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Inter-Individual Variation in Postprandial Glycemic Responses in Women Co-Ingesting Green Leafy Vegetables with a Carbohydrate Meal: Interactions with the Sirtuin System

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Scope: Green leafy vegetables (GLV) may improve postprandial glycemic responses (PGR) and metabolic health. However, inter-individual variations (IIV) preclude conclusive evidence. Sirtuin system is emerging as a key player in blood glucose control. This study investigates IIV in PGR in women co-ingesting GLV with a carbohydrate meal and interactions with the sirtuin system.

Methods and Results: Volunteers ($n = 31$ women) consume rice, rice with bok choy, or spinach (75g available carbohydrate) on separate occasions. Postprandial glucose, insulin, adropin, and lipid levels are measured. Anthropometric measurements and sex hormones are measured. GeXP assay measures whole blood postprandial gene expression profiles of 25 markers involved in sirtuin signaling. GLV consumption has no significant effect on PGR, which shows high variation. PGR correlated with age, but no other consistent associations are observed. Sirtuin gene expression profiles reveal distinct stratified subgroups associated with PGR, lipid, insulin, fat mass, waist/hip circumferences, and adropin levels.

Conclusion: PGR to co-ingesting GLV with a carbohydrate meal are highly variable in this cohort and fail to reveal a significant reduction in PGR. Variable responses are largely independent of menopausal status and meal consumed. However, lower expression of sirtuin gene targets is associated with higher PGR and with markers linked to health status.

1. Introduction

Postprandial glycemia is an important indicator of metabolic health and a driver of chronic diseases such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2D). Despite interventions, incidence of these diseases is increasing with annual global CVD mortality currently at 17.9 million[1] and T2D prevalence at 425 million.[2] Further research is required to achieve global reversal targets.[3] Postprandial hyperglycemia, is a critical factor for optimizing glycemic control[4] with individuals spending most of their day in a postprandial state. Studies indicate linear relationships between postprandial glucose concentrations and CVD in individuals with normal fasting glucose[5,6] suggesting increasing postprandial glucose increases CVD risk.[7] However, many of these studies are confounded by inter-individual variation (IIV) in postprandial glycemic responses (PGR) which preclude firm conclusive evidence. Possible factors responsible for these variations include genetics,[8] biological

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sex,[9,10] menopausal status,[11] age[8,9] and insulin sensitivity.[12] Hormones such as adropin are also implicated in the regulation of metabolic health,[13] glucose regulation, and CVD.[14]

Studies report that pre-menopausal women have a lower incidence of CVD compared to men of same age, attributed partly to physiological estrogen levels.[15] However, obesity and its associated abnormalities could reverse these protective effects leading to increased CVD risk even in pre-menopausal women. The risk of CVD rapidly increases after menopause,[16] and by age 70, women show CVD rates similar to that of men.[17]

The quantity and quality of carbohydrate consumed is a major predictor of PGR.[18] Non-carbohydrate dietary constituents could reduce PGR in healthy and diabetic individuals, particularly green leafy vegetables (GLV).[19–22] Acute phase investigators, however, have reported mixed results, potentially due to IIV in PGR among healthy adults.[23,24] Sun et al. concluded that co-ingesting bok choy with rice, attenuated participants' PGR.[30] However, IIV was highlighted in their responses, demonstrating both sex specific (slower postprandial blood glucose reduction in women) and within sex IIV in PGR.[10] Research implies that multiple factors influence PGR. However, limited work has been carried out to elucidate reasons for these variations particularly in pre-, peri-, and post-menopausal women.

Previous studies in our lab have used blood gene expression profiling as a means of investigating IIV in metabolic responses to interventions.[25,28,34] Gene expression profiling technologies present opportunities to gain an insight on outputs from the human genome, permitting investigation of IIV in response to diet and associated influences on health and prevention of nutrition associated chronic diseases.[34] Studies identified variable levels of *SIRT1* in whole blood from apparently healthy human participants.[25] Further interrogation revealed that participants with low *SIRT1* expression, had poorer metabolic profiles with higher levels of plasma inflammatory markers, deregulated metabolic responses to food consumption and low levels of HDL, markers implying compromised metabolic health status.[25] Notably, this revealed that whole blood gene expression profiles can be used to characterize individual responses to dietary intervention.

Sirtuins and associated genes involved in producing the chemical nicotinamide adenine dinucleotide (NAD) play a pivotal role in aging-related metabolic diseases, in metabolic health, and glucose homeostasis.[25,26] Reduced sirtuin expression due to obesity and ageing is correlated with T2D pathogenesis[27] and sex differences are apparent.[28] The sirtuin/NAD system is also reported to be differentially regulated in response to glucose intolerance during consumption of a high fat diet.[25]

This study hypothesized that co-ingesting GLV with carbohydrates would not significantly reduce PGR due to IIV in responses and that IIV would be associated with sirtuin gene expression signatures. With much of the prevailing research on PGR excluding females due to perceived complications linked to menstruation,[29] this study aimed to investigate IIV in PGR in women co-ingesting GLV with a carbohydrate meal. The study also aimed to determine whether these IIV were associated with modulation of the sirtuin system.

2. Experimental Section

2.1. Study Population and Design

VegGI (Impact of Vegetables on Acute Glycemia and Glycemia-Induced CVD risk in women: metabolic effects and IIV) study was a randomized controlled crossover study to assess the acute effects of consuming GLV on PGR in women (**Figure 1**A,B). Inclusion criteria included written informed consent, females aged between 18 and 75 years, BMI in the range of 25–35 kg m^{-2}, HbA1c < 6.5%, total cholesterol < 5 mmol L^{-1}, systolic blood pressure (BP) < 139 mmHg and diastolic BP < 89 mmHg, no chronic health conditions. Smokers, athletes, those with food intolerances, eating disorders, pregnant or breastfeeding, using prescription medication including hormonal contraceptives, thyroid medications, or hormonal replacement therapy were excluded.

The study recruited 31 participants between July 2017 and June 2019. Power calculations based on effect sizes seen in Sun et al.[30] showed 10 women were adequate to give the study 90% power to detect differences in blood glucose (measured as incremental area under the curve: iAUC for 180 min) of about 20% in magnitude. Therefore, the number used for the study was enough to detect treatment effects and study IIV. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Rowett Institute. VegGI study is registered at Research Registry (researchregistry3117).

Participants were provided a standardized meal the evening prior to all sessions, consisting of a vegetarian paella (made in-house), a soft white roll (big bite soft white roll, Tesco, UK) and a toffee yoghurt (Muller Light Smooth Toffee Yoghurt, Tesco, UK). They attended the Human Nutrition Unit (HNU) at the Rowett Institute in the morning after an overnight fast having avoided strenuous exercise and alcohol for 24 h. Menopausal status was assessed, where pre-menopause was defined as currently menstruating; peri-menopause as experiencing noticeable changes in the length, duration, or amount of flow in the menstrual cycle; and post-menopause as not having a menstrual cycle in the last 12 months (not due to hormonal contraceptives).[31] Pre-menopausal women attended the visits during the luteal phase to minimize sex hormone variations.

2.2. Interventions and Sample Collection

A glucose drink was prepared using 75 g of anhydrous glucose (Bulk Powders, UK) dissolved in 300 mL of water (room temperature) and consumed prior to oral glucose tolerance test (OGTT). Food-based interventions were as follows: white boiled rice (R), white rice with bok choy (RB), and white rice with spinach (RS) (Figure 1A). A portion of R (Thai Fragrant Rice, Sainsbury's UK) was defined as the amount containing 75 g of available carbohydrate and was boiled in water. Bok choy (Bok Choy, Tesco, UK) and spinach (Baby Spinach, Tesco, UK) consisted of a fresh portion size of 150 g and steamed for 25 and 10 min, respectively, and pureed before serving. Volunteers were instructed to eat half the portion of GLV provided and then consume the rice and vegetable

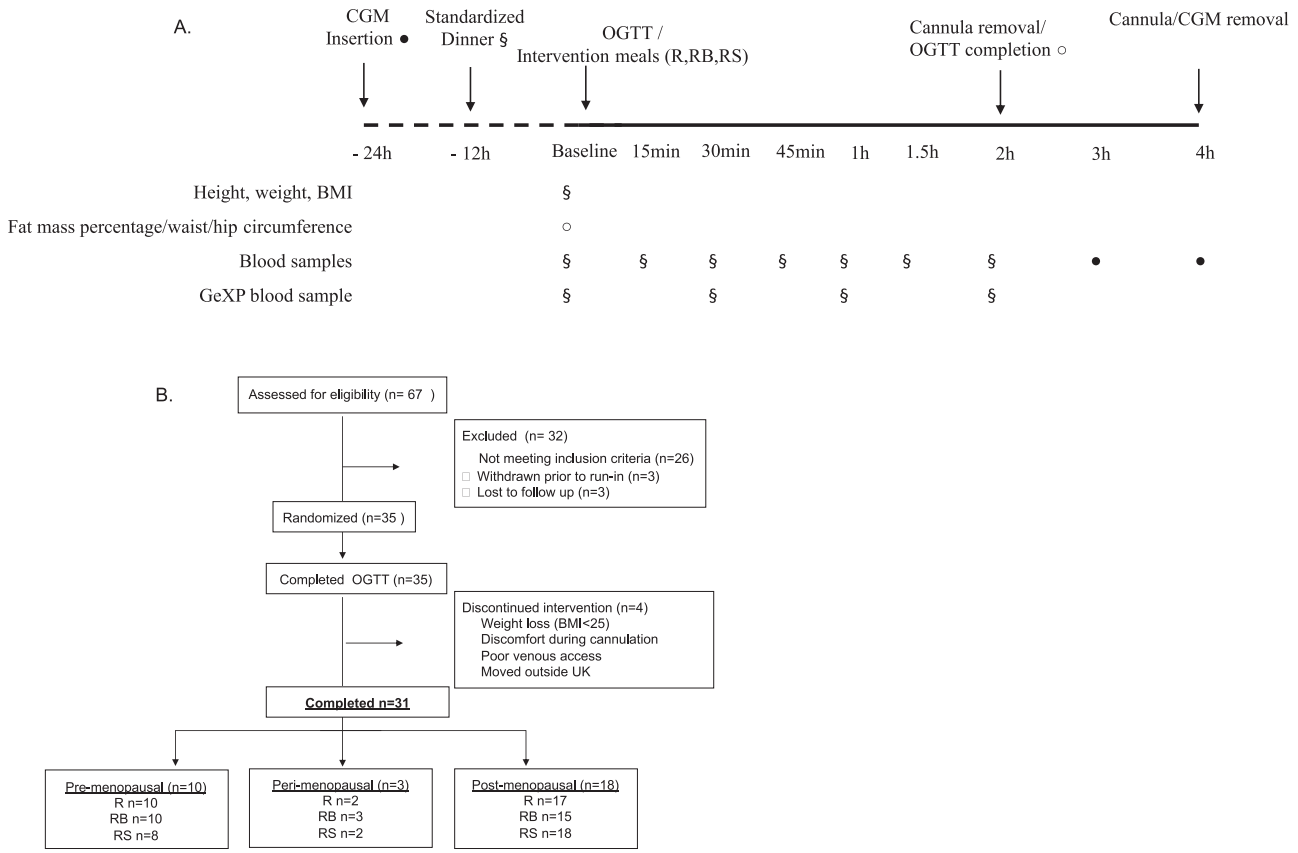

Figure 1. VegGI study overview and flow chart. A) An overview of the study involvement for each participant. Participants completed OGTT session and then randomly were assigned to consume each of the intervention meals, R, RB, and RS. B) VegGI recruitment and retention. CGM, continuous glucose monitoring system; GeXP, gene expression; OGTT, oral glucose tolerance test; R, rice; RB, rice with bok choy; RS, rice with spinach; § completed in all sessions; ● completed in R, RB, and RS; ○ completed in OGTT.

interchangeably within 15 min and the glucose drink within 5 min. Compositional information for the food-based interventions can be found in the Table S1, Supporting Information.

During the first study session (OGTT), participant anthropometric measurements (weight, waist and hip circumference, and body fat composition using BODPOD) were collected. Baseline blood samples were collected to determine fasting glucose and insulin levels and an OGTT was completed following WHO Guidelines.[32] A cannula was inserted into the antecubital fossa in either the left or right arm and kept patent with sterile saline (0.9% NaCl). Venous blood samples were collected at baseline and at 15 min, 30 min, 45 min, 1 h, 1.5 h, and 2 h postprandially into 3 mL EDTA plasma tubes (Becton Dickinson, USA) and at 30 min, 1 h, and 2 h into PaxGene tubes (PreAnalytiX). Blood samples were also collected into a 4 mL serum tube (Becton Dickinson, USA) for sex hormones analysis (Figure 1A).

Participants were randomly assigned to consume each of the intervention meals, R, RB, and RS during the course of three visits. Blood glucose levels for these intervention visits were measured using a continuous glucose monitoring system (CGM) (iPro2, Medtronic MiniMed, Northridge, USA), which measured interstitial glucose concentrations every 5 min giving 48 data points per treatment per volunteer. CGM was fitted the day prior to interventions and worn for approximately 24 h. The monitor was calibrated using finger prick blood samples analyzed by a glucose meter (Bayer Contour NEXT, Ascensia Diabetes Care, USA). Blood samples were collected as shown in Figure 1A. Four hours after the test meal was consumed, the cannula was removed (Figure 1A).

Plasma and serum were prepared by centrifugation at 3000 rpm, 4 °C for 15 min (Fisherbrand GT2 Benchtop Centrifuge Packs, Fisher Scientific, UK), aliquoted, and stored at −70 °C. Insulin, adropin, lipids, and sex hormone analysis were measured as described below. Blood collected in PaxGene tubes were stored according to the manufacturer's instructions prior to total RNA extraction (see below).

2.3. Plasma Analyses

Glucose levels during OGTT were measured using the HemoCue 201 Glucose Analyser (HemoCue AB, Sweden). Plasma insulin was measured using a two-site enzyme immunoassay kit (Mercodia human insulin ELISA, Uppsala, Sweden). iAUC was calculated for both insulin and glucose levels after intervention using the trapezoid rule.[33] Sex hormones, estradiol, progesterone, follicular stimulating hormone (FSH), and testosterone were measured in serum using an immunoassay system

(ADVIA Centaur XPT, Siemens Healthcare Ltd, UK). An ELISA kit (Cusabio USA) was used to measure plasma adropin. Lipid profiles were quantified in plasma samples using a Thermo Konelab clinical analyser (Thermo Electron Oy, Vantaa, Finland) and kits for HDL, LDL, total cholesterol, and triglycerides (Microgenics GmbH Passau, Germany).

2.4. Blood Expression Profiling of Sirtuin and NAD Biosynthetic Enzyme Genes

Total RNA was extracted from human whole blood samples using a PAXgene Blood RNA Kit (Qiagen, Crawley, UK) and 50 ng was assayed for sirtuin and NAD biosynthetic enzyme gene targets using the GenomeLab GeXP Genetic Analysis System (Beckman Coulter) and an in-house custom designed assay, the hSIRTNADPlex as described previously.[28] Details of the GenomeLab System for design of in-house custom designed multiplex assays and application to conduct blood gene expression profiling have been published previously.[25,28,34]

2.5. Statistical Analysis

Postprandial glucose and insulin responses were expressed as iAUC ignoring the area below the baseline. Glucose, insulin, and sex hormones were analyzed using linear mixed models where meals and menopausal status were fixed factors and volunteer was a random factor. Lipid profiles and adropin levels were analyzed using three-way mixed ANOVA with meals and time point used as within-subject factors and menopausal status as between-subject factor. Post hoc tests were performed to check for significant differences using Bonferroni adjustment. Pearson correlations were calculated to assess potential associations between PGR and metabolic, anthropometric, and gene expression dataset. Principal component analysis (PCA) and partial least squares (PLS) were performed on the hSIRTNAD gene expression (Y) dataset, and metabolic (fasting glucose, glucose and insulin iAUC, plasma lipid, adropin levels) and anthropometric markers (fat mass, waist and hip circumference, waist-to-hip ratio) (X) using SIMCA-P+ 14.1 software (MKS Instruments UK Ltd.). The assumptions of the statistical test have been assessed and deviations were not substantial.

Table 1. Baseline anthropometric and plasma characteristics of the study population.

	All participants ($n = 31$)
Age [years]	51.3 ± 14.8 (20–70)
Height [m]	1.6 ± 0.1 (1.5–1.8)
Weight [kg]	73.9 ± 7.7 (59.9–86.7)
BMI [kg m^{-2}]	27.9 ± 1.6 (24.8–30.5)
Total cholesterol [mmol L^{-1}]	5.4 ± 1.1 (3.0–7.0)
HbA1c [%]	5.3 ± 0.3 (4.8–5.8)
Systolic BP [mmHg]	125.4 ± 17.5 (95–171)
Diastolic BP [mmHg]	74.3 ± 8.4 (60–92)
Fat mass [%]	39.1 ± 6.8 (26.1–56.6)
Waist circumference [cm]	85.9 ± 5.7 (75.4–94)
Hip circumference [cm]	103.8 ± 6.8 (77.5–114.9)
Waist:hip	0.8 ± 0.1 (0.7–1.1)
Fasted glucose OGTT [mmol L^{-1}]	4.8 ± 0.9 (3.8–8.3)
Fasted glucose R [mmol L^{-1}]	5.3 ± 0.7 (3.4–6.9)
Fasted glucose RB [mmol L^{-1}]	5.4 ± 0.7 (3.5–6.6)
Fasted glucose RS [mmol L^{-1}]	5.5 ± 0.8 (3.3–6.7)
Fasted total cholesterol R [mmol L^{-1}]	4.6 ± 0.8 (3.2–5.8)
Fasted total cholesterol RB [mmol L^{-1}]	4.8 ± 1.1 (3.0–7.1)
Fasted total cholesterol RS [mmol L^{-1}]	4.7 ± 0.9 (3.1–6.9)
Fasted HDL R [mmol L^{-1}]	1.4 ± 0.4 (0.8–2.1)
Fasted HDL RB [mmol L^{-1}]	1.4 ± 0.4 (0.8–2.2)
Fasted HDL RS [mmol L^{-1}]	1.4 ± 0.3 (0.9–2.2)
Fasted LDL R [mmol L^{-1}]	2.6 ± 0.7 (1.4–4.3)
Fasted LDL RB [mmol L^{-1}]	2.7 ± 0.8 (1.5–4.2)
Fasted LDL RS [mmol L^{-1}]	2.8 ± 0.8 (1.5–4.6)
Fasted TRG R [mmol L^{-1}]	1.2 ± 0.5 (0.4–2.4)
Fasted TRG RB [mmol L^{-1}]	1.3 ± 0.5 (0.5–2.8)
Fasted TRG RS [mmol L^{-1}]	1.2 ± 0.5 (0.6–2.4)
Fasted adropin R [ng mL^{-1}]	1.3 ± 0.7 (0.2–2.9)
Fasted adropin RB [ng mL^{-1}]	1.4 ± 0.6 (0.2–3.5)
Fasted adropin RS [ng mL^{-1}]	1.3 ± 0.7 (0.3–3.4)
Fasted adropin OGTT [ng mL^{-1}]	1.5 ± 0.8 (0.3–3.6)

Data presented as mean ± SD (range); BP, blood pressure; OGTT, oral glucose tolerance test; R, rice; RB, rice with bok choy; RS, rice with spinach; TRG, triglyceride.

3. Results

3.1. Study Participant Anthropometric Data

Thirty-one women were recruited to the VegGI study and completed the four study sessions (10 pre-menopausal, 3 peri-menopausal, and 18 post-menopausal). The mean ± SD age was 51.3 ± 14.8 years, height 1.63 ± 0.08 m, weight 73.9 ± 7.7 kg, and BMI 27.9 ± 1.6 kg m^{-2}. Baseline anthropometric measures are shown in **Table 1**.

3.2. Postprandial Glucose and Insulin Responses to GLV

Due to CGM technical issues, PGR was measured on 29 participants in R visit (10 pre-menopausal, 2 peri-menopausal, and 17 post-menopausal), on 28 participants in RB (10 pre-menopausal, 3 peri-menopausal, and 15 post-menopausal), and on 28 in RS (8 pre-menopausal, 2 peri-menopausal, and 18 post-menopausal). Mean postprandial plasma glucose and insulin changes from baseline during the 4 h postprandial period for the three intervention sessions are shown in **Figure 2**A,B. There were no significant differences in glucose iAUC in the study cohort in response to R (175.8 ± 107.6 mmol × min L^{-1} 0–240 min), RB (182.8 ± 130.9 mmol × min L^{-1} 0–240 min) or RS (171.7 ± 127.3 mmol × min L^{-1} 0–240 min) ($p = 0.487$) (**Table 2**). Likewise, there were no significant differences in insulin iAUC in response to R (4143.3 ± 2544.7 mmol × min L^{-1} 0–240 min), RB (3476.8 ± 2415.6 mmol × min L^{-1} 0–240 min), or RS (3870.0 ± 2699.5 mmol × min L^{-1} 0–240 min) ($p = 0.104$) (Table 2). Postprandial glucose and insulin were then assessed separately in the three menopausal groups (Figure 2C–H). No significant

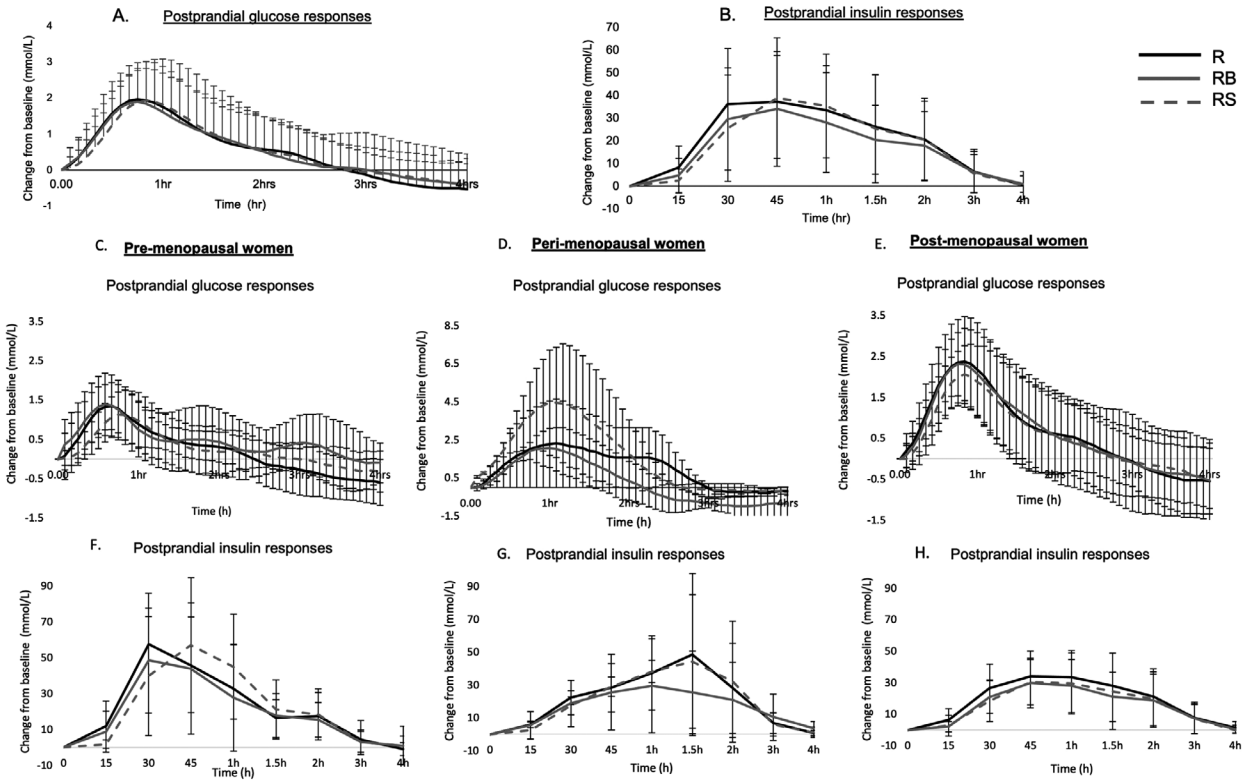

Figure 2. Postprandial plasma glucose responses using continuous glucose readings from CGM and insulin responses to R, RB, and RS. Mean change in A) blood glucose using CGM monitor and B) insulin responses in all participants. Mean change in blood glucose using CGM in C) pre-menopausal (R, RB $n = 10$, RS $n = 8$), D) peri-menopausal (R, RS $n = 2$, RB $n = 3$), and E) post-menopausal (R $n = 17$, RB $n = 15$, RS $n = 18$) women and in insulin responses in F) pre-menopausal, G) peri-menopausal, and H) post-menopausal women. Data presented as mean ± SD. Linear mixed model analysis was used on mean levels at a significance level of $p < 0.05$ using meal and menopausal status as fixed factors and volunteers as random factor. CGM, continuous glucose monitor; iAUC; incremental area under the curve; R, rice; RB, rice with bok choy; RS, rice with spinach.

interaction effects on glucose iAUC were found between the three intervention sessions and menopausal status ($p = 0.140$) (Table 2). There was no significant menopausal status effect or meal × menopausal status interaction for insulin (Table 2). However, marked IIV was observed within the three menopausal groups for both glucose and insulin iAUC in response to all test meals (**Figure 3**A,B). Postprandial glucose responses significantly and positively correlated with age ($r = 0.39$–0.44, $p < 0.05$). No other consistent correlations were observed for PGR.

3.3. Sex Hormones

Sex hormone concentrations were measured at each study visit prior to each test meal (0 h). This confirmed significantly higher FSH and lower estradiol in post-menopausal women (**Table 3**). There was no consistent significant association between menopausal status and PGR.

3.4. Lipid Profiling

Mean postprandial lipids for all participants following the three interventions are summarized (Table S2, Supporting Information). Repeated measures ANOVA confirmed significant time ($p \leq 0.001$) and time × meal ($p = 0.034$) effects for cholesterol levels. Cholesterol levels between the meals appeared to increase from 1 to 4 h (Table S2, Supporting Information). A significant time effect ($p \leq 0.001$) for HDL, LDL, and triglyceride levels was observed with no other significant interaction effects (Table S2, Supporting Information).

3.5. Adropin

There was no statistically significant interaction between intervention meals and time on plasma adropin in the cohort ($p = 0.950$). However, there was a time × meal interaction ($p = 0.041$) and time effect ($p < 0.001$) (**Figure 4**). Post hoc Bonferroni correction applied indicated that OGTT resulted in higher average adropin levels compared to R (1.37 ± 0.07 ng mL^{-1} and 1.21 ± 0.06 ng mL^{-1}, respectively) but not compared to RB (1.21 ± 0.08 ng mL^{-1}) and RS (1.22 ± 0.06 ng mL^{-1}).

3.6. Sirtuin System Gene Expression Profiling

The hSIRTNADPlex gene expression profiles were measured at baseline and postprandial time points 30 min, 1 h, and 2 h in all study sessions using whole blood total RNA

Table 2. Incremental areas under the curve (iAUC) for 240 min of postprandial plasma glucose and insulin in VegGI cohort.

	All (n = 31)			Pre-menopausal			Peri-menopausal			Post-menopausal		
	R	RB	RS	R (n = 8)	RB (n = 10)	RS (n = 10)	R (n = 2)	RB (n = 3)	RS (n = 2)	R (n = 17)	RB (n = 15)	RS (n = 18)
Glucose iAUC	175.8 ±	182.8 ±	171.7 ±	104.0 ± 52.0	141.8 ± 96.2	90.1 ± 55.5	270.2 ±	159.2 ± 83.3	383.3 ±	206.9 ±	214.9 ±	184.4 ±
[mmol × min L^{-1} 0–240 min]	107.6	130.9	127.3				142.1		245.6	110.0	153.9	111.1
Insulin iAUC	4143.3 ±	3476.8 ±	3870.0 ±	4061.6 ±	3575.3 ±	4241.0 ±	4865.7 ±	3886.6 ±	48 169 ±	4068.2 ±	3353.8 ±	3506.0 ±
[mmol × min L^{-1} 0–240 min]	2544.7	2415.6	2699.5	2273.7	2601.0	2946.0	3654.3	3933.2	3124.5	2649.0	2201.0	2595.6

Data presented as mean ± SD, data were analyzed by linear mixed model at a significance level of $p < 0.05$ using meal and menopausal status as fixed factors and volunteers as random factor. No significant differences were observed; iAUC, incremental area under the curve; R, rice; RB, rice with bok choy; RS, rice with spinach.

(RIN values 5.2–8.9). Gene expression was normalized using ubiquitin-conjugating enzyme E2 D2 (*UBE2D2*) as Normfinder (https://moma.dk/normfinder-software)[35] analysis of the hSIRTNADPlex gene expression data indicated that UBE2D2 exhibited stable expression in the blood samples. Several sirtuin system gene responses revealed significant changes in expression when determined using linear mixed model analysis with meal (OGTT, R, RB, and RS), time, and meal × time interactions assigned as fixed factors. *HIC1* expression levels remained skewed following log transformation of the data and were excluded from further statistical analysis.

Sixteen genes altered significantly post-consumption of the meals with increased (*SIRT1, SIRT2, SIRT3, SIRT6, SIRT7, NMRK1, NAMPT, PNP*, and *ABCA1*) or decreased expression (*SIRT4, SIRT5, NMNAT3, PARP1, PPIB, NMNAT1*, and *QPRT)* (Figure S1, Supporting Information). Responses to meals were significantly different for nine sirtuin genes (*SIRT2, SIRT3, SIRT6, SIRT7, NMNAT3, PNP, PPIB, NAMPT*, and *PARP1*). However, changes in relevant expression were small (Figure S1, Supporting Information). Comparison of pre- and post-menopausal groups using repeated measures ANOVA revealed significant differences only in the expression levels of *NAMPT*, which showed a time × meal × menopausal status interaction (p = 0.014) with no other significant effects (Figure S2, Supporting Information).

PCA biplot was applied to identify patterns that could be linked to variable PGR. PCA was conducted on the entire hSIRTNAD gene expression dataset (excluding *HIC1*) to determine associations between the sirtuin system and PGR variation (**Figure 5**A,B). The first two PCA components accounted for 46.2% of the dataset variation (Figure 5A,B). This variation did not seem to be explained by the menopausal status (Figure 5A) nor by the meal consumed (Figure 5B). The PCA biplot identified that the participants located on the right side of the central vertical axis of the plot (subsequently termed subgroup A) exhibited higher expression of the sirtuin system genes. Further analysis of gene expression data using repeated measures ANOVA, showed gene markers *SIRT2, SIRT3, SIRT6, ABCA1, PARP1, PPIB, NMNAT1, SIRT1, NADSYN1 (*all $p < 0.001$), *NMRK1* (p = 0.009), *NAMPT* (p = 0.001), and *SIRT5* (p = 0.010) (Supporting Information S3) were significantly differently expressed in subgroups A and B, indicating these genes contributed to stratification of the cohort. However, most of the anthropometric and plasma baseline characteristics for subgroups A and B did not demonstrate any significant differences (Table S3, Supporting Information).

PLS analysis was conducted on the hSIRTNAD gene expression dataset, and metabolic and anthropometric markers (X) to identify factors associated with the observed variation of the sirtuin system in the cohort (Figure 5C,D) and PGR. The PLS plot revealed that subgroup B was associated with higher baseline glucose levels, iAUC for glucose and insulin, higher waist and hip circumference, fat mass percentage, and triglyceride level. In addition, these individuals seemed to have lower adropin and HDL levels. This association was not associated with menopausal status (Figure 4C) nor by the meal consumed (Figure 4D). Notably, correlation analysis also revealed a positive association between HDL and adropin in the pre-menopausal women but not in the whole cohort or in the post-menopausal

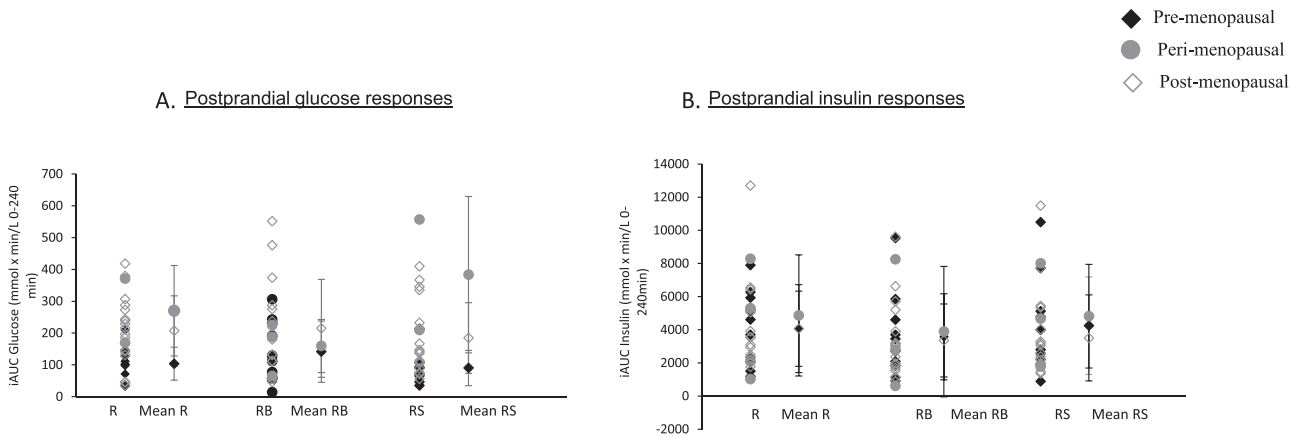

Figure 3. Mean and individual postprandial glucose and insulin responses to R, RB, and RS. A) Sum and mean (±SD) iAUC for blood glucose using CGM monitor in pre-menopausal (black diamond) (R, RB $n = 10$, RS $n = 8$), peri-menopausal (gray circle) (R, RS $n = 2$, RB $n = 3$), and post-menopausal (gray open diamond) (R $n = 17$, RB $n = 15$, RS $n = 18$) and B) sum and mean ($n = 31$) ± SD iAUC for insulin levels in pre-menopausal (black diamond), peri-menopausal (gray circle), and post-menopausal women (gray open diamond) following consumption of R, RB, and RS. CGM, continuous glucose monitor; iAUC; incremental area under the curve; R, rice; RB, rice with bok choy; RS, rice with spinach.

Table 3. Plasma sex hormone concentrations in each menopausal group across all interventions.

		Pre-menopausal	Peri-menopausal	Post-menopausal
FSH [IU L^{-1}]	OGTT	8.7 ± 8.8^{a}	37.4 ± 57.2^{a}	78.3 ± 26.2^{b}
	R	5.1 ± 2.1^{a}	24.6 ± 21.9^{a}	78.2 ± 27.3^{b}
	RB	5.5 ± 3.4^{a}	$41.6 \pm 46.3^{a,b}$	77.4 ± 25.3^{b}
	RS	4.5 ± 3^{a}	58.4 ± 51.3^{b}	80.6 ± 24.7^{b}
Estradiol [nmol L^{-1}]	OGTT	0.7 ± 0.3^{a}	0.5 ± 0.4^{a}	0.09 ± 0.07^{b}
	R	0.4 ± 0.2^{a}	$0.4 \pm 0.4^{a,b}$	0.09 ± 0.1^{b}
	RB	0.6 ± 0.5^{a}	$0.4 \pm 0.3^{a,b}$	0.08 ± 0.07^{b}
	RS	0.4 ± 0.2^{a}	$0.2 \pm 0.2^{a,b}$	0.09 ± 0.1^{b}
Progesterone [nmol L^{-1}]	OGTT	18.7 ± 25.3^{a}	19.7 ± 32.1^{a}	1.4 ± 2.9^{b}
	R	13 ± 12.9^{a}	$11.8 \pm 18^{a,b}$	0.7 ± 0.6^{b}
	RB	11.1 ± 11.7	11.4 ± 17.4	0.7 ± 0.6
	RS	18.4 ± 20.0^{a}	$11.3 \pm 17.2^{a,b}$	0.9 ± 0.7^{b}
Testosterone [nmol L^{-1}]	OGTT	1.1 ± 0.2	1 ± 0.1	0.8 ± 0.5
	R	0.9 ± 0.3	0.9 ± 0.1	0.7 ± 0.3
	RB	1.0 ± 0.3	0.9 ± 0.02	0.7 ± 0.3
	RS	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.3

Data presented as mean ± SD; Linear mixed model analysis at a significance level of $p < 0.05$ using meal and menopausal status as fixed factors and volunteers as random factors; Post hoc analysis were done by pairwise comparison; Different superscript letters indicate significant differences between the means across the menopausal groups within each visit; FSH, follicle stimulating hormone; OGTT, oral glucose tolerance test; R, rice; RB, rice with bok choy; RS, rice with spin.

women. However, a degree of IIV was still apparent within subgroups A and B (Figure 5A–D).

4. Discussion

The findings from this study did not provide conclusive evidence on GLV reducing acute phase glycemia within the specific study parameters. Based on other studies,[19–22] there could be beneficial effects of GLV on glycemia, perhaps in the long term, when other types of GLV are given, or when quantities and method of preparation are changed. Indeed, this supports why some studies that gave GLV whole saw effects whilst we did not when given pureed. However, there was notable IIV in PGR, suggesting multiple factors in addition to meal composition influencing PGR. Studies report inter- and intra-individual variations between 25% and 56% in subjects consuming identical test meals.[23,36,37] Individual genetics may be a factor determining individual responses to diet and the sirtuin/NAD system was investigated to determine associations with the observed PGR variations.

The sirtuin system consists of seven sirtuin genes (*SIRT* 1–7) and genes encoding enzymes that are involved in the de novo synthesis of NAD from dietary tryptophan and vitamin B3. In addition, several enzymes are involved in recycling NAD to regulate sirtuin activity to maintain homeostasis and respond to metabolic

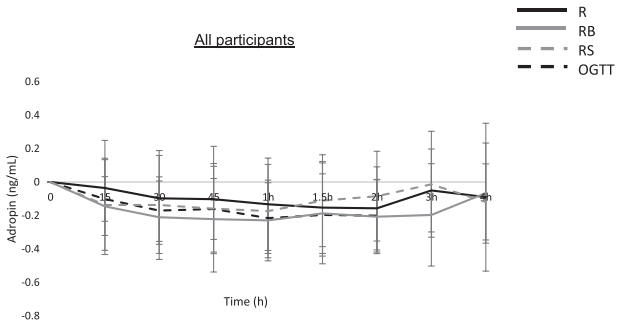

Figure 4. Plasma postprandial adropin levels. Mean ± SD change from baseline plasma adropin levels in response to R (black line), RB (gray line), RS (gray dotted line), and OGTT (black dotted line) in all participants ($n = 31$). Repeated measures ANOVA were used at a significance level of $p < 0.05$. OGTT, oral glucose tolerance test; R, rice; RB, rice with bok choy; RS, rice with spinach.

stress.[25,26] This study measured whole blood profiles of the sirtuin system in women following the consumption of R, RB, RS to determine whether PGR variation was associated with SIRT system profile in each participant as opposed to focusing on acute effect of meal interventions. Subsequent analysis of sirtuin gene expression profiles measured in whole blood samples identified factors associated with IIV in PGR in VegGI cohort.

PCA analysis indicated sirtuin system gene expression patterns were not ascribed by menopausal status or the meal consumed except for reduced *NAMPT* in post-menopausal women. *NAMPT* is involved in many important biological processes, including metabolism, stress response, and aging[39] (Figure S2, Supporting Information). However, gene expression measures used as input for PCA revealed stratification of the cohort into two subgroups, designated subgroups A and B. Subgroup A had higher expression of several sirtuin system gene targets compared to subgroup B. Gene expression profiles characterizing subgroup A were higher levels of *NMRK1, SIRT5, NADSYN1, NMNAT1, SIRT6, PPIB, SIRT1, SIRT2, ABCA1, SIRT3, NAMPT, and PARP1* expression, and lower levels of *NMRK2 and NMNAT2*. PLS analysis revealed that the sirtuin gene expression profiles that distinguish subgroups A and B were associated with markers linked to metabolic health status. Subgroup A was associated with a lower basal glucose and insulin and correspondingly lower iAUC for glucose and insulin. These results are in line with those of previous studies showing that *SIRT1*

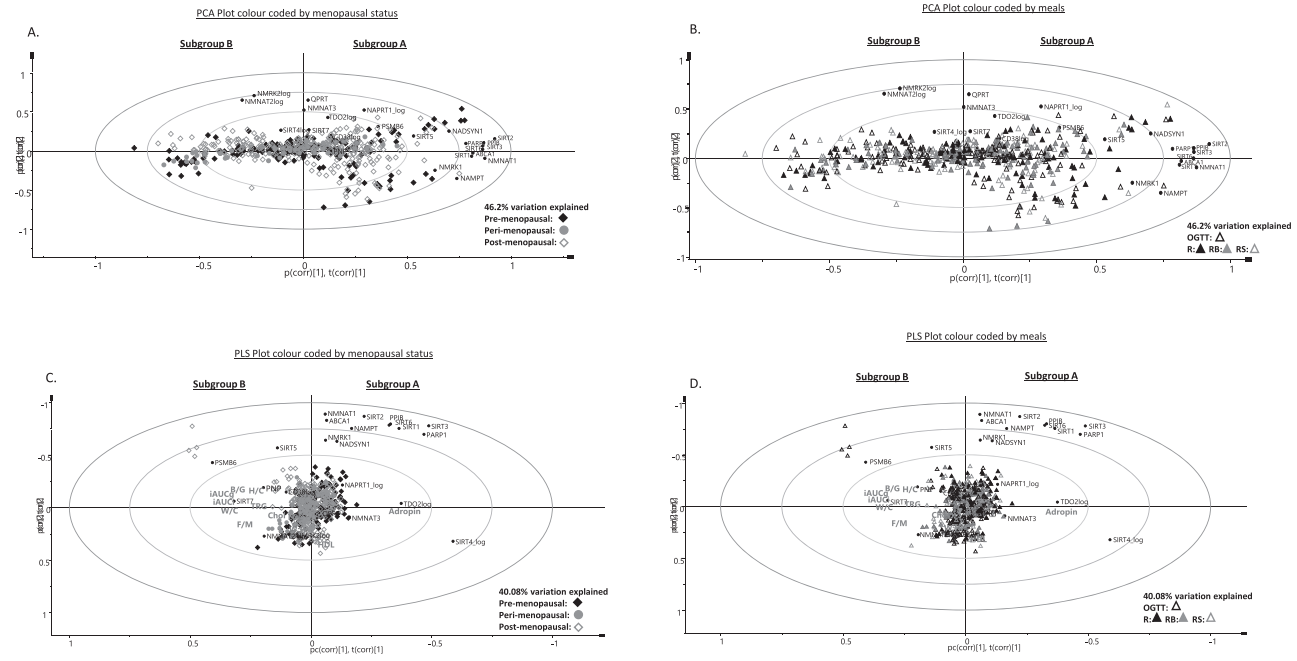

Figure 5. Principal component analysis (PCA) biplot and partial least squares (PLS) analyses of UBE2D2 normalized GeXP hSIRTNAD data from human whole blood at baseline (0 h) and at 30 min, 1 h, 2 h following OGTT (△), R (▲), RB (▲), and RS (△). Pre- (◆), peri- (●), and post-menopausal (◇). PCA biplot color coded by A) menopausal status and B) meals consumed. Gene expression data positions, denoted by • with the gene symbol placed next to it (black font), indicate associations with study subjects. The measurements all fall within the Hotelling T2 95% confidence limit. PLS plot of gene expression data (Y) denoted by • with gene symbol indicated in black font and anthropometric and metabolic markers (X) indicated in gray font coded by C) menopausal status and D) meals consumed. Skewed data was log-transformed before using in the plots. ABCA1, ATP-binding cassette, sub-family A, member 1; B/G, baseline glucose; CD38, cyclic ADP-ribose hydrolase 1; chol, cholesterol; F/M, fat mass%; H/C; hip circumference; iAUCg, incremental area under the curve for glucose responses; iAUCi, incremental area under the curve for insulin responses; NAD, nicotinamide adenine dinucleotide; NADSYN1, NAD synthetase 1; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT1, nicotinate phosphoribosyltransferase domain containing 1; NMNAT1, nicotinamide nucleotide adenylyltransferase 1; NMNAT2, nicotinamide nucleotide adenylyltransferase 2; NMNAT3, nicotinamide nucleotide adenylyltransferase 3; NMRK, nicotinamide riboside kinase; OGTT, oral glucose tolerance test; PARP1, poly (ADP-ribose) polymerase 1; PNP, purine-nucleoside/orthophosphate ribosyltransferase; PPIB, peptidylprolyl isomerase B (cyclophilin B); PSMB6, proteasome (prosome, macropain) subunit, beta type, 6; R, rice; RB, rice with bok choy; RS, rice with spinach, QPRT, quinolinate phosphoribosyltransferase; SIRT, sirtuin; TDO2, tryptophan 2,3-dioxygenase; TRG, triglycerides; UBE2D2, ubiquitin-conjugating enzyme E2D 2; W/C; waist circumference; W:H, waist to hip ratio.

expression is lower in individuals who are insulin resistant and glucose intolerant.[40] Previous research from our lab revealed compromised metabolic status in individuals with low levels of *SIRT1*.[25] Notably, *SIRT1*, *SIRT2*, and 6, expressed at higher levels in subgroup A, have been shown to regulate metabolism, with beneficial effects on glucose metabolism and improved insulin resistance.[41] Lower postprandial expression levels of these genes in subgroup B may imply compromised sirtuin activity. Subgroup B exhibited lower sirtuin system activation, which was associated with higher PGR, iAUC insulin, plasma lipid, fat mass and waist/hip circumferences and lower adropin, independent of menopausal status and test meal consumed. This is consistent with reports of adropin's involvement in preventing insulin resistance and impaired glucose tolerance.[42] In the VegGI study, a time and meal-dependent postprandial decrease in plasma adropin was observed. These results agree with Stevens et al., (2016), who reported significant meal effects of adropin and a decrease over time compared to baseline following a mixed meal containing 60% energy as carbohydrate. However, in the VegGI study, we did not find any consistent correlation between adropin and PGR. The current study also identifies a relationship between adropin concentrations, glucose regulation, and HDL levels in pre-menopausal women. However, the current analysis was performed on a small sample size of pre-menopausal women and requires cautious interpretation.

Although epidemiological studies report body composition as risk factors for metabolic diseases,[38] no correlation between body composition and PGR was found in the VegGI cohort. Lack of expected links with metabolic health markers may be attributed to the "high-risk" participants recruited. Since VegGI participants all have a BMI > 25, this may be linked to poorer glycemic control compared to individuals with a "healthy" BMI. Declining estrogen levels during menopause lead to redistribution of fat from the lower body to abdominal compartments and are linked to increased T2D and associated risk factors.[38] However, PGR was not linked to waist/hip circumference in post-menopausal women. This may lead to compromised metabolic health in our cohort of women regardless of menopause status and lessening differences between pre- and post-menopausal groups.

Overall, PLS analysis suggests that PGR variation is partly associated with body composition, adropin and sirtuin system gene expression. PLS analysis clearly identifies that neither incorporation of GLV in test meals or menopause status are unequivocal predictors of PGR. The stratification of the VegGI cohort based on sirtuin gene expression profiles does identify factors linked to metabolic health that influence PGR.

Three peri-menopausal women were recruited, making it difficult to draw conclusions for this group. However, metabolic variability in these women (Figure 5A–D) warrants more research to comprehend the impact of menopause on glucose regulation and metabolic health. Nevertheless, menopausal comparisons were still performed with sufficient numbers in pre- and post-menopausal women ($n = 10$ and 18, respectively), as it was considered appropriate to report all different comparisons.

In conclusion, VegGI study does not support evidence that co-ingestion of GLV with a carbohydrate meal reduces PGR in the acute phase. This is not in agreement with epidemiological studies reporting a potential beneficial effect of high GLV consumption on T2D.[20,21] As hypothesized, there was a high degree of IIV in PGR, which was not attributed to menopausal status or the meal consumed. Gene expression profiling in our previous studies[25] and in VegGI study have proved useful in characterizing IIV. Sirtuin gene expression profiles identified stratified subgroups in the VegGI cohort and associated metabolic and anthropometric markers providing links to differing metabolic health within the cohort. The study supports links between adropin and metabolic control, with higher PGR associated with lower adropin levels. Our findings emphasize the need for improved study designs, analyses, and reporting of variation in nutrition studies. Grouping individuals based on criteria assumed to generate homogenous groups is often overly simplistic and leads to misreporting of findings and inaccurate interpretations. The VegGI study demonstrated that gene profiling could prove a useful tool to generate accurate and detailed data on study cohorts. This may be used to stratify participants and identify associated metabolic and anthropometric markers to assist in identifying determinants of variation in nutritional responses. Subgroup-based approaches based on metabolic phenotype were also recently suggested in order to deal with IIV.[43] Future nutrition studies should accurately report IIV within study cohorts and further investigate the causes of IIV in PGR.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.S., V.R., and J.E.D. designed research; M.S. and A.J.F. conducted research; M.S., A.J.F., and G.H. analyzed data and performed statistical analysis; J.H. and M.S. wrote paper; M.S. had primary responsibility for final content; M.S, V.R., J.E.D., A.J.F., and G.H. interpreted data; M.S., V.R., J.E.D., A.J.F., and G.H. discussed and edited the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

carbohydrates, inter-individual variation, postprandial glycemia, sirtuins, women

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