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Variable Glycemic Responses to Intact and Hydrolyzed Milk Proteins in Overweight and Obese Adults Reveal the Need for Precision Nutrition

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ABSTRACT

Background: Dietary modifications can contribute to improved pancreatic β cell function and enhance glycemic control.

Objectives: The objectives of this study were as follows: *1*) to investigate the potential of milk protein hydrolysates to modulate postprandial glucose response; *2*) to assess individual responses; and *3*) to explore the inter- and intraindividual reproducibility of the response.

Methods: A 14-d randomized crossover study investigated interstitial glucose levels of participants in response to 12% w/v milk protein drinks (intact caseinate and casein hydrolysate A and B) consumed in random order with a 2-d washout between treatments. Milk protein drinks were consumed immediately prior to study breakfast and evening meals. Twenty participants (11 men, 9 women) aged 50 $\pm$ 8 y with a body mass index (in kg/m^2) of 30.2 $\pm$ 3.1 were recruited. Primary outcome was glucose levels assessed at 15-min intervals with the use of glucose monitors.

Results: Repeated-measures ANOVA revealed that for breakfast there was a significant difference across the 3 treatment groups ($P = 0.037$). The ability to reduce postprandial glucose was specific to casein hydrolysate B in comparison with intact caseinate ($P = 0.039$). However, despite this significant difference, further examination revealed that only 3 out of 18 individuals were classified as responders ($P < 0.05$). High intraclass correlation coefficients were obtained for glucose response to study meals (intraclass correlation coefficient: 0.892 for breakfast with intact caseinate). The interindividual CVs were higher than the intraindividual CVs. Mean inter- and intraindividual CVs were 19.4% and 5.7%, respectively, for breakfast with intact caseinate.

Conclusion: Ingestion of a specific casein hydrolysate successfully reduced the postprandial glucose response; however, at an individual level only 3 participants were classified as responders, highlighting the need for precision nutrition. Exploration of high interindividual responses to nutrition interventions is needed, in combination with the development of precision nutrition, potentially through an *n*-of-1 approach. This clinical trial was registered as ISRCTN61079365 (https://www.isrctn.com/). *J Nutr* 2019;149:88–97.

Keywords: hydrolysate, postprandial glycemic response, glucose monitoring, precision nutrition, interindividual responses

Introduction

Over the past decade the prevalence of type 2 diabetes (T2D) has continued to rise globally (1, 2). The prevalence of diabetes in adults worldwide was estimated to be 8.8% in 2015, and is predicted to increase to 10.4% in 2040, with the majority of these individuals being affected by T2D (3). As a result, T2D is a huge economic burden (4). The risk of T2D can be decreased by $\leq$58% by implementing diet and exercise interventions (5). Therefore, research is needed to identify and implement novel strategies to optimize glycemic control.

There is an increasing acknowledgment that nutritional interventions are not applicable or successful for entire study populations, leading to increased interest in profiling individual participant characteristics, and ultimately highlighting the need for precision nutrition (6). Consequently, there is heightened interest in exploring responses at an individual level, and in identifying responders and nonresponders in intervention

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settings (7, 8). Furthermore, the use of "omic" techniques may allow for advances in precision nutrition by exploring the variability in responses (9). The use of objective, data-driven signatures may have potential for identifying individuals who will benefit from dietary interventions in the future (7, 10). A recent intervention study observed high interindividual variability in glucose response to whey protein ingestion, highlighting that not everyone will benefit from supplementation, and promoting the need to tailor the diet to individual responses (8, 11).

Dairy is a food group which has received attention in relation to T2D risk and glycemic control. Total dairy intake, and in particular low-fat dairy, was inversely associated with T2D risk according to several meta-analyses (12–14); however, a meta-analysis by Chen et al. (15) identified that an increased intake of yogurt only was associated with a reduced risk of T2D. Furthermore, meta-analyses have produced mixed results in terms of milk consumption and its association with T2D incidence, with several publications identifying no significant association between total milk intake and T2D risk (16, 17), and others between only low-fat or skimmed milk and T2D risk (13, 14, 18, 19). Evidence from intervention studies is emerging on the benefit of milk protein consumption in aiding glycemic control. A number of studies have examined the potential of casein and whey proteins to increase insulin secretion in healthy, obese, prediabetic, and T2D individuals (20–24). In particular, hydrolyzed casein and whey protein–derived peptides have shown particular efficacy in optimizing glycemic management (25). Jonker et al. (24) determined that 12 g of casein hydrolysate added to 50 g of carbohydrate load increased insulin secretion and decreased glucose response over time compared with a carbohydrate load alone. Collectively, these studies support the potential of milk proteins in glycemic management in an acute setting. Although the potential exists for a glycemic management role for milk proteins, it is necessary to be cognizant of the emerging literature in terms of the high interindividual responses with respect to glucose regulation (26, 27). Therefore, this study had the following objectives: *1*) to investigate the potential of milk protein hydrolysates to modulate postprandial glucose response; *2*) to assess postprandial glucose response to milk protein hydrolysates at an individual level; and *3*) to explore the inter- and intraindividual reproducibility of the postprandial glucose response to study meals measured by continuous glucose monitoring.

Methods

Study population

All experimental procedures were in accordance with the Declaration of Helsinki and were approved by the Human Research Ethics Committee—Sciences (LS), ethics number LS16-13-Brennan, and all participants provided written informed consent before taking part in the intervention. The study was registered at the ISRCTN registry under ISRCTN61079365. Participants were recruited via poster advertisement and media such as email, online notice boards, and word of mouth.

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Supplemental Tables 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
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Abbreviations used: CH-A, casein hydrolysate A; CH-B, casein hydrolysate B; ICC, intraclass correlation coefficient; RI, reliability index; T2D, type 2 diabetes.

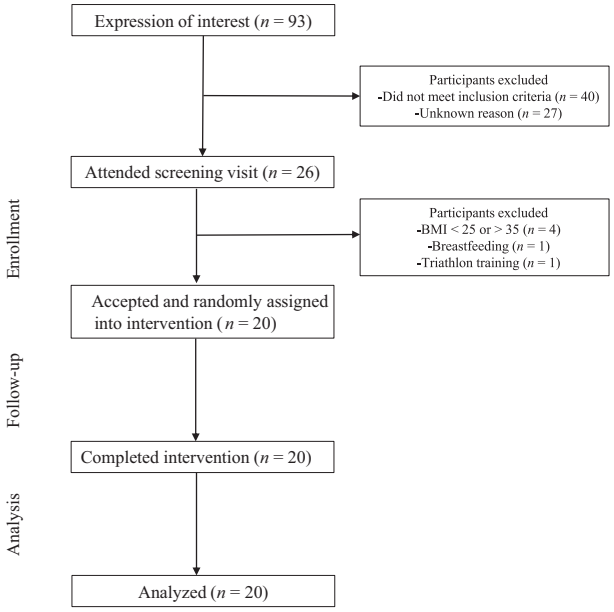

FIGURE 1 Flow diagram displaying the recruitment process for the intervention study.

Recruitment started on 1 October 2016 and ended on 1 September 2017. Inclusion criteria included individuals aged 40–65 y, with a BMI (in kg/m^2) of >25 and <35, taking no prescription medication, and free of milk allergies or lactose intolerance. Exclusion criteria included the presence of any chronic or infectious diseases, taking prescribed medication, not meeting the criteria for BMI or age, being pregnant or lactating, or having a known allergy to medical-grade adhesive. Estimation of the sample size for this study was based on a previously published glucose monitoring study, in which a follow-up 2-arm randomized controlled trial examined personalized dietary interventions to improve postprandial glycemic responses (26). Using the data presented for postprandial glycemic responses, a sample size of 17 is required to see differences between groups at a power of 80% and an α of 5%. Twenty healthy participants were accepted onto the Food for Health Ireland Glucose Monitoring Study, to allow for a dropout rate, following an initial screening visit at the Institute of Food and Health in University College Dublin, Ireland (**Figure 1**).

Study design

This was a randomized crossover study investigating postprandial glucose levels of participants in response to milk protein hydrolysates, using continuous glucose monitoring in a free-living setting. Participants attended the intervention suites at the Institute of Food and Health at the beginning and end of the 2-wk study. Participants were randomly assigned to receive the following in random order: *1*) intact sodium caseinate (control), *2*) casein hydrolysate A (CH-A), and *3*) B (CH-B); these milk products were given in a 12-g serving in the form of a protein shake. An online, open-access tool (www.randomizer.at) was used to handle randomization of the study population. CH-A was prepared by the University of Limerick from a 1% (w/w) sodium caseinate [89.2% (w/w) protein, Kerry Group] solution, which was hydrolyzed at pH 7.0, 50°C for 240 min with a food-grade commercial preparation as previously outlined (28). CH-B was prepared as previously described; a different temperature and hydrolysis time were used than for CH-A (29–31). Laboratory-scale hydrolysates were scaled-up sequentially to 50- and 1000-L volumes. At each increase in scale the effect of processing on physical properties, microbial quality, and biofunctionality was determined. The finalized process for manufacture of casein hydrolysates consisted of the following steps: *1*) dissolution of sodium caseinate (Kerry Group) to 10% protein (w/w) in reverse osmosis treated water; *2*) hydrolysis with a food-grade enzymatic preparation at a defined temperature

and pH; *3*) heat-induced enzyme inactivation; *4*) evaporation to 40% total solids; and *5*) spray drying (Niro TFD 20 Tall-Form Dryer, GEA). Powders were agglomerated to ensure quick rehydration during preparation of the protein drinks. The protein drinks were consumed 2 times/d for 3 d, directly before ingestion of study breakfast and evening meals. Participants were given individual sachets of protein powder and made up the drinks in 100 mL of cold water in a shaker according to the instructions provided (12% w/v solution). Lunch was standardized in the middle of the day of each arm. A washout period of 2 d was employed between treatments, during which time participants followed their normal diet. All study meals were provided to participants at intervals throughout the study to prepare and eat at home or work.

Anthropometric measurements, blood pressure, and a fasting blood sample were taken on day 0 of the study. Glucose was measured continuously throughout the 14-d study period through the use of the FreeStyle Libre System (Abbott Diabetes Care). During the study period, participants were asked to record all food and drinks consumed in the diary provided. The glucose levels were the primary outcome and were assessed with the glucose monitor. Control throughout a 24-h period was assessed as the secondary outcome; by assessing average 24-h glucose, 24-h SD, daytime glucose, night-time glucose, SD within 1 and 4 h [SD within time series of respectively 1 (0800–0900) and 4 h (2000–2400)] and the *J*-index.

Measurements

Interstitial glucose measurements were collected with the use of the FreeStyle Libre system. Glucose readings were taken every 15 min over the 14-d study period via a small sensor worn on the back of the upper arm. The sensor measured, captured, and stored glucose data throughout this period. The FreeStyle Libre Flash Glucose Monitoring System uses a delivery applicator that places a sensor 0.5 cm into the skin. The well-being of participants was closely monitored by regular contact from the study co-coordinators throughout the study period. The participants were provided with general information and instructions on how to deal with the sensors and to avoid self-removal. The sensor was scanned with the reader provided at least every 8 h to obtain data for the previous 8 h. Participants were asked to scan the sensor first thing in the morning, last thing at night, and once in the mid-afternoon, in order to capture maximum glucose data. After 14 d of wearing the sensor, the sensor was removed in the Institute of Food and Health either under the supervision of researchers or by the researcher (preference of participant). Data were extracted from the reader through the use of the FreeStyle Libre software. Data were imported into Microsoft Excel (Microsoft) for subsequent analysis.

Participants attended the intervention suite fasting for visit 1 and were previously instructed to avoid any food or drink for 12 h before testing. Height was measured with a wall-mounted Harpenden stadiometer (Holtain Limited) to the nearest 0.1 cm, and a Tanita scale (Tanita Corporation of America) was used to measure weight/bioimpedence. Waist and hip circumference were measured with a nonstretch tape. Blood pressure was measured with an Omron M6 Comfort digital automatic blood pressure monitor (Omron Healthcare Europe), with participants in a seated position. Venous blood samples were collected at visit 1 by a trained phlebotomist. Fasting serum and plasma samples were collected in serum tubes containing a clot activator coating and in tubes containing lithium heparin. Lithium heparin tubes were inverted 8–10 times to ensure that the coagulant was mixed throughout, and then placed directly on ice. Serum samples were inverted 8–10 times and allowed to sit at room temperature for 30 min. All blood samples were processed within 30 min of collection. Samples were centrifuged at 1800 × *g* for 10 min at 4°C, and 500 μL aliquots were stored at –80°C for subsequent analysis. An overview of the study protocol is outlined in **Supplemental Table 1**.

Dietary details for intervention period

The participants were randomly assigned to receive, in random order, 12 g/serving of the 3 different intact/hydrolyzed milk protein samples in the form of a protein shake. Participants were asked to store and return empty sachets as a measure of compliance. All study meals were provided to participants to be consumed in a free-living setting. Breakfast consisted of 2 slices of toasted white bread, 40 g of strawberry jam and 125 mL of orange juice. The meal contained ∼78 g of carbohydrate, and was consumed immediately after drinking the test drink. Participants were instructed to consume breakfast immediately after their night fast, not to modify the meal, and to refrain from eating or performing strenuous physical activity for 2 h before and after meal consumption. Participants were asked to consume the study breakfast within a 10-min period.

The study evening meal consisted of tomato and basil spaghetti and an apple. Participants were asked to avoid food or drink for 2 h before and after the evening meal. They were requested to consume the study evening meal within 20 min. The meal was consumed immediately after drinking the intact/hydrolyzed protein drink. A study lunch was consumed on 3 d of study period. These 3 d were called standardized days (the middle day of each of the 3 study arms). No snacking was allowed during these 3 d.

The composition of the meals and milk proteins are displayed in **Supplemental Tables 2** and **3**. Participants were asked to record the time at which they consumed the study meals, and the details and times of food eaten outside of the study meals in a diary provided to them. Sleep/wake times and physical activity were self-recorded in a diary for the 2-wk period.

Biochemical and clinical chemistry analyses

Biochemical analyses were performed on fasting serum and plasma samples. Clinical chemistry analyses were performed with an Rx-Daytona chemical autoanalyzer (Randox Laboratories) and Randox reagents. The analytes were glucose (glucose oxidase), triacylglycerol (lipase/glycerol kinase), and total cholesterol (cholesterol oxidase). Glucose was measured in plasma, whereas triacylglycerol and total cholesterol were measured in serum. Insulin was measured in plasma samples with the use of a Mercodia insulin ELISA kit (Mercodia AB). Total serum adiponectin was assessed with the use of a human adiponectin ELISA kit (EMD Millipore), and serum resistin was measured with a human resistin ELISA kit (EMD Millipore).

A Phenomenex EZ: faast kit for amino acid analysis was used to determine the concentration of amino acids in plasma samples (**Supplemental Table 4**). The procedure was followed as stated in the manufacturer's protocol. In brief, the protocol consists of a solid-phase extraction followed by a derivatization and then a liquid/liquid extraction. Volumes of 100 μL of serum and 100 μL of internal standard were passed through a sorbent tip attached to a 1.5-mL syringe. The amino acids were bound to this sorbent tip and any impurities were passed through the syringe. The sorbent tip was then washed to desorb the amino acids, which were transferred into a vial for derivatization with propyl chloroformate. A 150-μL portion of the organic layer containing the amino acids was evaporated under nitrogen. Samples were resuspended and analyzed with a GC-MS system comprising an Agilent 7683 autosampler and a 7890A gas chromatograph interfaced to a 5975C mass spectrometer (Agilent). Amino acids were identified by comparing the mass spectra with those in the National Institute of Standard Technology library (software version 1.7). Automatic peak detection was carried out with an Agilent Chemstation MSD, and deconvolution of mass spectra was performed with an automated mass spectral deconvolution and identification system (AMDIS version 2.65, 20 December 2006). To obtain accurate peak areas for the internal standard and specific compounds, 1 quant mass was chosen as a target and 3 masses were used as qualifiers. Each data file was manually analyzed for false positives/negatives in Agilent Chemstation. Quantification of amino acids was performed against known standards (EZ: faast AAA) with Agilent Chemstation (software version E.02.00.493).

Lipidomic analysis was performed on plasma samples (BIOCRATES Life Sciences). Absolute concentrations were obtained for metabolites from various lipids classes including (lyso-) glycerophosphocholines, -ethanolamines, -serines, -glycerols, sphingomyelins, and ceramides, measured with the use of an in-house lipid assay as previously described (32). Standard quality-control

procedures were followed on all platforms to ensure integrity of the data.

Statistical analysis

Demographic data are presented as means ± SDs. GraphPad Prism 6.0 (GraphPad Prism Software) was used to calculate the total AUCs for the postprandial glucose response of all study meals; these calculations used glucose data from 30 min prior to ingestion of the study meal, and intact/hydrolyzed casein protein data from 120 min after the study meal ingestion. Missing glucose data points for study meals (due to the sensor not being scanned) were noted. Normality was assessed by the Shapiro-Wilks normality test, and data that were not normally distributed underwent inverse transformation. Repeated-measures ANOVA was performed with SPSS Statistics 20 (IBM) to analyze the potential effect of milk proteins on total glucose AUC values for study breakfast and evening meals between each of the 3 study arms, with the use of paired comparison tests to determine where a significant difference lies. P values of <0.05 were considered statistically significant. For data that did not follow a normal distribution on inverse transformation, the Friedman test was used to determine differences between treatments, and the Wilcoxon signed rank test was used to determine where the difference was. Independent t tests, in addition to graphical representation of postprandial glucose responses, were used to explore if individuals were responders or nonresponders to either CH-B or CH-A compared with intact caseinate supplementation, at an individual participant (n-of-1) level ($P < 0.05$). The SEM was calculated based on available data from 3 repeats of breakfast on 3 study days of each arm. For further assessment of responders compared with nonresponders for CH-B supplementation, nonparametric Mann-Whitney tests were used to investigate potential differences in baseline characteristics between the two groups. P values of <0.05 were considered statistically significant.

Intraclass correlation coefficients (ICCs) and CVs were calculated between the total AUCs for glucose levels obtained from study meals with SPSS. Reliability indexes (RIs) were calculated with the use of the following formula:

$$\mathrm{RI} = \sqrt{(n \times \mathrm{ICC})} \div \sqrt{[1 + (n-1) \times \mathrm{ICC}]} \quad (1)$$

where n represents the number of repeated samples. The RI measures the correlation between a mean of n measurements and the true underlying value of an exposure of interest.

Results

The study consisted of 20 participants aged 50 ± 8 y with a BMI of 30.2 ± 3.1. Baseline characteristics are displayed in Table 1.

Glucose control and glycemic variables

Protein sachets were returned to study researchers as a measure of compliance; 98% of them were empty. Repeated-measures ANOVA revealed that for the study breakfast there was a significant difference across the treatment groups ($P = 0.037$), with a reduction in the glucose AUC for CH-B in comparison to intact caseinate ($P = 0.039$). With respect to the evening meal, there was no significant difference between the 3 treatments for glucose AUC (Table 2). Consumption of milk proteins with study breakfast and evening meal on fully standardized days resulted in no significant differences across treatment groups (Table 2). However, a similar trend emerged, with an overall treatment effect of $P = 0.051$. In addition, the subsequent response to study lunch was investigated; however, no significant difference in glucose response was observed across treatments. Illustrations of the glucose responses on fully standardized days for breakfast, lunch, and evening meal are displayed in Figure 2.

TABLE 1 Baseline characteristics of the Food for Health Ireland Glucose Monitoring Study population ($n = 20$)[1]

Variable	Mean ± SD
Sex, M/F	11/9
Age, y	50 ± 8
Weight, kg	90.5 ± 14.9
BMI, kg/m^2	30.2 ± 3.1
Body fat %	32.1 ± 10.3
Waist, cm	101 ± 9.9
Hip, cm	110 ± 6.4
WHR	0.92 ± 0.08
BP systolic, mm Hg	124 ± 11.9
BP diastolic, mm Hg	79 ± 6.5
Total serum cholesterol, mmol/L	5.19 ± 0.85
Serum triglycerides, mmol/L	1.17 ± 0.69
Fasting plasma glucose, mmol/L	5.45 ± 0.57
Fasting plasma insulin, μIU/mL	8.60 ± 3.28
Serum resistin, ng/mL	13.5 ± 4.31
Serum adiponectin, μg/mL	8.09 ± 4.76
HOMA-IR	2.11 ± 0.91

[1] All values are means ± SDs. BP, blood pressure; WHR, waist-to-hip ratio.

Peak glucose during the postprandial response to study meals was also investigated (Table 3). A statistically significant difference in peak glucose for study breakfast between treatments was observed, $\chi^2(2) = 14.39$, $P = 0.001$. A significant decrease in peak glucose for study breakfast was observed for both CH-A and CH-B compared with intact caseinate. No differences were observed for the study lunch or evening meal. Glycemic variables were calculated for each of the 3 fully standardized study days with no significant differences observed across treatment groups for 24-h, daytime, and nighttime glucose (Table 3). CH-B reduced maximum range glucose (millimoles per liter) over a 24-h period compared with CH-A ($P = 0.039$). No significant differences across treatments for the J-index, a measure of quality of glycemic control, were observed ($P = 0.28$).

TABLE 2 Total AUC for interstitial glucose following ingestion of the different intact/hydrolyzed protein drinks for study meals[1]

	Intact caseinate	CH-A	CH-B	P value
Three study days[2]				
Breakfast, min mmol/L	990 ± 189[b]	943 ± 135[a,b]	931 ± 154[a]	0.037
Evening meal, min mmol/L	889 ± 125	861 ± 104	847 ± 129	0.08
Standardized day only[3]				
Breakfast, min mmol/L	994 ± 217	939 ± 145	907 ± 150	0.05
Evening meal, min mmol/L	872 ± 146	866 ± 149	841 ± 164	0.44
Lunch, min mmol/L	938 ± 133	993 ± 158	930 ± 129	0.07

[1] Values represent means ± SDs for total AUC for interstitial glucose for 3 d of treatment for study breakfast and evening meal, and for glucose on fully standardized days for breakfast, evening meal, and lunch. Labeled means without a common superscript letter differ, $P < 0.05$. CH-A, casein hydrolysate A; CH-B, casein hydrolysate B.

[2] Repeated-measures ANOVA was carried out to determine differences between the 3 treatments based on the mean of 3 study days ($n = 18$ participants for study breakfast, $n = 19$ participants study evening meals). Overall $P = 0.037$ for study breakfast. Significance lies between intact caseinate and CH-B for study breakfasts based on pairwise comparisons ($P = 0.039$).

[3] Repeated-measures ANOVA was carried out to determine differences between the 3 treatments on fully standardized days only, for $n = 16$ participants for study breakfast, $n = 13$ participants for study evening meals, and $n = 17$ participants for study lunches. A P value of <0.05 was considered significant.

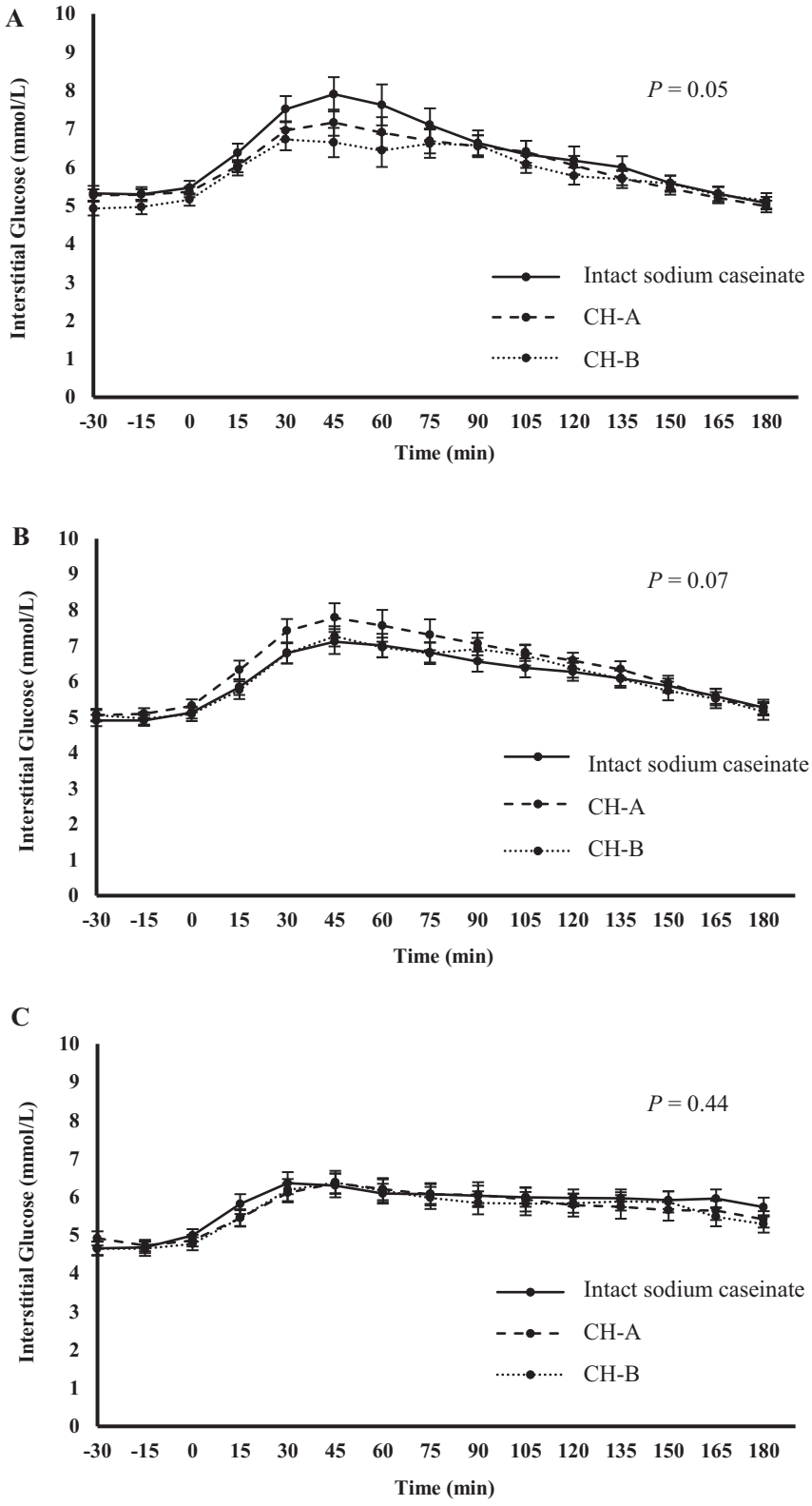

FIGURE 2 Illustration of mean glucose responses of study population to meals supplemented with intact sodium caseinate, CH-A, and CH-B, on fully standardized days. Data points represent mean interstitial glucose levels every 15 min, and error bars represent SEMs, based on data from $n = 20$ participants on fully standardized days. Time point 0 min represents time of ingestion of study meal with milk protein. An extended 3-h postprandial glucose response is illustrated. Repeated-measures ANOVA was carried out to determine differences between the 3 treatments on fully standardized days only. A P value of <0.05 was considered significant. The AUC was calculated from glucose data from 30 min prior to ingestion of study meal and milk protein to 120 min after study meal ingestion. (A) Mean interstitial postprandial glucose response to study breakfast. (B) Mean interstitial postprandial glucose response to study lunch. (C) Mean interstitial postprandial glucose response to study evening meal. CH-A, casein hydrolysate A; CH-B, casein hydrolysate B.

TABLE 3 Comparison of peak interstitial glucose, estimates of glycemia, and glycemic variability between treatments[1]

	Intact caseinate	CH-A	CH-B	*P* value
Breakfast peak glucose,[2] mmol/L	8.42 ± 1.83[a]	7.84 ± 1.45[b]	7.57 ± 1.64[b]	0.001
Evening meal peak glucose,[2] mmol/L	7.10 ± 1.05	6.79 ± 0.94	6.66 ± 1.09	0.21
Lunch peak glucose,[2] mmol/L	7.68 ± 1.35	8.20 ± 1.89	7.58 ± 1.06	0.17
24-h glucose, mmol/L	5.53 ± 0.74	5.54 ± 0.56	5.38 ± 0.72	0.22
Daytime glucose, mmol/L	5.68 ± 0.74	5.71 ± 0.63	5.51 ± 0.73	0.08
Night-time glucose, mmol/L	5.18 ± 0.83	5.22 ± 0.57	5.13 ± 0.82	0.83
24-h SD	0.97 ± 0.35	0.94 ± 0.32	0.88 ± 0.23	0.50
SDws1	0.49 ± 0.39	0.39 ± 0.27	0.42 ± 0.37	0.23
SDws4	1.03 ± 0.64	0.83 ± 0.52	0.83 ± 0.44	0.36
Range, min	4.05 ± 0.74	4.20 ± 0.52	3.95 ± 0.67	0.39
Range,[3] max	8.73 ± 2.08[a,b]	8.50 ± 1.75[b]	7.97 ± 1.59[a]	0.027
Coefficient of variation, %	17.5 ± 4.77	16.7 ± 4.47	16.3 ± 2.91	0.84
J-index	14.0 ± 4.55	13.8 ± 3.56	13.0 ± 3.60	0.28

[1]Values are means ± SDs. Labeled means without a common superscript letter differ, $P < 0.05$. CH-A, casein hydrolysate A; CH-B, casein hydrolysate B; J-index, $J = 0.324 \times (\text{mean} + \text{SD})^2$; SDws1 and SDws4, standard deviation within time series of respectively 1 (0800–0900) and 4 h (2000–2400).
[2]Peak glucose was defined as the highest glucose data point in the 2 h following consumption of study meals on the 3 study days in each arm. The Friedman test was used to determine differences between treatments for peak glucose, and the Wilcoxon signed rank test was used to determine where the difference was. Overall $P = 0.001$ for peak glucose for study breakfast, with a difference between intact caseinate and CH-B ($P = 0.001$) and intact caseinate and CH-A ($P = 0.002$).
[3]Repeated-measures ANOVA revealed a significant overall difference between 3 treatments for range (max) ($P = 0.027$). Pairwise comparisons reveal significance lies between CH-A and CH-B ($P = 0.039$). Comparison of estimates of glycemia and glycemic variability between treatments based on fully standardized days only.

Glucose responses on an individual level

When the data were analyzed at an individual level (*n*-of-1), only 3 individuals demonstrated a decreased postprandial glucose response to breakfast when CH-B was ingested compared with intact caseinate ($P < 0.05$) (**Figure 3**, **Supplemental Table 5**). A further 3 participants displayed a trend towards reduced postprandial glucose response with CH-B supplementation compared with intact caseinate ($P = 0.051$, $P = 0.053$, $P = 0.088$). When CH-A glucose responses to breakfast were compared with intact caseinate supplementation, 4 individuals displayed a significantly decreased postprandial glucose response; however, for 1 responder only data for 1 CH-A breakfast were available. Furthermore, 1 participant had a significantly increased postprandial glucose response to CH-A supplementation compared with intact caseinate supplementation ($P = 0.030$).

Because a significant postprandial glucose-lowering effect at an average study population level was observed with CH-B supplementation, the baseline characteristics of individuals who responded to CH-B supplementation were examined to investigate potential differences between responders and nonresponders (**Table 4**). This aspect of the analysis was explorative in nature, due to the sample size being low for a detailed comparison of responders and nonresponders. Individuals who responded to CH-B supplementation with study breakfast had lower total cholesterol and serine concentrations than non-responders. Five lipids comprising ceramides, phosphatidylcholines, and a sphingomyelin [N C12:0 (OH) Cer, N C28:0 Cer, PC aa 30:1, PC aa 30:2, and SM C16:0] were significantly different between the 2 groups.

Intra- and interindividual variability

Intra- and interindividual reproducibility were examined for study meals (**Table 5**). High ICCs were obtained for all study meals, indicating excellent intraindividual reproducibility. High RIs were also obtained, indicating a high correlation between the mean of a number of repeat measurements and the true underlying value. The intraindividual CVs were low, showing that there was low variation in the same person's response to the same study meal. Interindividual CVs were also calculated and found to be higher than intraindividual CVs; for example, the mean intraindividual CV for the intact caseinate study breakfast was 5.66%, whereas the interindividual CV for the same meal was 19.41%.

Discussion

Consumption of a specific casein hydrolysate resulted in reduced postprandial glucose levels following consumption of a breakfast meal. The significant effect was unique to a particular casein hydrolysate, clearly indicating specificity of the increased bioactivity. Interestingly, examination of the postprandial glucose responses at an individual level revealed that only 3 individuals significantly responded, demonstrating that the positive effects of the particular casein hydrolysate are not applicable to the general population. Furthermore, the glucose response for study meals was highly reproducible at an individual level, but high interindividual variability in glucose response was observed for study meals. Further understanding of this variability will be important for the development of precision nutrition.

This study investigated the effect of intact and hydrolyzed milk proteins, when consumed with a standardized meal, on postprandial glucose control. CH-B significantly reduced postprandial glucose response with no significant reduction observed with CH-A. This emphasizes that the glucose-lowering effect was specific to a particular casein hydrolysate at an average study population level. Based on previous research, the casein hydrolysate stimulated insulin secretion, which provides further evidence of the effect of a casein hydrolysate on insulin release and action (29, 31). A study by Geerts et al. (33) demonstrated that, compared with an intact protein, a casein hydrolysate (15 g) reduced plasma

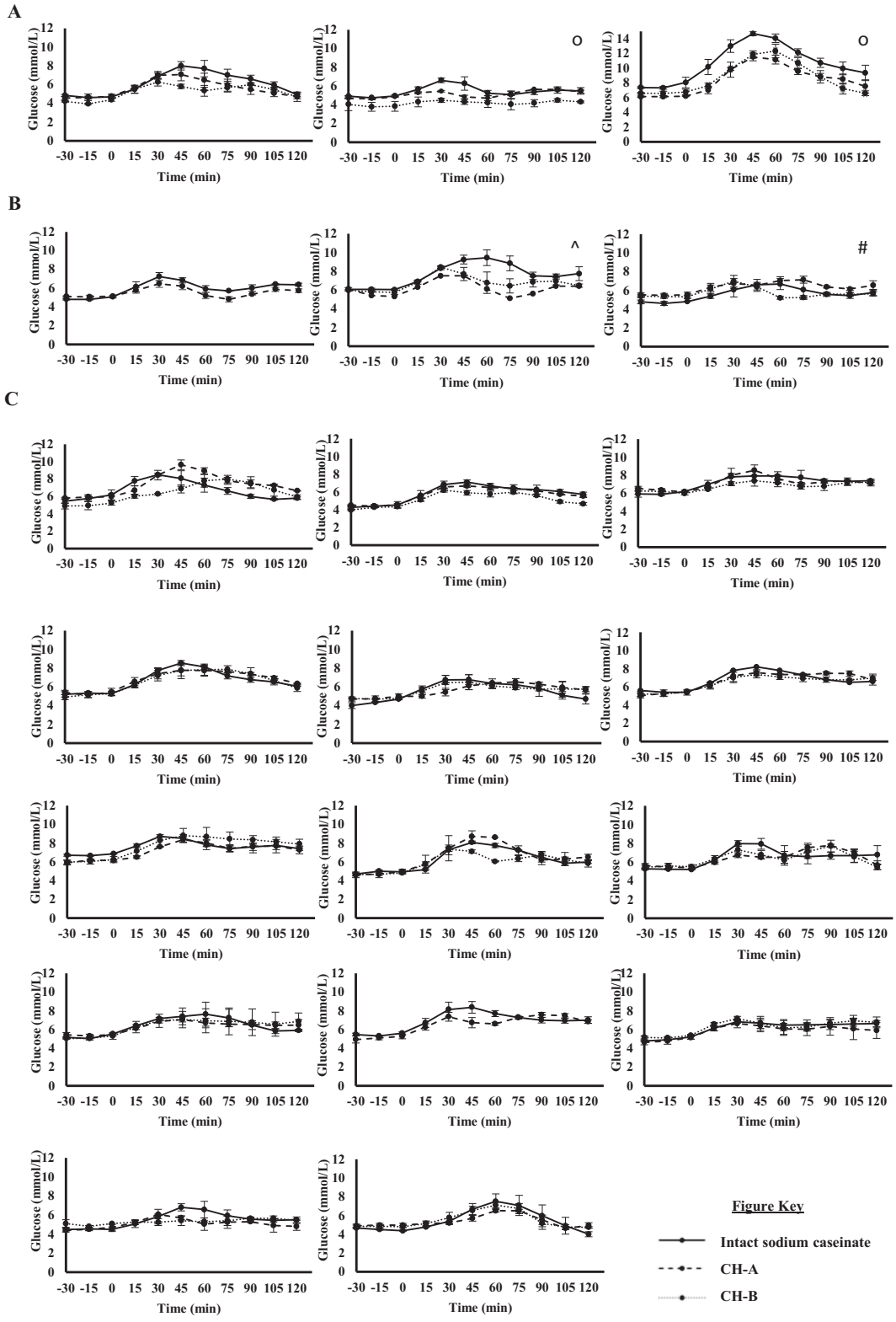

FIGURE 3 Postprandial glucose responses to study breakfasts on the individual level. Twenty individual postprandial glucose responses to study breakfast supplementation with intact sodium caseinate, CH-A, and CH-B. Time point 0 min indicates ingestion of study breakfast with intact/hydrolyzed protein drink (intact caseinate, CH-A, and CH-B). Mean interstitial glucose (mmol/L) and SEM displayed at 15-min intervals, based on available data from 3 repeats of breakfast on 3 study days of each arm. Corresponding data and *P*- values are present in Supplemental Table 5. (A) Individual responders to CH-B supplementation compared with intact caseinate. (B) Individual responders to CH-A supplementation compared with intact caseinate. (C) Individual nonresponders to casein hydrolysate (CH-A or CH-B) supplementation compared with intact caseinate. Responders and nonresponders to either CH-B or CH-A compared with intact caseinate were defined by independent *t* tests ($P < 0.05$). °Participants responded to both CH-B and CH-A supplementation compared with intact caseinate. #Participant significantly increased postprandial glucose response with CH-A supplementation compared with intact caseinate. ^Participant had only 1 study breakfast with CH-A supplementation available. CH-A, casein hydrolysate A; CH-B, casein hydrolysate B.

TABLE 4 Differentiation of responders compared with nonresponders for CH-B based on baseline characteristics[1]

	Responders ($n = 3$)	Nonresponders ($n = 15$)	P value[2]
Total serum cholesterol, μM	4390 ± 340	5310 ± 840	0.039
Plasma serine, μM	83.9 ± 10.1	112.1 ± 22.6	0.039
SM C16:0, μM	107 ± 7.77	124.9 ± 11.7	0.017
PC aa C30:1, μM	36.8 ± 2.76	42.4 ± 3.86	0.038
PC aa C30:2, μM	2.21 ± 0.10	3.18 ± 0.69	0.002
N-C12:0(OH) Cer, μM	0.01 ± 0.003	0.01 ± 0.002	0.039
N-C28:0 Cer, μM	0.03 ± 0.001	0.03 ± 0.001	0.010

[1] Values are means ± SDs. Variables assessed: age (y), BMI (kg/m^2), body fat %, waist (cm), waist-to-hip ratio, systolic and diastolic blood pressure (mm Hg), total cholesterol (mmol/L), glucose (mmol/L), insulin (μIU/mL), TGs (mmol/L), adiponectin (nmol/L), resistin (ng/mL), HOMA-IR, quantitative insulin sensitivity check index, alanine, glycine, aba, valine, leucine, isoleucine, threonine, serine, proline, aspargine, aspartic, methionine, glutamic, phenylalanine, glutamine, ornithine, lysine, histidine, tyrosine, tryptophan, cystine. Plasma lipid classes including (lyso-) glycerophosphocholines, -ethanolamines, -serines, -glycerols, SMs, PCs, and Cers were assessed (μM). Cer, ceramide; CH-A, casein hydrolysate A; CH-B, casein hydrolysate B; PC, phosphatidylcholine; SM, sphingomyelin.
[2] P value obtained from nonparametric Mann-Whitney U tests. Only significant variables are included in the table.

TABLE 5 Intra- and interreproducibility for study breakfasts, study evening meals, and study lunches[1]

	ICC	95% CI	RI[2]	Intra-CV (%)[3]	Inter-CV (%)[3]
Breakfast					
Intact caseinate	0.89	0.78 - 0.95	0.99	5.66 ± 3.51	19.4 ± 0.49
CH-A	0.80	0.61 - 0.92	0.99	5.20 ± 4.41	14.8 ± 1.50
CH-B	0.76	0.55 - 0.90	0.99	7.65 ± 5.46	17.7 ± 3.82
Evening meal					
Intact caseinate	0.74	0.49 - 0.90	0.99	10.8 ± 10.6	16.3 ± 3.92
CH-A	0.78	0.56 - 0.92	0.99	6.53 ± 5.69	13.7 ± 3.03
CH-B	0.84	0.64 - 0.95	0.99	7.62 ± 8.42	15.5 ± 1.60
Lunch	0.62	0.41 - 0.85	0.99	7.00 ± 4.37	14.0 ± 1.12

[1] CH-A, casein hydrolysate A; CH-B, casein hydrolysate B; ICC, intraclass correlation coefficient; inter-CV, interindividual CV (%); intra-CV, intraindividual CV (%); RI, reliability index.
[2] $RI = \sqrt{(n \times ICC)} \div \sqrt{[1 + (n-1) \times ICC]}$.
[3] Values are means ± SDs.

glucose concentrations by enhancing the carbohydrate-induced insulin response. Elsewhere, Koopman et al. (34) randomly assigned 10 healthy men to receive hydrolyzed casein (35 g) or a phenylalanine-labeled intact casein. Postprandial insulin levels were significantly increased after ingestion of the casein hydrolysate, but no significant reduction in plasma glucose was observed. The authors described ingestion of relatively high servings of casein at 35 g, as opposed to the 12-g serving used in the current study. No significant difference across the 3 treatments for study evening meal glucose AUC was observed. Lack of significance may be due to the difference in nutrient profile of the evening meal, which contained 29.4 g of total fat as opposed to 1.4 g in the breakfast meal. In addition, although it contained a similar amount of carbohydrate, the study breakfast may have elicited a cleaner glucose response due to the overnight fast and being the first meal of the day.

A novel and interesting aspect of this study explored postprandial glucose responses at an individual level. Individual participant studies, or *n*-of-1 studies, can identify the optimal treatment for an individual, in contrast to typical randomized controlled trials that explore parameters at a population level (35). Potential for *n*-of-1 studies exist in the area of precision nutrition, but novel strategies in terms of study design and analytic techniques are required, which differ from those used in the past (10). Owing to the use of continuous glucose monitoring technology in the present study, and having a study design with a number of measurements per individual per treatment arm, the data permitted analysis at an individual participant level. This provided a proof-of-concept aspect to the study, following analysis at the traditional average study population level. For the present study, a subgroup of 3 participants displayed significantly reduced postprandial glucose for the study breakfast when this was supplemented with CH-B rather than with intact caseinate. This subgroup had lower total cholesterol and serine concentrations, and alterations in 5 lipids. Interestingly, no differences in HOMA-IR or in the quantitative insulin sensitivity check index were observed. A recent study by Almario et al. (8) concluded that the ability of whey proteins to decrease glucose levels in a T2D cohort was dependent upon baseline characteristics of individuals, and the glucose-lowering benefit of whey protein consumption was not evident for a general T2D population. This conclusion, in combination with the findings herein, strongly reinforces the need for precision nutrition. Furthermore, Almario et al. (8) demonstrated that individuals with high triglyceride levels were less likely to display a glucose lowering effect with whey protein supplementation. Our study observed that individuals who responded to casein hydrolysate supplementation had lower total cholesterol concentrations; therefore, a favorable lipid profile may increase the probability of individuals successfully responding to hydrolyzed casein supplementation.

A metabolomic analysis was employed to gain a more in-depth understanding of the difference between those individuals who responded to the intervention and those who did not. Amino acid analysis identified decreased serine concentrations in the subgroup of responders to casein hydrolysate supplementation. Serine provides a 1-carbon unit for methylation reactions that arise through the formation of *S*-adenosylmethionine, with the control of methyl group transfer being important in regulating many cellular processes (36). The concentrations of 5 lipids significantly differed between CH-B responders compared with nonresponders. An increase in ceramide C12:0(OH) concentration was observed in the responder group. This is of interest due to ceramides having a direct effect on insulin signaling, which may contribute to pancreatic β cell apoptosis (37). C16:0 sphingomyelin was significantly decreased in the responder group, and a sphingomyelin that contains palmitate (16:0) can interact with cholesterol in structured lipid domains (38). Furthermore, PC aa C30:1, PC aa 30:2, and C28:0 Cer were significantly reduced in the responder group. These specific lipid results warrant further investigation and validation. Interestingly, when individual participant responses were assessed for CH-A supplementation, a mixed result emerged, with 4 participants displaying a decreased postprandial glucose response and 1 participant having a significantly increased response, compared with intact caseinate. Overall, the results highlight the need for caution: not all supplementation with hydrolyzed milk proteins results in a positive or neutral impact on postprandial glycemia, in particular at an individual level.

Continuous glucose monitoring has been used to successfully investigate postprandial glucose responses to meals in several intervention studies, primarily examining meals varying in carbohydrate content (26, 39–42). Postprandial glucose responses

are critical in assessing glycemic control, with alterations often associated with disease risk (43). The present results indicate that the glucose response for study meals was extremely reproducible for each individual, but high interindividual variability in glucose responses were observed. This is in line with findings from Zeevi et al. (26), which demonstrated high reproducibility of the same person's glucose response to the same food, and increased variability of postprandial glucose responses of different individuals to the same food. Furthermore, Matthan et al. (27) reported high interindividual variability among 63 participants in response to a fixed amount of white bread, indicating the limited applicability of the glycemic index to predict postprandial glucose responses. These findings demonstrate the urgent need to tailor dietary advice to the individual level, and to address the high interindividual variation observed in response to nutrition interventions.

The strengths of the present study include the multiple measurements obtained in response to the same study meal and protein supplementation. Furthermore, continuous glucose monitoring enabled a detailed glucose profile to be obtained, allowing glucose excursions to be identified and glycemic variability to be assessed throughout the study. Exceptional compliance across treatments was observed, due to the provision of foods and the frequent contact of researchers with participants. The limitations of this study include a relatively small sample size, in particular for analysis of baseline characteristics of responders compared with nonresponders.

In conclusion, the present study demonstrated the ability of a specific casein hydrolysate to improve glycemic function at an average study population level. However, only 3 individuals significantly reduced postprandial glucose response to the casein hydrolysate, highlighting the need for precision nutrition. The results from this study and the emerging literature support the urgent need to address the high interindividual response to nutrition interventions, and highlight the importance of the development of precision nutrition, potentially through an *n*-of-1 approach.

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