxidation; UCCS, uncooked cornstarch; VCO_2, rate of carbon dioxide production.

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1. Introduction

Glycogen storage disease type I (GSD I; von Gierke disease) is inherited in an autosomal recessive manner affecting ~1:100,000 births. The most common and severe form of the hepatic GSDs [1–3]. GSD Ia [OMIM 232200] represents the majority, ~ 80% of GSD I cases, and is caused by deficiency of glucose-6-phosphatase (G6Pase) enzyme that catalyzes the production of glucose from glucose-6-phosphate (G6P), a crucial step that contributes to glucose homeostasis [4,5]. G6Pase deficiency results in inadequate production of glucose, leading to severe fasting hypoglycemia and shunting of G6P into alternative pathways, resulting in hyperlactatemia, hyperuricemia and hyperlipidemia [6–8].

Dietary therapy is the mainstay of GSD Ia treatment [9,10]. The major goal is to maintain normal blood glucose concentrations (> 70 mg/dL or 4 mmol/L) in order to avoid neuroglycopenia and secondary metabolic decompensations [11]. Prevention of fasting and the provision of small frequent meals high in complex carbohydrates is key [11–14]. The introduction of uncooked cornstarch (UCCS) in the 1980s helped to maintain normoglycemia and improved quality of life in GSD Ia [15]. UCCS can maintain normoglycemia for 2.5–6 h in GSD Ia [16]. Despite the beneficial effects of this treatment for GSD Ia, UCCS has several issues including unpalatable taste, bloating, diarrhea, sleep interruption, exhaustion and risk of delay in the administration of treatment; issues which have been associated with hypoglycemia, seizures, and even death [16,17]. *Glycosade*® (Vitaflo International Ltd., Liverpool, UK), a novel waxy maize heat-modified cornstarch, was developed and approved in the United Kingdom in 2009, and in 2012 in North America [8]. *Glycosade*® has been shown to maintain normoglycemia and to improve metabolic control, sleep, and quality of life in GSD Ia [17–20]. To date, clinical trials have primarily studied overnight periods of use, with few and limited data on daytime use [8,10,19]. However, the primary dilemma in the dietary treatment of GSD Ia is the choice of the exogenous carbohydrate sources to achieve a desirable glycemic control in individual patient management [10,21,22]. In addition, energy demands are higher during the day and may not be sufficiently covered with a slower release starch [10]. Traditional monitoring of patients with GSD Ia is done by measuring finger prick blood glucose levels, which is invasive and does not offer dynamic details of in vivo glucose metabolism.

An in vivo ^{13}C-breath test may provide a minimally invasive, sensitive, and robust tool for the evaluation of nutrient utilization reflected in digestion, absorption, and integrated metabolism [23,24]. Nutrients need to be labeled either artificially by using stable isotopes, or naturally with ^{13}C atoms [25]. Using [U-$^{13}C_6$] D-glucose, breath tests have been developed to test glucose metabolism in type 2 diabetes [26–29]. Other breath tests, based on the hydrolysis of the test starch into glucose that is absorbed, and is subsequently released in $^{13}CO_2$ in breath, have been used to identify glucose oxidation from UCCS and *Glycosade*® in GSD Ia [18,25]. The earlier breath tests were invasive [18,25], requiring several blood samples along with breath samples, and were based on a starch-loading test (~ 6–10 h) that increased the risk of hypoglycemia in GSD Ia. In order to implement a routine breath test for carbohydrate management during the daytime in GSD Ia patients there is a need to develop a minimally invasive and sensitive ^{13}C-glucose breath test (^{13}C-GBT).

The current study was designed in twosteps: 1) to establish the use of ^{13}C-GBT and its oxidation to $^{13}CO_2$ as a minimally invasive technique for examining in vivo glucose metabolism in healthy adults, and 2) to measure the utilization of UCCS and *Glycosade*® in patients with GSD Ia and healthy controls using ^{13}C-GBT, based on the natural enrichment of ^{13}C in UCCS and *Glycosade*®. We hypothesized that 1) the oxidation of $^{13}CO_2$ from [U-$^{13}C_6$] D-glucose could be detected in expired air, and $^{13}CO_2$ oxidation would be a sensitive measure to examine glucose metabolism within a short test period and 2) $^{13}CO_2$ oxidation of glucose from *Glycosade*® would be lower compared to UCCS, in patients with GSD Ia and healthy controls.

2. Material and methods

2.1. Study principle

The current study has two experiments. The study design for the 1st experiment was based on the oxidation of labeled [U-$^{13}C_6$] D-glucose, a stable isotope tracer, in comparison with the carbohydrate source naturally enriched with ^{13}C glucose used for OGTT (Thermo Scientific™ NERL™ Trutol™ Glucose Tolerance Test Beverages, CA) to $^{13}CO_2$ to examine glucose metabolism. The principle of the ^{13}C-glucose breath test (^{13}C-GBT) is based on the ingestion of a dose of [U-$^{13}C_6$] D-glucose to label CO_2 exhaled, along with the oral dose of naturally labeled glucose on one day, versus naturally labeled (enriched) glucose only on the other day. This undergoes absorption, and insulin mediates the ^{13}C-labeled glucose uptake by a variety of cells [30,31]. Therefore, ^{13}C-glucose enters cellular metabolism, the glycolysis and tricarboxylic acid cycle, and is subsequently excreted via breath CO_2 [26,27]. This whole-body response includes glucose absorption, glucose-induced insulin response, and tissue uptake of glucose, and is a non-invasive marker of glucose metabolism [29] within a short time interval of 4 h [27].

The study design for the 2nd experiment was based on the utilization of naturally labeled ^{13}C-glucose from UCCS and *Glycosade*®. The utilization in this study relates to the rate and extent of starch digestion, absorption, and glucose disposal through oxidation [24]. The principle of the breath test in this case is based on the fact that the release $^{13}CO_2$ in breath includes digestion and hydrolysis of the test starches (UCCS and *Glycosade*®) into glucose that is absorbed and subsequently oxidized [23,32].

2.2. Experiment 1 – Pilot study to establish the use of ^{13}C-glucose breath test in healthy adults

2.2.1. Subjects

Ten healthy adults (4 M: 6F; 22-33y) (Table 1) underwent ^{13}C-GBT protocols as a proof of principle at BC Children's Hospital Research Institute within our Clinical Research and Evaluation Unit (CREU) in Vancouver, Canada. At the time of the study, all participants were ensured to be free of any concurrent illness (cold, flu-like symptoms), with no recent history of medical conditions, smoking, use of medication, or use of oral contraceptives or hormonal birth control for adult females. The participants were studied twice: once with naturally labeled glucose only and once with naturally labeled glucose and stable isotope tracer [U-$^{13}C_6$] D-glucose. A non-probability sampling technique, convenience sampling, was used. Advertisements with study details and contact information were distributed in community areas such as BC Children's and Women's Hospital, the University of British Columbia and coffee shops, to recruit healthy adults. All participants provided written informed consent before participating. Participants were assigned an alphanumeric code to maintain confidentiality.

Table 1
Experiment 1 – Subject characteristics.

Characteristics	Value[a] $n = 10$	Male $n = 4$	Female n = 6
Age (y)	26.8 ± 4.3	27.8 ± 5.0	26.2 ± 4.1
Weight (kg)	69.0 ± 11.4	75.3 ± 10.1	64.7 ± 10.8
Height (cm)	169.5 ± 9.3	177.3 ± 5.4	164.3 ± 7.6
BMI[b] (kg/m^2)	24.0 ± 3.9	23.9 ± 2.0	24.2 ± 5.0
Fat-free mass[c] (kg)	48.2 ± 10.3	57.6 ± 9.3	41.9 ± 4.6
Fat mass (%)	30.1 ± 8.5	23.8 ± 2.9	34.3 ± 8.5
REE[d] (kcal/d)	1479.6 ± 405.6	1755.3 ± 534.1	1295.8 ± 153.7

[a] All values are means ± SDs; n = 10 (4 M: 6F).
[b] Body mass index (BMI).
[c] Fat-free mass measured using bioelectrical impedance analysis (BIA).
[d] Resting energy expenditure (REE) measured using open-circuit indirect calorimetry.

Compensation was provided in the form of an honorarium for each completed study day. All procedures were reviewed and approved by the University of British Columbia / Children's and Women's Health Centre of British Columbia Research Ethics Board (CW16–0377 / H16–03050) and registered through clinicaltrials.gov (NCT03218904).

2.2.2. Preliminary and eligibility assessment

A preliminary assessment (pre-study day) was done, after an overnight fast (~12 h) at the CREU to evaluate eligibility. Basic anthropometry (weight and height) was measured using a digital scale and a stadiometer. Resting energy expenditure (REE) was measured by a continuous, open-circuit indirect calorimeter (Vmax Encore, Viasys Healthcare Inc. Yorba Linda, CA), which was calibrated prior to use. Body composition (fat free mass, FFM and fat mass, FM) was determined using bioelectrical impedance analysis (BIA-Quantum IV, RJL Systems, MI). A general questionnaire was used to collect participants' medical history, nutritional status, supplement intake and physical activity.

2.2.3. Study protocol

Participants were instructed to arrive for the study day after a fast (~4 h) to standardize measurements. Basic anthropometric measurements (body weight and height) were recorded, and a brief study day questionnaire was administered to collect information on medical, diet and physical activity history. Two baseline breath samples were collected to determine natural background ^{13}C abundance. Participants randomly received on one day an oral dose of 75 g of glucose with orange flavoring, used for OGTT (Thermo Scientific™ NERL™ Trutol™ Glucose Tolerance Test Beverages, CA), and on the other day an oral dose of 75 mg of universally labeled [U-$^{13}C_6$] D-glucose (99 atom% ^{13}C enrichment, Cambridge Isotope Laboratories Inc., Andover, MA) plus the 75 g of glucose [33]. Each study was separated by ≥1 week for males and once a month for females since we controlled for the phases of menstrual cycle. Participants remained fasting and resting in the unit for each study visit to eliminate variability in CO_2 production. Breath samples in triplicate were collected every 20 min for 240 min after oral administration of glucose; the 4 h protocol was based on Dillon et al., 2009. During the study visit, the rate of carbon dioxide production (VCO_2) was measured for 20 min, two hours after the oral glucose dose using an indirect calorimeter (Vmax Encore, Viasys Healthcare Inc. Yorba Linda, CA). Hourly finger-prick blood glucose was measured using a glucometer (OneTouch® Ultra®2 LifeScan, Canada Ltd).

2.3. Experiment 2 – Utilization of different exogenous carbohydrate sources in patients with glycogen storage disease type Ia

2.3.1. Subjects

A flow chart detailing the enrolment of patients with GSD I is provided in (Fig. 1). We studied three patients with GSD Ia (1F: 2 M) aged 12, 13, and 28 years (Table 2) who are followed by the Biochemical Diseases Clinic at BC Children's Hospital and Adult Metabolic Diseases Clinic at Vancouver General Hospital. Six healthy controls (2F: 4 M; 10-32y) (Table 2) underwent ^{13}C-GBT twice, and each study was separated by ≥1 week. All participants were ensured to be free of any concurrent illness (cold, flu-like symptoms) at the time of the study. Patients were recruited using advertisements via the Biochemical Diseases staff at BC Children's Hospital and the Adult Metabolic Diseases Clinic at Vancouver General Hospital. Healthy participants were recruited from the community using posters. Written informed consent was obtained from adults, and assent from children. At the completion of each study day an honorarium was provided to participants.

2.3.2. Experimental design

The study day protocol was similar to the 1st experiment (see section 2.2.3. Study protocol). In this 2nd experiment, the heat-moisture processed cornstarch *Glycosade*® (Vitaflo International Ltd., Liverpool, UK) was compared with uncooked Fleischmann's® brand cornstarch, the standard starch preparation used in Canada (ACH Food Companies, Inc. Mississauga, ON). For each study day, the test starch dose of 42 g for

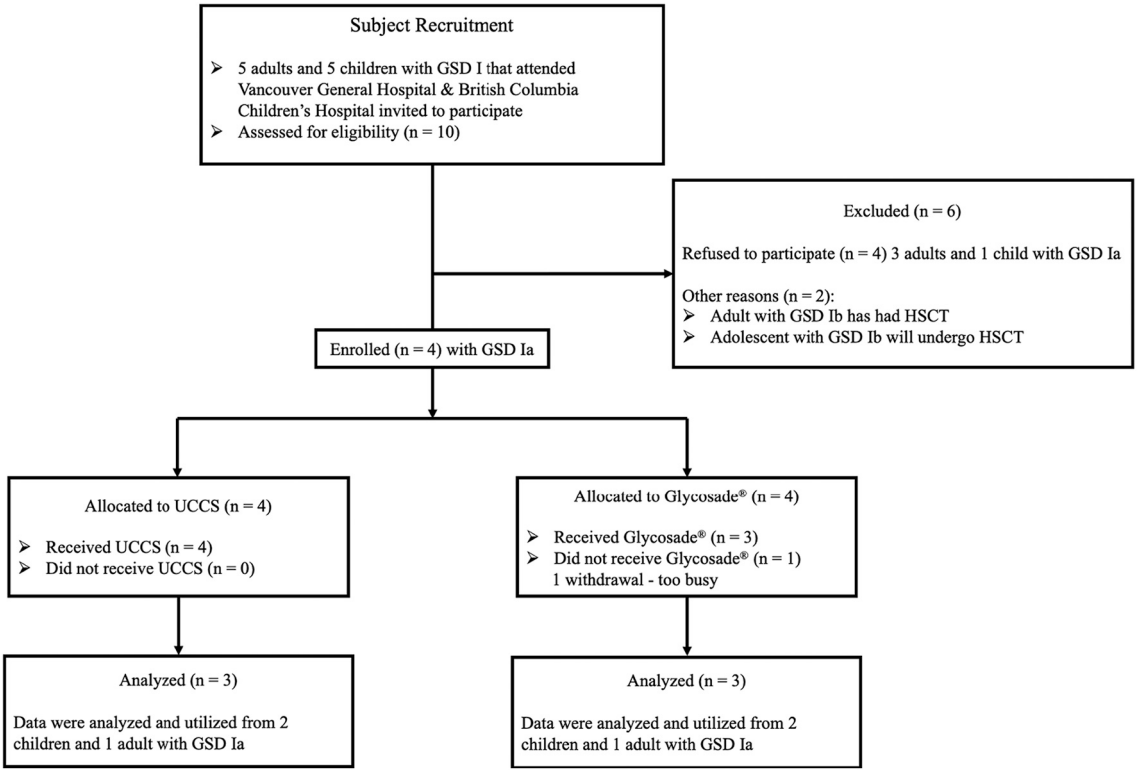

Fig. 1. CONSORT flow chart depicting enrollment and allocation of patients with glycogen storage disease type I (GSD I).
GSD Ia, glycogen storage disease type Ia; GSD Ib, glycogen storage disease type Ib; HSCT, hematopoietic stem cell transplantation; UCCS, uncooked cornstarch.

Table 2
Experiment 2 – Subject characteristics.

Subject	Age (y)	Gender	Pre-dose fast (h)	Weight (kg)	Height (cm)	BMI (kg/m^2)	BMI percentile (%)	Fat-free mass (kg)	Fat mass (%)
Patients with glycogen storage disease type Ia (GSD Ia)									
BTGSD02	12	F	3.5	46.8	145.6	–	85th - 97th[a]	29.8	36.3
BTGSD03	28	M	4	100	169.5	34.8	–	65.5	34.5
BTGSD05	13	M	3.5	44.4	153.5	–	50th - 85th[b]	31.3	29.5
Healthy controls[c]									
BTHC01	25	M	4	90.3	183.4	26.8	–	70.2	22.3
BTHC02	24	M	4	64.8	170.2	22.4	–	48.1	25.8
BTHC03	32	M	4	77.1	177.6	24.4	–	56.2	27.1
BTHC05	10	F	3.5	36.7	143.0	–	50th- 85th[d]	23.6	35.8
BTHC06	10	F	3.5	30.5	139.1	–	15th- 50th[e]	24.2	20.7
BTHC07	11	M	3.5	46.1	153.5	–	At 85th[f]	34.0	26.2

BMI, body mass index; GSD Ia, glycogen storage disease type Ia.
[a] The BMI is 22.1, placing the BMI-for-age between 85th - 97th percentile for girls aged 12 years. This child is overweight [45].
[b] The BMI is 18.8, placing the BMI-for-age between 50th - 85th percentile for boys aged 13 years. This child has healthy weight [45].
[c] Healthy controls were selected to represent the wide range in age of patients with GSD Ia recruited for the study.
[d] The BMI is 17.9, placing the BMI-for-age between 50th - 85th percentile for girls aged 10 years. This child has healthy weight [45].
[e] The BMI is 15.8, placing the BMI-for-age between 15th - 50th percentile for girls aged 10 years. This child has healthy weight [45].
[f] The BMI is 19.6, placing the BMI-for-age at 85th percentile for boys aged 11 years. This child has healthy weight [45].

BTGSD02, 100 g for BTGSD03, and 43 g for BTGSD05 was administered orally (based on patient's current prescribed dose (Table 3)), dissolved in 100–150 mL water and added to an artificially sweetened, carbohydrate-free, raspberry drink (Crystal-Light; Kraft Foods Inc., Northfield, IL) to enhance flavor (Fig. 2). The same doses as above were administered to the age-matched controls.

2.4. Sample collection and analysis

Breath samples were collected in disposable Exetainer® tubes (Labco Limited, Buckinghamshire, UK) using a collection mechanism that permits removal of dead air space (Single use collection bags, Easy Sampler System, QuinTron, Terumo Medical) [34]. Baseline breath samples were collected −0 and − 5 min prior to oral glucose or starch dose. After oral administration of test dose, breath samples were collected every 20 min for 240 min for the 1st experiment and every 30 min for the 2nd experiment. All breath samples were stored at room temperature until analyzed. Expired $^{13}CO_2$ enrichment was measured using a continuous flow isotope ratio mass spectrometer (CF-IRMS IsoPrime100, Cheadle, UK) and expressed as atom percent excess (APE) for the 1st experiment and δ per mil (δ ‰) for the 2nd experiment.

Finger-prick blood glucose measurements were performed at 60 min intervals, mainly to prevent hypoglycemia in GSD Ia patients using a glucometer (OneTouch® Ultra®2 LifeScan, Canada Ltd). Self-monitoring using finger-prick blood glucose has been part of our center's protocol to ensure safety before physical activity or meals. Therefore, we decided to follow the same protocol, rather than continuous glucose monitor (CGM) to reduce burden for study participation.

Table 3
Starch treatment in patients with GSD Ia.

Subjects	UCCS (doses/day)	Total UCCS (g/day)	*Glycosade*® (doses/night)	Total *Glycosade*® (g/night)
BTGSD02	6	213	1	130
BTGSD03	N/A	N/A[a]	1	150
BTGSD05	5	215	1	110

GSD Ia, glycogen storage disease type Ia; UCCS, uncooked cornstarch.
[a] Sometimes using daytime UCCS – amounts quite variable.

2.5. Calculations

2.5.1. Experiment 1 – Calculations

Rate of ^{13}C-glucose oxidation ($F^{13}CO_2$, μmol/kg/h) with and without [U-$^{13}C_6$] D-glucose was calculated as:

$$F^{13}CO_2 = (FCO_2)\,(ECO_2)\,(44.6)\,(60)/(W)\,(0.82)\,(100)$$

where FCO_2 is the CO_2 production rate (mL/min) on each study day as measured by indirect calorimetry, ECO_2 is the $^{13}CO_2$ enrichment above baseline (APE) obtained from breath samples at each time point, W is the body weight (kg) of each subject, 44.6 (μmol/mL) and 60 (min/h) are constants used to convert FCO_2 to μmol/h, 0.82 is the correction factor for carbon dioxide retained by the body due to bicarbonate fixation, and 100 is used to convert APE to a fraction [35].

2.5.2. Experiment 2 – Calculations

The oxidation of glucose from UCCS and *Glycosade*®, representing starch utilization was calculated from the ^{13}C enrichment in expired CO_2 with the formula [25]:

$$\text{Glucose oxidation}\,(\text{mg/kg/}min) = [(\delta‰CO_2\,(t) - \delta‰CO_2\,(t_0))/(\delta‰\text{starch} - \delta‰CO_2\,(t_0))] \times [(VCO_2\,(t) \times 180)/(22.4 \times 6 \times \text{lean body mass})].$$

where δ ‰ starch is the δ value of UCCS and *Glycosade*® obtained from previous literature, which is measured after complete combustion (δ ‰ = −11.34 and − 10.75, respectively) [18,25], substituting the UCCS and *Glycosade*® enrichments in the formula. δ ‰ CO_2 (t_0) is the δ value of expired CO_2 at baseline, and δ ‰ CO_2 (t) is the δ value of expired CO_2 at a specific time [36]. VCO_2 (t) is expressed in milliliters per minute measured by indirect calorimetry. 180 is the molecular weight of glucose, 1/22.4 is for the conversion in moles of CO_2, and 1/6 is for the conversion to moles of glucose. Lean body mass or FFM is expressed in kilograms.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.1.0

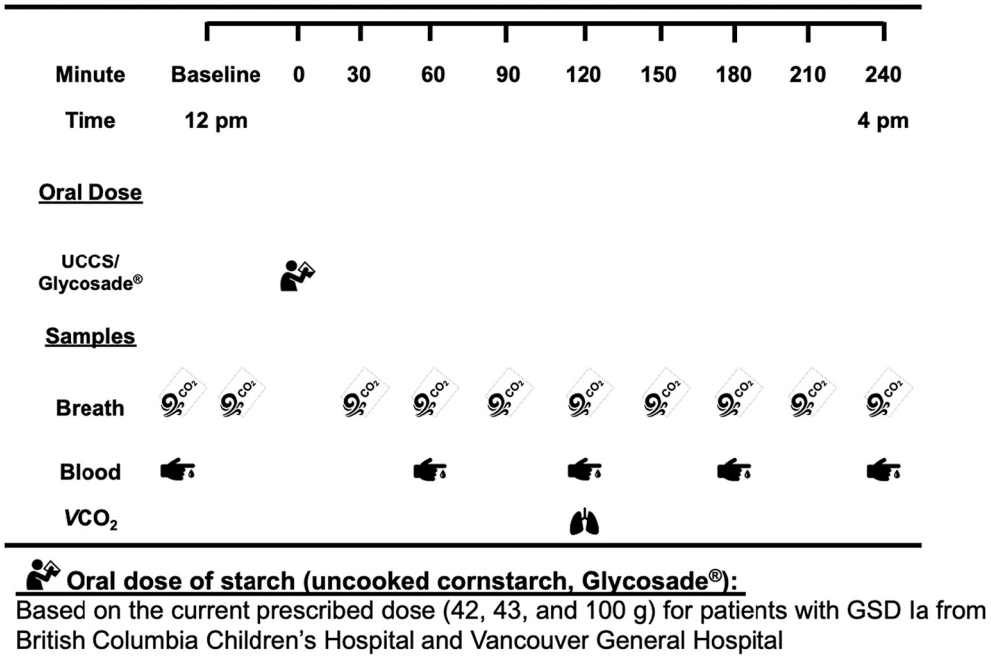

Fig. 2. Study day protocol for the 2nd experiment in patients with GSD Ia and healthy controls using the ^{13}C-glucose breath test (^{13}C-GBT), based on the natural enrichment of ^{13}C in UCCS and *Glycosade*®.

(GraphPad Software Inc., CA). Normality of data was assessed by the Shapiro-Wilk test. For the 1st experiment, subject characteristics are presented as mean ± SD. $F^{13}CO_2$ was the primary outcome measure. A paired *t*-test was used to compare the $^{13}CO_2$ oxidation without and with oral isotope [U-$^{13}C_6$] D-glucose dose. For the 2nd experiment, subject characteristics are presented for individual subjects. Starch utilization, represented as glucose oxidation from UCCS and *Glycosade*® (mg/kg/min), was the primary outcome measure. Area under the curve (AUC) for the patient's $^{13}CO_2$ oxidation from t_0 to t_{240}, the time to reach maximum $^{13}CO_2$ oxidation (t_{max}), and the maximum peak enrichment in $^{13}CO_2$ oxidation (C_{max}) were calculated. A paired t-test was used to compare the $^{13}CO_2$ oxidation derived from UCCS and *Glycosade*® for each patient separately. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1 – Results

3.1.1. Subject characteristics

Ten healthy adults (26.8 ± 4.3 y; 4 M: 6F) completed a total of 20 study days. Subject anthropometric measures (Table 1) were in the normal range [37], and were healthy during each study day.

3.1.2. Rate of ^{13}C-glucose oxidation

There were statistically significant differences ($P < 0.001$) in the rate of ^{13}C-glucose oxidation ($F^{13}CO_2$) in all healthy adults from glucose with [U-$^{13}C_6$] D-glucose compared to without stable isotope dose, as expected (Fig. 3A). The time to reach maximum enrichment (t_{max}) however

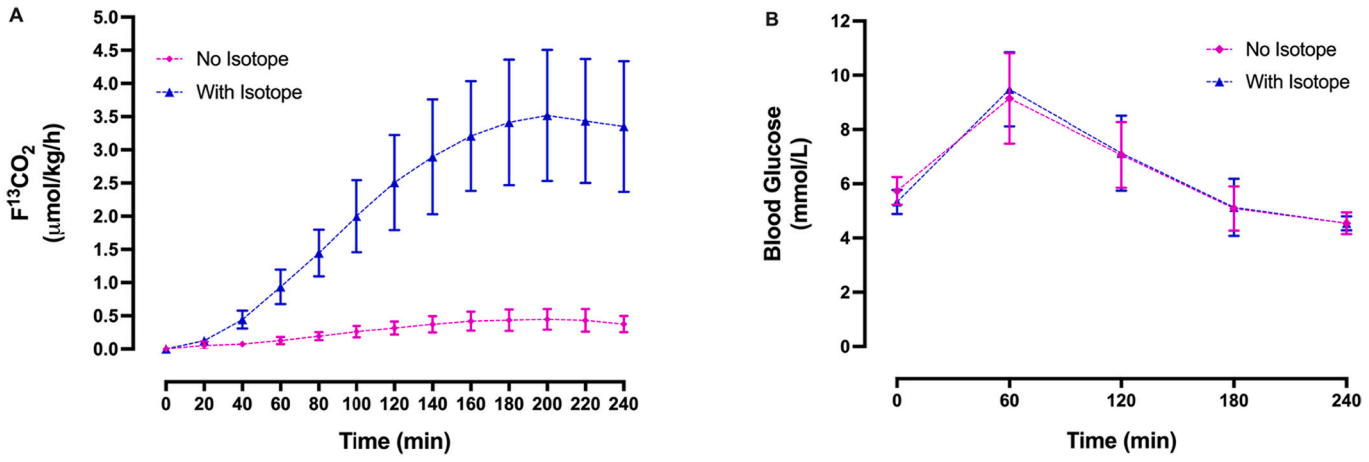

Fig. 3. ^{13}C-glucose breath test (^{13}C-GBT) and finger-prick blood glucose concentrations in healthy adults.
A. Rate of ^{13}C-glucose oxidation ($F^{13}CO_2$) in healthy adults without vs. with stable isotope [U-$^{13}C_6$] D-glucose for 240 min study day protocol.
B. Finger-prick blood glucose concentrations in healthy adults from glucose without vs. with stable isotope [U-$^{13}C_6$] D-glucose. Values are means ± SDs.

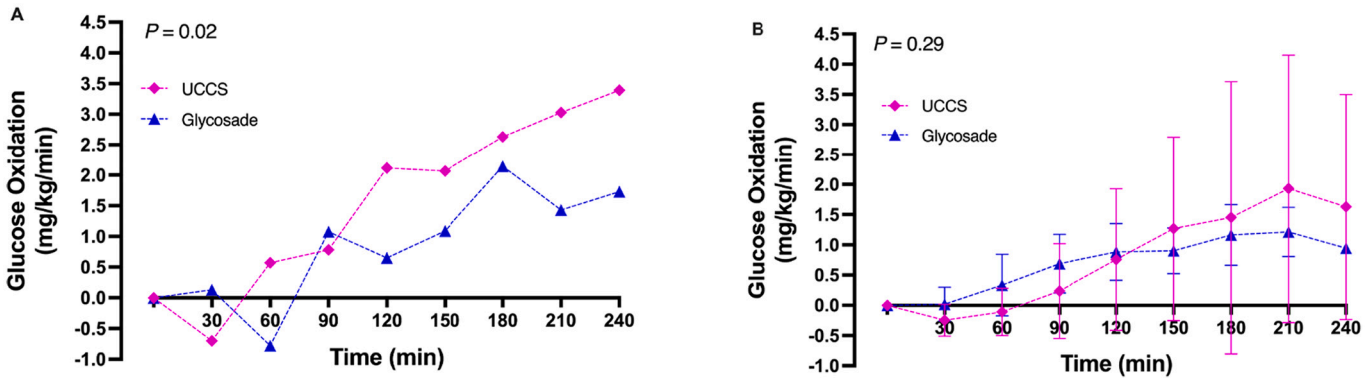

Fig. 4. ^{13}C-glucose breath test (^{13}C-GBT) in the 12y GSD Ia and healthy age-matched controls.
A. Glucose oxidation in GSD Ia (12y, F) who received UCCS vs. *Glycosade*® (dose 42 g).
B. Glucose oxidation in healthy children who received UCCS vs. *Glycosade*® (dose 42 g).

occurred at t_{200} in all healthy adults without and with [U-$^{13}C_6$] D-glucose. These results suggest that the natural enrichment of the glucose drink (Thermo Scientific™ NERL™ Trutol™ Glucose Tolerance Test Beverages, CA), made from maize starch is similar to the [U-$^{13}C_6$] D-glucose approach, and sensitive within the 4 h test to explore glucose metabolism.

3.1.3. Finger-prick blood glucose concentrations

There were no statistically significant differences ($P = 0.96$) noted in the blood glucose concentrations at any time point in all healthy adults (Fig. 3B). The mean peak glucose concentrations was 9.15 ± 1.67 and was 9.48 ± 1.37 mmol/L, with and without isotope tracer, respectively and occurred at 60 min after the oral administration of glucose. This suggests that the traditional blood glucose measurements rely on the accumulation of substrate in the circulation, compared to the ^{13}C-GBT results, which provide a more dynamic measure of glucose metabolism since $F^{13}CO_2$ represents the metabolic end point for measurement of glucose oxidation.

3.2. Experiment 2 – Results

3.2.1. Subject characteristics

Three patients with GSD Ia (12, 13, and 28 y; 1F: 2 M) and six healthy controls (10–32 y; 2F: 4 M) completed a total of 18 study days (Table 2). Among pediatric participants, BMI percentiles were within normal values in the pediatric controls and the 13y GSD Ia; the 12y GSD Ia (BTGSD02) had BMI-for-age in the overweight category. However, the body composition measures were comparable among all children, and were within normal ranges for their ages [37]. Among adult participants, body composition measures were comparable except for the 28y GSD Ia (BTGSD03) who had BMI under the obese classification and high FM when compared with reference values [37]. A healthy control (BTHC01) had BMI within the overweight category but had high FFM and normal FM [37].

3.2.2. Glucose oxidation (UCCS / Glycosade® utilization)

Glucose oxidation (utilization) from 42 g *Glycosade*® was significantly lower than glucose oxidation from 42 g UCCS in the 12y GSD Ia ($P = 0.02$) (Fig. 4A). These results are likely due to higher amylopectin content in *Glycosade*® that delays digestion, absorption, and the subsequent oxidation of glucose. Healthy children who received the same 42 g dose showed that *Glycosade*® utilization was somewhat lower than UCCS utilization, although the differences were not statistically significant (Fig. 4B).

The 13y GSD Ia and his healthy age-matched control who received the same 43 g dose displayed a lower *Glycosade*® utilization than UCCS utilization, although not significant ($P > 0.05$) (Fig. 5A, B). There was a subsequent return of glucose oxidation from both starches to ~ baseline value by end of 4 h in the 13y GSD Ia (Fig. 5A). These findings indicate that oral 43 g dose of UCCS or *Glycosade*® reached the intracellular metabolism and was fully oxidized by 4 h; and dosage adjustment may be necessary for this 13y GSD Ia child.

In the 28y GSD Ia there were no statistical differences noted in the 100 g UCCS and *Glycosade*® utilization since the glucose oxidation from

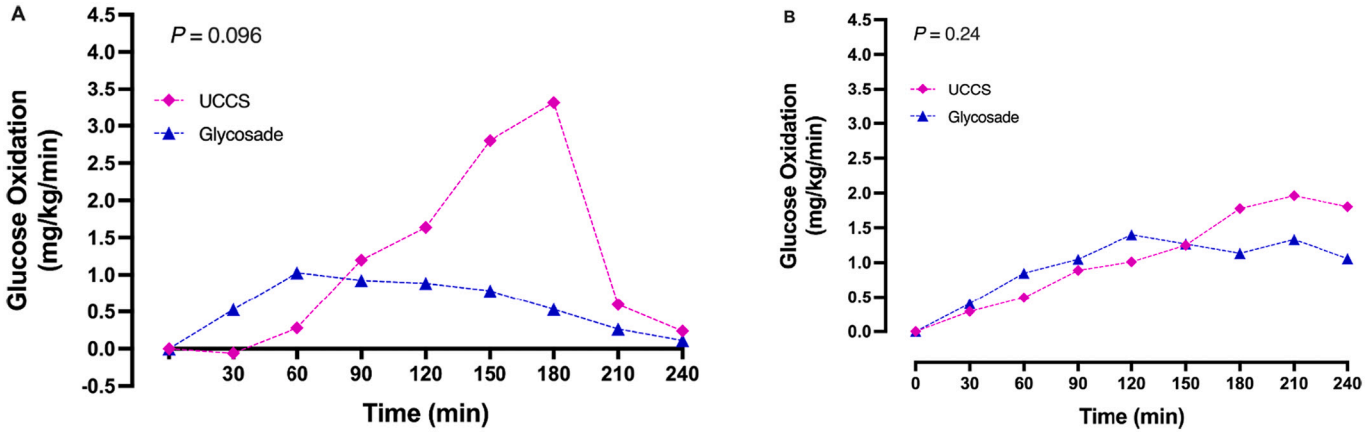

Fig. 5. ^{13}C-glucose breath test (^{13}C-GBT) in the 13y GSD Ia and healthy age-matched control.
A. Glucose oxidation in GSD Ia (13y, M) who received UCCS vs. *Glycosade*® (dose 43 g).
B. Glucose oxidation in a healthy child who received UCCS vs. *Glycosade*® (dose 43 g).

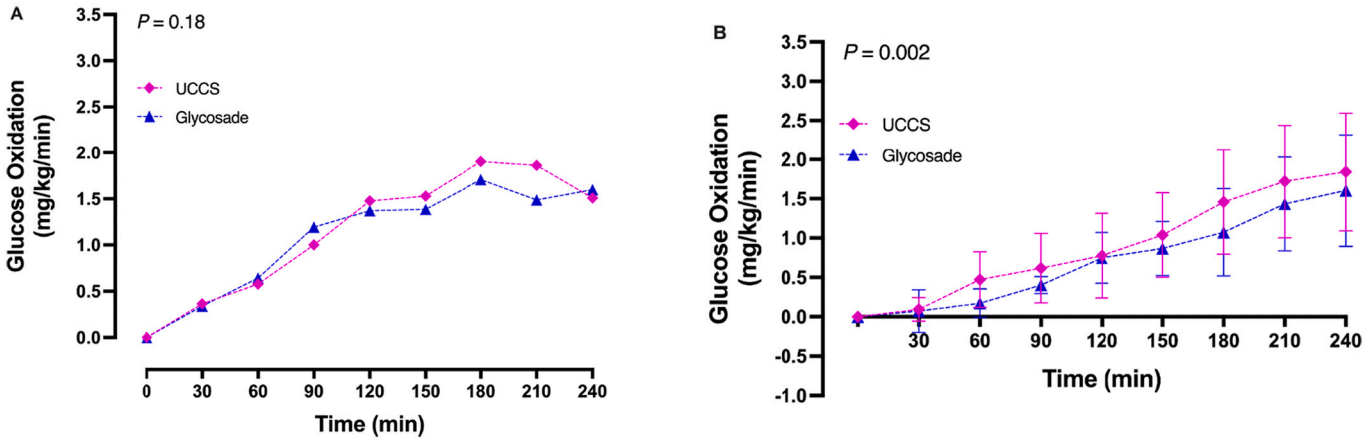

Fig. 6. ^{13}C-glucose breath test (^{13}C-GBT) in the 28y GSD Ia and healthy age-matched controls.
A. Glucose oxidation in GSD Ia (28y, M) who received UCCS vs. *Glycosade*® (dose 100 g).
B. Glucose oxidation in healthy adults who received UCCS vs. *Glycosade*® (dose 100 g).

both starches remained the same (Fig. 6A). However, there was a statistically significant decrease in utilization of *Glycosade*® compared to UCCS in the 3 healthy adult controls ($P = 0.002$) (Fig. 6B). More details regarding AUC for each subject's $^{13}CO_2$ oxidation from t_0 to t_{240}, t_{max}, and C_{max} are presented in (Table 4).

3.2.3. *Finger-prick blood glucose concentrations*

Finger-prick blood glucose measurements were performed at 60 min intervals until the test end, mainly to monitor hypoglycemia (glucose <4 mmol/L) in GSD Ia patients (Fig. 2), except for BTGSD05 who had measurements at 120 min intervals based on patient request. Blood glucose concentrations were not significantly different at any time point in patients (Fig. 7A, C, E), and in all healthy children from UCCS and *Glycosade*® (Fig. 7B, F); healthy adults (Fig. 7D) had a significant increase ($P = 0.01$) in the mean blood glucose from *Glycosade*® compared to UCCS. Our findings suggest that blood glucose measures are less sensitive compared to ^{13}C-GBT. Finally, both starches were capable of maintaining normoglycemia over a 4 h test (Fig. 7A, C, E).

4. Discussion

The objective of the 1st experiment was to establish a minimally invasive and sensitive test to examine in vivo glucose metabolism in a healthy adult population. The results from the healthy adults confirmed that the ^{13}C-GBT study protocol was sufficient and can provide valuable dynamic information about glucose metabolism over a 4 h study period. In all probants the breath test was sensitive with similar maximum enrichment (t_{max}) and we were able to use the natural enrichment of glucose, to get similar single-dose decay kinetics as with the stable isotope [U-$^{13}C_6$] D-glucose. The objective of the 2nd experiment was to measure the utilization of UCCS and *Glycosade*®, using their natural ^{13}C enrichment in patients with GSD Ia using the ^{13}C-GBT. When GSD Ia patients were tested at their current prescribed carbohydrate doses, the ^{13}C-GBT was able to identify different glucose oxidation patterns from the starches. Our primary goal was to use this principle as a minimally invasive method to improve dietary management, to individualize treatment, and to tailor carbohydrate supply during the daytime in GSD

Table 4
Glucose oxidation (mg/kg/min) area under the curve in patients with GSD Ia (n = 3) and healthy controls (n = 6)

Subjects	Patients with glycogen storage disease type Ia (GSD Ia)					
	$^{13}CO_2$ oxidation					
	AUC_{240}[a] (mg/kg/min)		t_{max}[b] (min)		C_{max}[c] (mg/kg/min)	
	UCCS	*Glycosade*®	UCCS	*Glycosade*®	UCCS	*Glycosade*®
BTGSD02	381.9	210.8	240	180	3.39	2.15
BTGSD03	284.0	267.8	180	180	1.90	1.71
BTGSD05	297.5	150.0	180	60	3.31	1.03

Subjects	Healthy controls					
	$^{13}CO_2$ oxidation					
	AUC_{240} (mg/kg/min)		t_{max} (min)		C_{max} (mg/kg/min)	
	UCCS	*Glycosade*®	UCCS	*Glycosade*®	UCCS	*Glycosade*®
BTHC01	133.7	163.7	240	240	1.25	1.53
BTHC02	336.2	249.8	240	240	2.69	2.36
BTHC03	169.4	93.9	240	240	1.58	0.94
BTHC05	17.70	112.6	150	240	0.192	1.00
BTHC06	386.9	234.0	210	180	3.50	1.52
BTHC07	257.0	238.9	210	120	1.96	1.40

AUC, area under the curve; GSD Ia, glycogen storage disease type Ia; UCCS, uncooked cornstarch.
[a] Area under the curve for $^{13}CO_2$ oxidation from t_0 to t_{240}.
[b] Time to reach maximum $^{13}CO_2$ oxidation.
[c] Maximum peak enrichment in $^{13}CO_2$ oxidation.

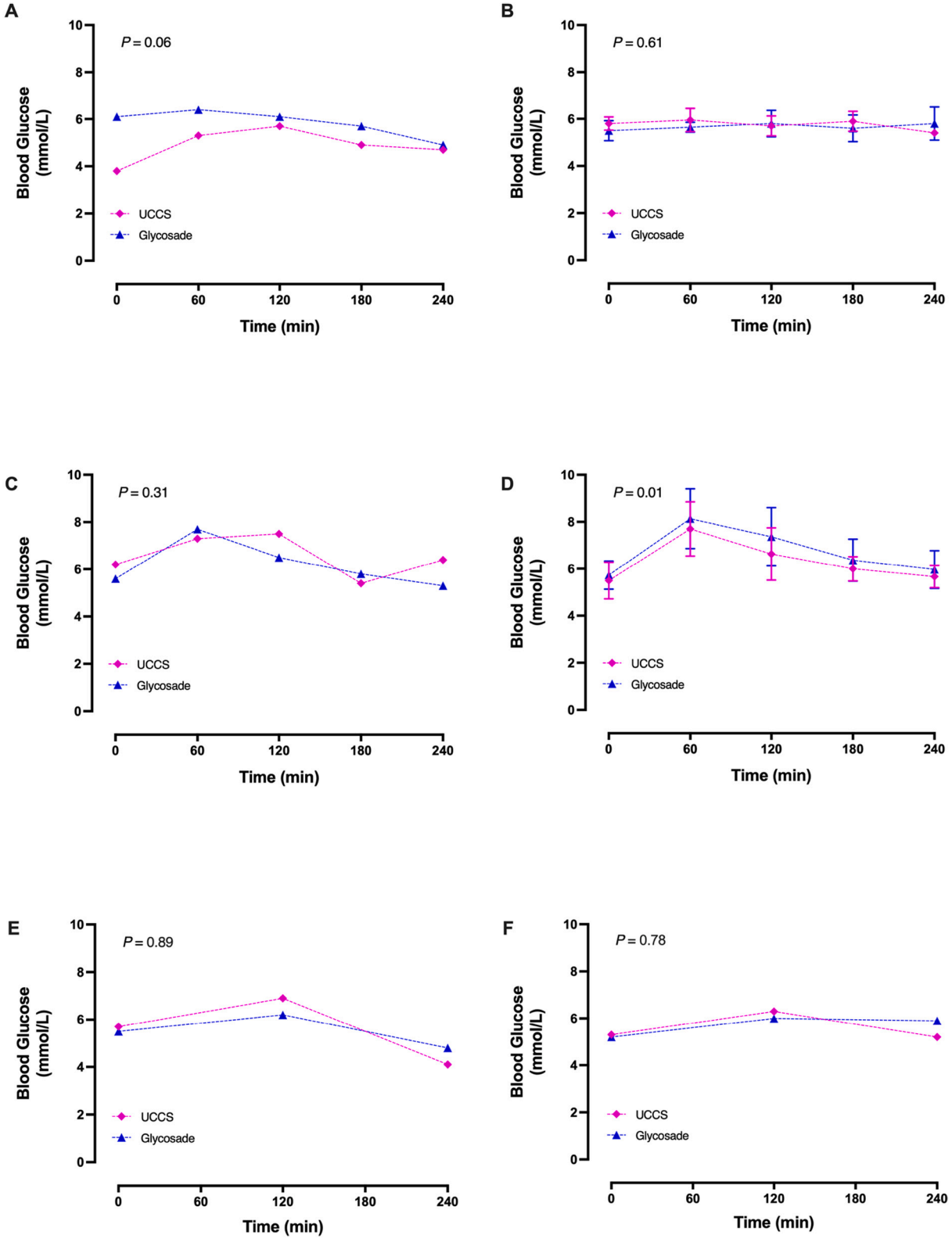

Fig. 7. Finger-prick blood glucose concentrations from UCCS vs. *Glycosade*®.
A. GSD Ia (12y, F), B. Healthy children's controls, C. GSD Ia (28y, M), D. Healthy adults, E. GSD Ia (13y, M), and F. Healthy child.

Ia patients.

Previously using stable isotope [U-$^{13}C_6$] D-glucose, ^{13}C-GBT has been developed to test glucose metabolism and insulin resistance in type 2 diabetes [27,33]. Dillon and colleagues [27] studied adults with normal glucose tolerance and with pre-diabetes and early-stage diabetes for a 10 h stable isotopically labeled OGTT; blood and breath samples were collected at 30 min intervals. The authors showed that ^{13}C-GBT may help in identification of pre-diabetes or early-stage diabetes in patients,

since breath $^{13}CO_2$ is lower compared to individuals with normal glucose tolerance, especially within the first 4 h of the study [27]. Ghosh and colleagues [33] studied 65 adults ($n = 25$ type 2 diabetes, $n = 22$ pre-diabetes, and $n = 18$ non-diabetic controls) aged 25–79 years and used 75 mg [U-$^{13}C_6$] D-glucose along with 75 g normal glucose. Similar to the above study, the results showed that $^{13}CO_2$ enrichment was significantly lower in type 2 diabetes compared to the other groups. It was suggested that the impaired glucose uptake is likely due to reduced insulin secretion from the pancreas or impaired insulin action, resulting in diminished glucose oxidation to $^{13}CO_2$ [33]. When compared to the traditional OGTT, which involves several blood samples to measure glucose concentrations as a marker of glucose disposal, the ^{13}C-GBT is minimally invasive, especially when the length of time can be shortened to 4 h, and is more informative (in vivo metabolism).

The ^{13}C-GBT in the 1st experiment is reflective of a whole-body response that includes glucose absorption, insulin response, and tissue uptake of glucose. Our test protocol of 4 h, with a peak enrichment occurring consistently at 200 min in all healthy adults with and without stable isotope, suggests that the oral dose of glucose was absorbed and reached the maximum intracellular disposal within 200 min; and that the natural enrichment of glucose was sufficient to reliably measure $^{13}CO_2$ (Fig. 3A). Thus, using different exogenous starches, and their natural ^{13}C-enrichment in GSD Ia over a 4 h protocol would allow individual patient responses to be characterized, and may be useful in dietary management.

Previous studies have used naturally enriched ^{13}C breath test in GSD Ia patients. Bodamer and colleagues [25] studied 8 adults with GSD Ia and 15 healthy controls who received a dose of UCCS (1 g/kg) followed by collection of blood and breath samples for 6 h. The results revealed that UCCS utilization was significantly higher in healthy controls for 4.5 h until the end of the study, compared to GSD Ia patients. The authors suggested that in GSD Ia patients a significant amount of absorbed glucose derived from UCCS is stored in the liver as glycogen instead of being oxidized, due to G6Pase deficiency [25]. Bhattacharya and colleagues [18] used a starch load test in GSD types Ia, Ib, and III ($n = 21$) age range (3–47 y). The participants received 2 g/kg, but had a maximum amount restriction (120 g) of UCCS or *Glycosade*® followed by collection of blood and breath samples hourly for 10 h or when blood glucose $\leq$3.0 mmol/L. The results showed that there is no significant increase in glucose oxidation of UCCS compared to *Glycosade*® [18]. However, the results were confounded by several factors including variability in test duration and pre-load fasting range ($2-12h$). Compared to our protocol, the earlier breath test [18,25] was relatively more invasive, requiring several blood samples along with breath samples, and was based on a starch load test that lasts for 6–10 h, which increases the risk of hypoglycemia in GSD Ia patients. Previous study has indicated that UCCS can prevent hypoglycemia for a median time 4.25 h in children (range 2.5–6 h) [16]. Also, the study by Bhattacharya and colleagues [18] did not measure actual CO_2 production rates but they relied on predicted CO_2. It has been shown previously that the endogenous CO_2 production rates of each participant could explain some of the differences in breath testing [38]. In our current study all values were corrected by measuring CO_2 production rates (using an indirect calorimeter) on each study day. Thus, our study protocol has some advantages: testing done as per each patient's current prescribed dose during daytime, measurement of actual CO_2 production rates, and standardized fasting condition (3.5–4 h, based on each patient's fasting tolerance), and the first potential step in making ^{13}C-GBT routine, as part of regular GSD Ia clinic visits.

The principle of the breath test in the 2nd experiment is based on the digestion of the 2 types of starches used, including hydrolysis into glucose that is subsequently absorbed and oxidized to be released as $^{13}CO_2$. This is unlike the 1st experiment where glucose was administered, which is readily absorbed and in contrast to starch does not undergo intestinal digestion [23]. Glucose oxidation from *Glycosade*® was lower compared to UCCS in GSD Ia children (12y & 13y) (Figs. 4A, 5A). This is likely due to higher amylopectin content in *Glycosade*® that delays digestion, absorption, and the oxidation of glucose (amylopectin content: 99.5% in *Glycosade*® vs. 72.8% in UCCS) [18]. However, the glucose oxidation remained the same for both starches in the 28y GSD Ia (Fig. 6A). The lack of difference in the adult GSD Ia patient is puzzling. It could be either due to a similar release of glucose from both starches in the intestine during a 4 h study period, or to similar oxidation of the glucose derived from both starches. The 28y GSD Ia was on metformin, because as a complication of his GSD Ia he had developed insulin resistance / type 2 diabetes; whether the medication influenced glucose uptake or oxidation rates of both is unknown. Recently, a randomized double-blind clinical trial by Koffert and coworkers [39] in 41 patients with type 2 diabetes showed that patients in a metformin group had a 2-fold increased intestinal glucose uptake [39]. Animal studies have shown similarly that metformin increased glucose uptake in intestinal mucosa [40], enhanced the amount of GLUT2 transporter in the small intestine, particularly in the apical enterocyte membranes of insulin resistant mice [41]. Still this known effect of metformin on mucosal glucose uptake does not explain why in our patient *Glycosade*® and UCCS-derived glucose uptake and oxidation rates were similar. The finger-prick blood glucose concentrations did not show the difference in glucose uptake for both starches in all patients with GSD Ia. Traditional measures (finger prick glucose) offer a more global picture of glucose metabolism, the breath test offers more dynamic and patient-specific details of in vivo glucose oxidation. Thus, the breath test adds value to the clinical and nutritional management of patients with GSD Ia.

Breath test based on stable isotopes has been used in several clinical conditions with the most popular and standardized test being the ^{13}C-urea breath test for the identification of *Helicobacter Pylori* infection in the stomach [42]. Previously our team was able to develop a 2-h ^{13}C-phenylalanine breath test to examine in vivo phenylalanine metabolism in response to sapropterin dihydrochloride (Kuvan®) treatment in phenylketonuria (PKU) children. Children who respond to sapropterin dihydrochloride treatment had increased $^{13}CO_2$ production from 1-^{13}C-phenylalanine, suggesting improved phenylalanine hydroxylase activity [43]. Similarly, the use of naturally enriched ^{13}C-breath tests to measure starch absorption in healthy populations [23], in obese women [44], and in patients with pancreatic disease [32], has been described before. Our current study adds to this literature with a focus on a breath test in GSD patients to benefit their dietary management. While the measurement of blood glucose concentrations will remain the key measure of clinical management and traditional monitoring of GSD patients, there is a need for a more routine sensitive test to improve management these patients. There are two key limitations to be considered, including the small sample size due to the rarity of the disease and the fact that most patients with GSD Ia were not living close to our hospital, that limit the generalizability of the results. Secondly, with such large differences in the age of the participants with GSD Ia (individualized management leading to different doses of UCCS or *Glycosade*®), it was necessary to stratify data by starch doses and age. This resulted in loss of statistical power in the 2nd experiment. Future work is necessary with a larger sample size, different hepatic GSD types, and validation studies of ^{13}C-GBT such as repeatability of the test within the same subject along with the use of continuous glucose monitor (CGM) for aiding in glucose follow up. In order to make the test more applicable in the clinic, prediction of CO_2 production values versus actual measured values needs to be validated. Further studies are needed to use ^{13}C-GBT in response to different exogenous carbohydrate doses to determine optimal glucose intakes in GSD Ia, since overtreatment with lifelong UCCS intake can put these patients at risk of insulin resistance and hyperinsulinemia [14].

In summary, the current study is a first step in establishing the ^{13}C-GBT as a minimally invasive and functional test that could potentially be used as a routine test, as part of each clinic visit in GSD Ia patients, to improve dietary therapy, to tailor UCCS supply during the daytime, and to examine glucose metabolism including new food sources like *Glycosade*®. The results indicate that the 4 h breath test with natural

enrichment of ^{13}C in starches provides valuable data on in vivo glucose metabolism. Our results can be used to tailor carbohydrate dose and type. We have shown the ^{13}C-GBT is more sensitive compared to blood glucose concentration measurements. Future studies should focus on further validation of the ^{13}C-GBT, in other GSD types, and on whether it could be useful in the nutritional and clinical management of GSD Ia.

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Declaration of Competing Interest

None.

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AT, RE, SS, SS contributed to the study design and development; AT collected and analyzed the data; RS, GH, JB, AR, TB contributed to patient recruitment; AT, SS, SS, and RE contributed to writing the manuscript; RE had primary responsibility for final content. All authors read and approved the final manuscript.

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