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Exploring the Diversity of Sugar Compounds in Healthy, Prediabetic, and Diabetic Volunteers

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Scope: Diabetes is thought to primarily represent a disturbance of carbohydrate metabolism; however, population studies employing metabolomics have mainly identified plasma amino acids and lipids, or their products, as biomarkers. In this pilot study, the aim is to analyze a wide spectrum of sugar compounds in the fasting state and during an oral glucose tolerance test (OGTT) in healthy, prediabetic, and type 2 diabetic volunteers.
Methods and results: The three volunteer groups underwent a standard OGTT. Plasma samples obtained in the fasting state, 30 and 90 min after the OGTT, are subjected to a semitargeted GC–MS (gas chromatography–mass spectrometry) sugar profiling. Overall, 40 sugars are detected in plasma, of which some are yet unknown to change during an OGTT. Several sugars (e.g., trehalose) reveal significant differences between the volunteer groups both in fasting plasma and in distinct time courses after the OGTT. This suggests an endogenous production from orally absorbed glucose and/or an insulin-dependent production/removal from plasma.
Conclusion: It is demonstrated that more sugars than expected can be found in human plasma. Since some of these show characteristic differences depending on health status, it may be worthwhile to assess their usability as biomarkers for diagnosing early-stage insulin resistance and type 2 diabetes.

1. Introduction

Although glucose and insulin, or HbA1c levels are routinely determined for type 2 diabetes management, they do not reveal which metabolic perturbations occur during glucose consumption, as simulated by an oral glucose tolerance test (OGTT).[1–5] Therefore, metabolomics has become popular as a more comprehensive profiling approach and biomarker discovery tool. This approach has revealed numerous differences in plasma or urine metabolites between healthy subjects and subjects with impaired glucose tolerance and/or type 2 diabetes.[4,6–12] Some studies have not only searched for differences in metabolite profiles during fasting, but also in time courses during an OGTT.[1,3,13–18] Remarkably, these studies mainly identified plasma amino acids, various lipid species, or bile acids as discriminating markers related to insulin resistance (IR) and type 2 diabetes. Considering diabetes is associated with marked alterations in carbohydrate metabolism, it is thus surprising that other sugars or sugar-derived intermediates have rarely been identified as associated with the metabolic impairments.

We recently established a semitargeted sugar profiling method and demonstrated a rather complex mixture comprising 55 different sugar compounds in urine samples of healthy human volunteers.[19] This finding suggested that the plasma sugar profile is equally complex. Metabolite profiling studies of human biofluids typically analyze only a few sugar compounds; this is mainly due to analytical limitations such as insufficient separation and identification of structural isomers.[19–22] Therefore, sugars or sugar-like candidates are

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Table 1. Basic characteristics of the three volunteer groups.

	Healthy			Prediabetic			Diabetic		
	All	Male	Female	All	Male	Female	All	Male	Female
n	—	15	—	15	10	5	11	3	8
Age [years][a)]	—	26.3 ± 2.5	—	62.1 ± 6.5	60.0 ± 5.9	66.2 ± 6.1	63.0 ± 6.2	65.3 ± 4.7	61.9 ± 6.6
BMI [kg cm^{-2}][a)]	—	24.0 ± 2.0	—	29.0 ± 5.9	28.5± 6.2	30.1 ± 5.6	29.4 ± 2.6	28.9 ± 1.8	29.5 ± 2.9

[a)] For age and BMI mean values ± standard deviations are reported.

often reported as unknowns,[8,14] or are represented as a group, for example "hexoses"[7,9,14] or "fructose/glucose/galactose,"[13,18] which clearly limits interpretability. In addition to glucose, sugar compounds identified as changing during the time course of an OGTT or that display altered levels in type 2 diabetes patients are 1,5-anhydroglucitol, mannose, maltose, and glucuronic acid.[11,12,14,15,20,23] These findings suggest that additional sugars, and intermediates of the metabolic conversion of glucose, may be found in plasma and urine when specific methods are employed.

We therefore applied our semitargeted sugar profiling method, previously used for urine analysis,[19] to assess whether novel sugar compounds can be identified in plasma of healthy, prediabetic, and diabetic subjects in a fasted state and after an OGTT.

2. Experimental Section

2.1. Study Design and Subjects

This pilot study was part of the MIPROMET cohort, which was performed at the Human Study Center of the Technical University of Munich at the Weihenstephan campus.[24] Study design, participant recruitment, and examination procedures for healthy and prediabetic participants were previously described by Hoefle et al.[24] Type 2 diabetic participants were additionally recruited with study design and research parameters identical to those of the healthy and prediabetic volunteers.[24] For patients with type 2 diabetes, oral medication (metformin alone) and/or insulin therapy was an additional inclusion criterion. Prediabetic and diabetic states were defined according to WHO criteria.[25] Altogether, 15 male healthy controls, 15 male and female prediabetic cases, and 11 male and female diabetic cases were recruited for this pilot study. However, distribution of males and females, as well as age class was unbalanced between volunteer groups (see **Table 1** for volunteer characteristics) because of difficulties with recruitment due to strict inclusion criteria (see flow chart in Figure S1, Supporting Information File 1).

Volunteers received a test drink containing 50 g of maltodextrin19 (starch hydrolysate; Berco Arzneimittel, Kleve, Germany), 10 g of lactulose (Hemopharm, Bad Vilbel, Germany), 2 g of 4-hydroxyproline (Sigma-Aldrich, Munich, Germany), or 1 g of acetaminophen (Paracetamol; Ratiopharm, Ulm, Germany) in the case of prediabetic and diabetic volunteers, and ten drops of lemon or vanilla flavor (Dr. Oetker, Bielefeld, Germany). Inclusion of non-digestible lactulose enabled the assessment of the oro-cecal transit time by detecting hydrogen exhalation in the breath as a result of fermentative production of hydrogen by the intestinal flora. The appearance of acetaminophen in plasma depended on the velocity of gastric emptying as the limiting step, allowing the rate of appearance of paracetamol in the blood to serve as marker for gastric emptying[26]; similarly 4-hydroxyproline can be applied. Maltodextrin19 rather than D-glucose was used since this prevents secondary effects of high osmolarity on gastric emptying. Maltodextrin19 could be considered equivalent to glucose due to its fast hydrolysis in the gut. With a dextrose equivalent of 19, maltodextrin19 was close to the typically used glucose syrup in commercial OGTT solutions.

Plasma samples were taken in fasted state, as well as 30 and 90 min after ingestion of the test drink. Blood was collected in monovettes coated with EDTA, then inverted gently ten times and centrifuged at 20 °C at 1000 × *g* for 10 min. Thereafter, plasma was aliquoted on ice, frozen on dry ice, and stored at −80 °C until analysis.

All procedures performed in the study were in accordance with the 1964 Helsinki declaration and its later amendments. The study was approved by the Ethics Committee of the Technical University of Munich (#2436/09) and was registered at the German Clinical Study Register (DRKS00005682). Informed consent was obtained from all individual volunteers included in the study.

2.2. Semitargeted GC-MS Sugar Profiling

For plasma sample preparation, 20 µL of the internal standard solution (see Table S1, Supporting Information File 1) were pipetted into a 1.5 mL Eppendorf reaction tube before adding 40 µL plasma sample. Then, 150 µL of ice-cold methanol were added and the tube mixed for protein precipitation. After centrifugation for 10 min at 16 100 × *g* and 4 °C, 120 µL of the supernatant were transferred to a second 1.5 mL Eppendorf reaction tube. For lipid extraction, 700 µL tert-butyl methyl ether (TBME) were added and samples vigorously mixed for 2 min. After the addition of 70 µL of water, samples were again vigorously mixed for 2 min and then shortly centrifuged for phase separation. The upper TBME-containing phase was discarded and the aqueous phase transferred to a GC screw-top vial with a 300 µL insert. After rinsing the reaction tube with 30 µL methanol, the wash solution was added to the aqueous phase in the GC vial. Samples were then evaporated for 90 min at 40 °C and <1 mbar. For removal of traces of water, 20 µL of methanol were added and samples evaporated again under the same conditions for 30 min. Afterward, samples were methoximated using 20 µL methoxylaminhydrochloride in pyridine (20 mg mL^{-1}), the reaction was carried out for

6 h at 60 °C and 1000 rpm. Trimethylsilylation was carried out by adding 40 µL *N*-(trimethylsilyl)-*N*-methyltrifluoroacetamide (MSTFA) with 1% trimethyl chlorosilane (TMCS) and incubation for 1 h at 75 °C without shaking. Analyses were performed on a Shimadzu GCMS QP2010 Ultra instrument (see Table S2, Supporting Information File 1) in scheduled Scan-/SIM (selected ion monitoring)-mode allowing the selective and sensitive detection of sugar compounds. Measurements were performed with helium as carrier gas in constant pressure mode on a 60 m Rxi-5SilMS column with 10 m integrated pre-column, 0.25 mm internal diameter, and 0.25 µm film thickness. 1.2 µL sample were injected in cold split mode and a split ratio of 1:5. For further details about instrument parameters, see Table S3, Supporting Information File 1. The GCMSsolution (version 4.1.4, Postrun Analysis) software was used to pre-process the data (details see Table S4, Supporting Information File 1). For each measurement day, along with the study samples, quality control (QC) samples (mixture of all study samples) were regularly measured to enable correction of drift and offset effects and a solvent blank was created using water instead of the sample.[27] A retention index marker solution was added to the solvent blank.[27] Each day, 24 study samples, seven QC samples, and a solvent blank were prepared and analyzed, beginning with the daily blank, followed by four blocks of six study samples surrounded by QC samples, and at the beginning and end of the day two QC samples were always measured. Each measurement week, the liner was changed and the MS was tuned. The injector septum was replaced every 100 runs.

2.3. Analysis of the Ingested Test Drink for the OGTT and Further Commercial OGTT Solutions

A 2 mL sample of the ingested test drink for the OGTT was evaporated and then 10 mg of the dried powder were solved in 500 µL of deionized water. Afterward, 50, 100, and 200 µL of the solution were evaporated for GC-MS analysis. Derivatization and GC-MS analysis were the same as described earlier for plasma samples (see also Methods Section 1.2.1, Supporting Information File 1).

An additional GC × GC-MS measurement was performed to explore the sugar profile of the ingested test drink and two commercial OGTT solutions (Accu-Chek Dextrose O.G-T., Roche and Rapilose OGTT solution, Galen) in more detail. For the ingested test drink 25 µL of the solution described earlier were evaporated, while in the case of both commercial OGTT solutions 50 µL of a 1:20 dilution were evaporated. Derivatization was as described earlier for plasma samples (see also Methods Section 1.2.1, Supporting Information File 1). The GC × GC-MS parameters can be found in Table S6, Supporting Information File 1.

2.4. Statistics

2.4.1. Test for Group and Time Differences After an OGTT

The pilot study consisted of repeated measurements of sugar compounds at non-equidistant time points (0, 30, and 90 min) in three independent groups (15 healthy, 15 prediabetic, and 11 diabetic participants). Due to the participant recruitment, the pilot study had unbalanced participant characteristics. Participant recruitment reflected the fact that older people were more prone to develop type 2 diabetes mellitus; the average age of healthy participants was ≈26 years in contrast to 63 years in diabetic participants (Table 1). Moreover, the distribution of sex in the three groups was uneven (Table 1). A general model accounting for the characteristics leading to the unbalanced design would describe each sugar compound dependent on: i) group, time, and interaction between both, ii) age, BMI, and sex (as competing covariates), and iii) with random slope and random intercept to account for correlations across time (longitudinal data). An example of such possible models can be found in File 2, Supporting Information.

However, according to the one-in-ten rule, it is not reasonable to fit such a general model with the limited sample size in this pilot study. To find a compromise between model complexity and feasibility, a simpler model (mixed linear model) was fitted where each sugar compound was described dependent on group and time with a random intercept (to account for correlation between repeated measurements at different time points for each participant). Testing was performed for group and time differences in order to identify and focus on sugar compounds, which change concentration differently in healthy, prediabetic, and diabetic participants after an OGTT. Specifically, the differences in sugar compounds were tested for i) healthy versus diabetic group, ii) healthy versus prediabetic group, and iii) prediabetic versus diabetic group, while keeping time constant. Moreover, the differences in sugar compounds were tested for i) 0 versus 30 min, ii) 0 versus 90 min, and iii) 30 versus 90 min, while keeping group constant (see File 3, Supporting Information).

For the sake of simplicity and to ensure comparability, normality for sugar compound variables was generally assumed, although literature has shown that this assumption is not always justified.[28,29] For model diagnostics, QQ-plots depicting the quantiles of the residuals against the theoretical normal were included to ascertain normality (File 3, Supporting Information). For those sugar compounds found to be significantly different either dependent on health status and/or on time, a visual analysis of these QQ-plots was performed. For the majority of sugar compounds, the assumption of normality was justified.

The software R (version 3.3.2 2016-10-31)[30] was used to perform these statistical analyses. The applied R packages can be found in[31–37] and were added as libraries in Files 2 and 3, Supporting Information. A detailed report (including the R script) of the main statistical analyses using R can be found in File 3, Supporting Information. Generally, when no value was reported due to the signal intensity being below the limit of detection, half of the minimal signal intensity for this sugar compound was considered.

2.4.2. Differentiation of Healthy, Prediabetic, and Diabetic Subjects in the Fasted State

Independently of the administration of maltodextrin19, some sugar compounds already differ between the healthy, prediabetic, and diabetic group in the fasted state. A principal component

analysis (PCA) and a one-way ANOVA were performed in order to identify such sugar compounds.

First, the PCA was performed to observe whether plasma sugar profiles in the fasted state enable a separation of healthy, prediabetic, and diabetic volunteers. Both the scores and the loadings plot were drawn. The loadings plot was evaluated to ascertain whether glucose was the main variable driving the separation of the three groups.

Second, to determine the sugar compounds differentiating between the three groups, an ANOVA-based response screening workflow was applied. Briefly, a first one-way ANOVA with correction for multiple testing was performed to screen for significant sugar compounds, which were then tested for normality and homogeneity of variances. A second one-way ANOVA and post-hoc tests (each depending on the distribution and homogeneity of variance) were performed to receive the final selection of sugar compounds significantly differentiating between the healthy, prediabetic, and diabetic group in the fasted state. Specifically, the second one-way ANOVA was a normal one-way ANOVA (in the case of normality and homogeneity of variances), a one-way ANOVA with Welch-correction (in the case of normality and inhomogeneity of variances), or a one-way ANOVA with Kruskal–Wallis-correction (in the case of non-normality). For a more detailed description of the screening workflow, the reader is referred to the Supporting Information by Weinert et al.[38] Box plots were plotted for the significant sugar compounds revealed by these procedures. The software JMP (version 13, SAS Institute Inc., Cary, NC, 1989–2007) was used to perform these statistical analyses.

2.5. Evaluation of Potential Confounders in Fasting Plasma Samples in an Independent Study

In the present study, the healthy group comprised solely male volunteers, the prediabetic group was 2/3 male, and the diabetic group was 2/3 female (Table 1). Moreover, in addition to sex, also age and body mass may represent important baseline covariates (Table 1). Due to the sample size limitation of this pilot study, another independent external study was considered to generally ascertain the influence of age, BMI, and sex on the sugar compounds of interest using correlation analyses and unpaired tests.

The sugar profile of a subset of fasting plasma samples from the cross-sectional KarMeN study was analyzed. Details about study design, participant recruitment, and examination procedures, as well as sample storage were described by Bub et al.[39] Sample preparation, data preprocessing, and treatment were exactly the same as described in Section 2.2 and in Section 1.2.1, Supporting Information File 1. Minor changes in the instrument parameters can be found in Table S3, Supporting Information File 1. For participant characteristics of the randomly selected subset of 58 fasting plasma samples (about ten participants for every 10 years with half of the participants being male) see Table S5, Supporting Information File 1.

In particular, to ascertain the influence of age, BMI, and sex on sugar compounds found to differentiate between healthy, prediabetic, and diabetic subjects, a correlation analysis (Spearman correlation coefficients), *t*-tests (in the case of normality and homogeneity of variances), Welch's *t*-tests (in the case of normality and inhomogeneity of variances), or Wilcoxon *t*-tests (in the case of non-normality) were performed. In the case of age, two groups of the KarMeN volunteers were defined covering the same age range as the healthy (21–30 years; $n = 10$) and prediabetic and diabetic volunteers (50–72 years; $n = 23$) of the MIPROMET study. For BMI, KarMeN participants were divided into two groups (19–23.9 kg m^{-2}, $n = 29$ and 24–30 kg m^{-2}, $n = 29$). In the case of sex, 28 males were compared with 30 females. The KarMeN study was registered at the German Clinical Study Register (DRKS00004890) and was in accordance with the 1964 Helsinki declaration and its later amendments.

The software JMP (version 13, SAS Institute Inc., Cary, NC, 1989–2007) was used to perform these statistical analyses.

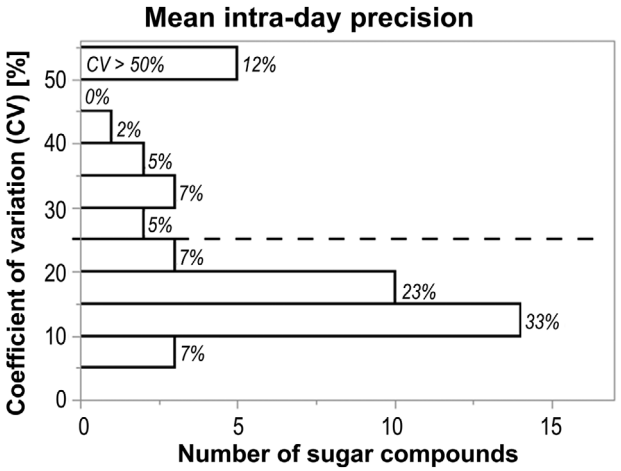

Figure 1. Distribution of the average intra-day coefficients of variation for all sugar compounds measured in the QC samples before drift correction. Dashed line indicates coefficients of variation below and above 25%.

3. Results

3.1. Plasma Sugar Profile

Overall, we detected 40 sugar compounds represented by mono- and disaccharides, polyols, sugar acids, and so far unknown sugar-like compounds with the semitargeted sugar profiling method. Based on characteristic masses in the scan mass spectra, unknown compounds were defined as sugar-like compounds (e.g., m/z 292 and 333 are characteristic for sugar acids, m/z 319 and 307 for aldoses, ketoses as well as polyols, m/z 305 and 318 for cyclitols, and m/z 361 is characteristic for disaccharides). Additionally, in some cases the chromatographic elution order allowed us to derive the number of C-atoms in the sugar compound (e.g., pentose or hexose). However, future absolute identification can only be ensured by comparing retention indices and mass spectra of the unknown sugar-like compounds (Figure S2, Supporting Information File 1) with those of known standard substances. Data on the quality of measurement (e.g., reproducibility of quality control samples and internal standards, identification level, median, and range from minimum to maximum of peak area in the study samples) and on selectivity can be found in Table S7, Supporting Information File 1. **Figure 1**

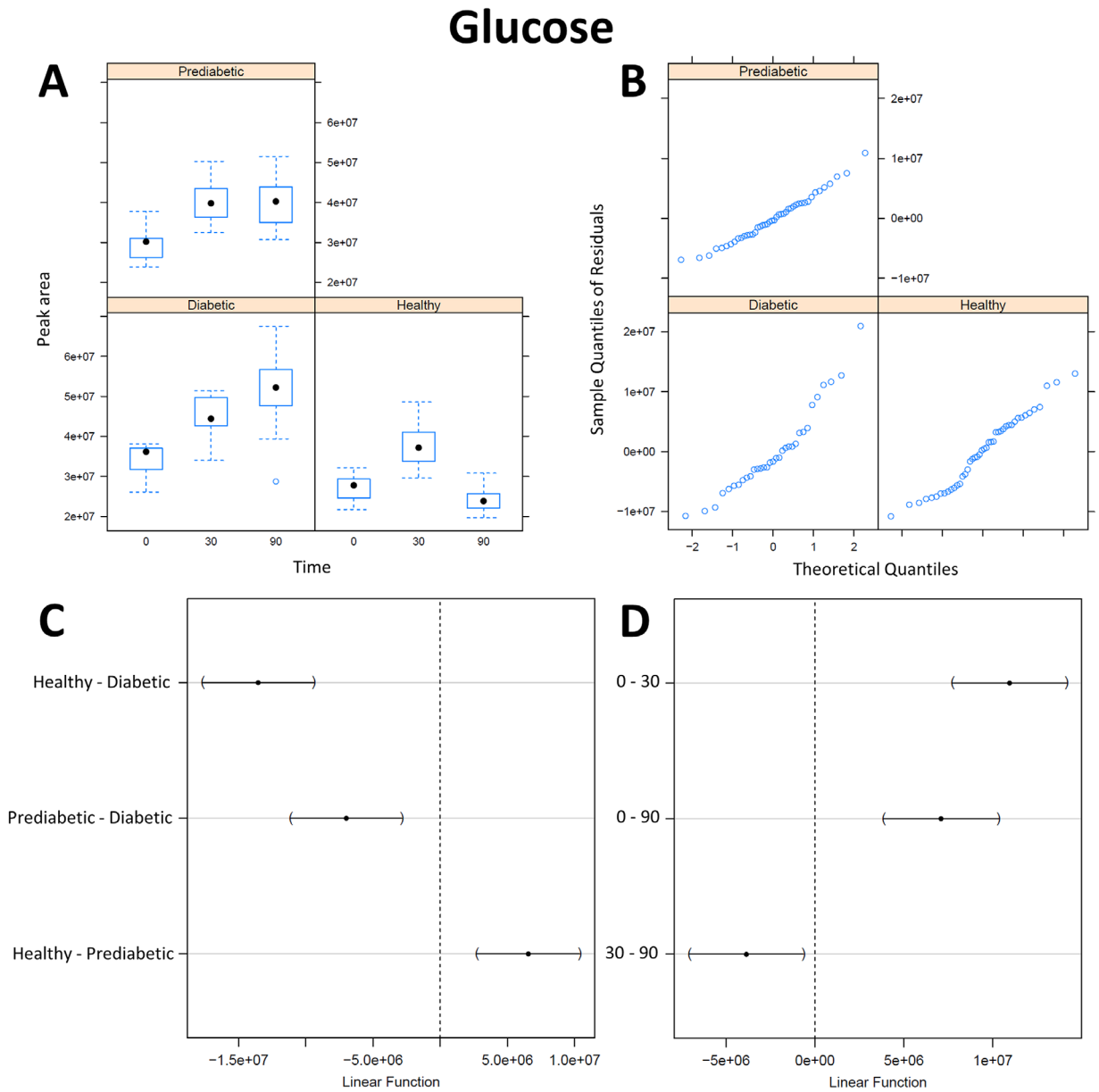

Figure 2. Glucose as example for the statistical workflow to detect group and time differences after an OGTT. Panel A) Box plots grouped by health status and time. Panel B) QQ-plots depicting the quantiles of the residuals against the theoretical normal. Panel C) Plot for the effect (dots) and confidence interval (lines) for differences in the health status (group). Panel D) Plot for the effect (dots) and confidence interval (line) for differences during the time points (time). Significance corresponds to the confidence interval line not crossing the vertical dotted zero-line.

depicts the distribution of the average intra-day coefficients of variation for all sugar compounds measured in the QC samples before drift correction. 70% of the sugar compounds had a coefficient of variation of less than 25% and could therefore be measured with good reproducibly. Interestingly, disaccharides such as sucrose, lactose, maltose, trehalose, and lactulose were detected in all plasma samples, even though carbohydrates are generally hydrolyzed into monosaccharides before absorption. Lactulose was only detected in the time course after ingestion of the test drink containing lactulose for transit-time measurement (Figure S3, Supporting Information File 1).

3.2. Results for Group and Time Differences After an OGTT

The longitudinal trend of each sugar compound was illustrated by box plots grouped by health status and time points. Each sugar compound was modeled by a mixed linear model on group and time (both as factor variables) with a random intercept. Then, we tested for all possible group and time differences. In Supporting Information File 3, each sugar compound is presented by box plots, the fitted model, test results, and QQ-plots (see e.g., glucose in **Figure 2**). **Table 2** summarizes the sugar compounds showing significant group and/or time differences. Moreover, in Table 2,

Table 2. Significant sugar compounds to evaluate health status-dependent changes in time courses during an OGTT.

Sugar compounds	*p*-values—Group			*p*-values—Time			Direction of time-dependent change[a]		
	Healthy—Diabetic	Prediabetic—Diabetic	Prediabetic—Healthy	0–30 min	0–90 min	30–90 min	Healthy	Prediabetic	Diabetic
Glucose[b),d)]	<0.001	<0.001	<0.001	<0.001	<0.001	0.013	↑↓	↑	↑
Mannose[c)]	<0.001		<0.001			<0.001	↓	–	↑
1,5-Anhydroglucitol	<0.001		<0.001	0.044			↑↓	–	–
U15	<0.001		0.004	<0.001	0.041		↑↓	↑	↑
Sedoheptulose[b),d)]	0.001		0.03				↑↓	–	–
Fructose[b),d)]	<0.001	<0.001		0.005	0.002		↑↓	↑	↑
Trehalose[c)]	<0.001	<0.001		<0.001	<0.001	0.004	↑↓	↑	↑
Sorbitol[b)]	<0.001	<0.001					↑↓	↑	↑
Arabinose[b)]	<0.001			0.014			↑↓	↑	↑
U01	0.001				0.035		–	↓	↓
U07	0.007			<0.001	0.013		↑↓	↑	↑
U16	0.004			0.018			–	↑↓	↑
meso-Erythritol	0.038						↑↓	–	–
Glucuronic acid[d)]		0.027					↓	–	–
Psicose				<0.001	<0.001	<0.001	↑	↑	↑
U18[b)]	0.015	0.012		<0.001	<0.001	<0.001	↑	↑	↑
Threitol				<0.001	<0.001		↑	↑	–
Lactulose[b),d)]				0.009	<0.001	<0.001	↑	↑	↑
scyllo-Inositol				0.039	0.002		–	↑	↑
Xylose[b)]	0.049				<0.001	<0.001	↓	–	–
Erythronic acid				0.036			↑↓	↑	↑
Maltose[b),d)]					0.032		↑↓	↑	↑
U17					0.034		↓	–	↓
Threonic acid						0.002	↑↓	↑	–

Significance threshold: *p*-value < 0.05. [a)] The direction of the time-dependent changes for the different volunteer groups is given, ↑↓: increase from 0 to 30 min and decrease from 30 to 90 min; ↑ increase from 0 to 30 to 90 min; ↓ decrease from 0 to 30 to 90 min;—remain unchanged over time; [b)] Detectable in the OGTT test drink; [c)] Presence in OGTT drink not confirmed due to low concentration and huge amounts of glucose or maltose; [d)] For readability only one of two detected derivatives is listed.

the direction of time-dependent changes of sugar compounds is described. Box plots of these sugar compounds are depicted in **Figure 3**.

Specifically, the selected sugar compounds could be classified depending on whether they mainly show a health status-dependent difference, either dependent or independent of the time course during an OGTT, or they mainly show a difference in time course independent of health status. For example, i) trehalose clearly differentiated between health status and also between the time points (group and time effect), ii) sorbitol showed a distinct difference only in health status (only group effect), and iii) psicose distinctly increased during the OGTT independent of the health status (only time effect). For the effects of all other sugar compounds see Table 2.

3.3. Differentiation of Healthy, Prediabetic, and Diabetic Subjects in the Fasted State

To ascertain whether healthy, prediabetic, and diabetic volunteers can be separated based on their fasting plasma sugar profile, we compared PCA scores A) and loadings B) plots (**Figure 4**). A separation of healthy and diabetic subjects was observed in the scores plot, while the prediabetic volunteers were in between. In the loadings plot it is obvious that, in addition to glucose, a range of other sugar compounds similarly added to the separation of the volunteer groups observed in the scores plot.

In addition to glucose, significant differences between healthy, prediabetic, and diabetic volunteers were revealed for maltose, trehalose, an unknown sugar compound (U15), fructose, mannose, 1,5-anhydroglucitol, and sedoheptulose (**Figure 5**). The highest median fold change with a factor of almost three between healthy and diabetic volunteers (diabetic volunteers had a higher level) was found for maltose. Sedoheptulose concentration was almost twofold lower in diabetic volunteers than in healthy individuals.

3.4. Evaluation of Potential Confounders in Fasting Plasma Samples in an Independent Study

To assess potential confounder effects of age, BMI, and sex across the volunteer groups in the set of sugars with significant

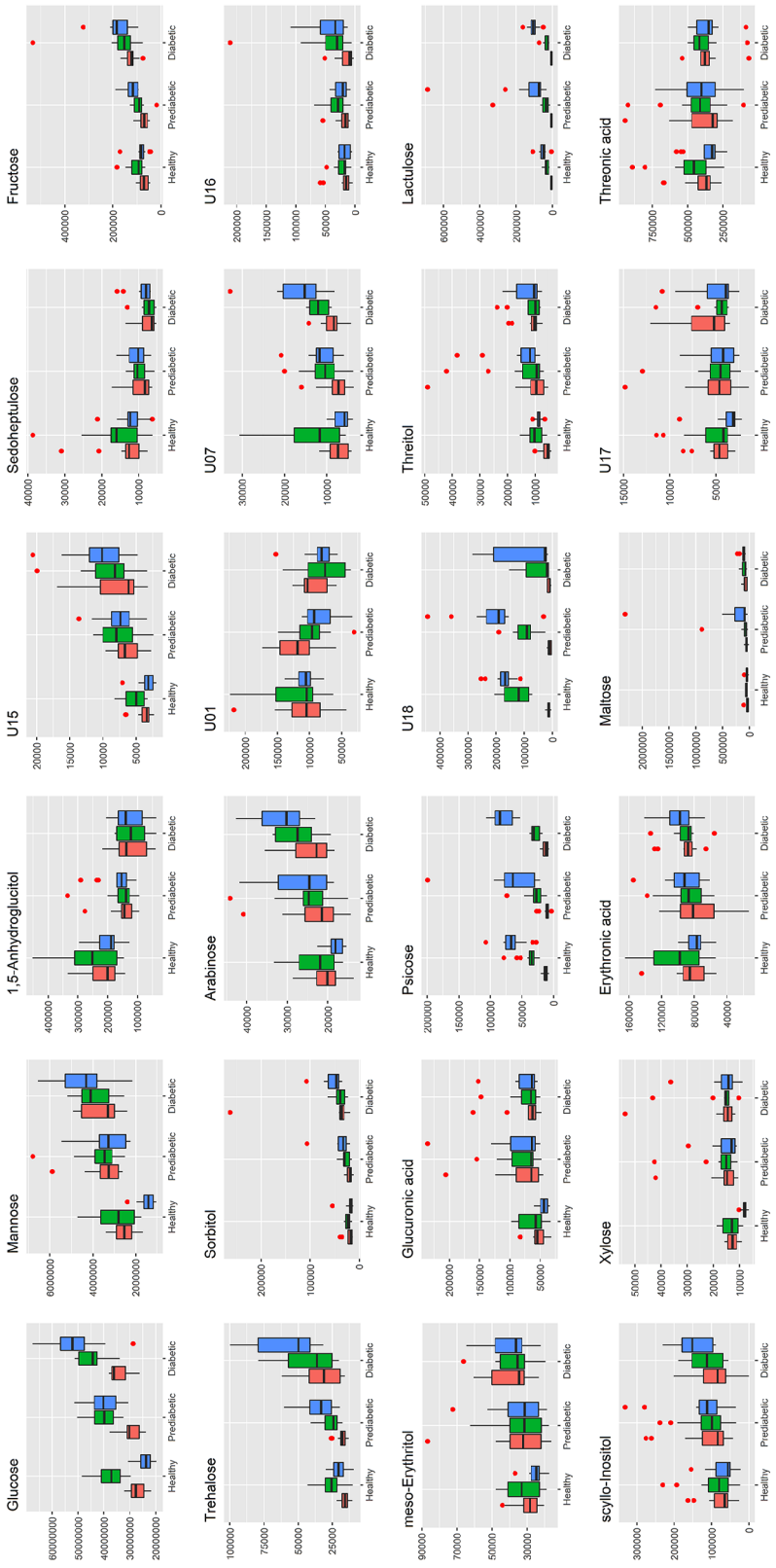

Figure 3. Box plots of peak areas (*y*-axis) for significant sugar compounds listed in Table 2 showing similar and different patterns to glucose after an OGTT, grouped into healthy ($n = 15$), prediabetic ($n = 15$), and diabetic ($n = 11$) volunteers (*x*-axis). Red box plot: time point 0 min, green box plot: time point 30 min, and blue box plot: time point 90 min.

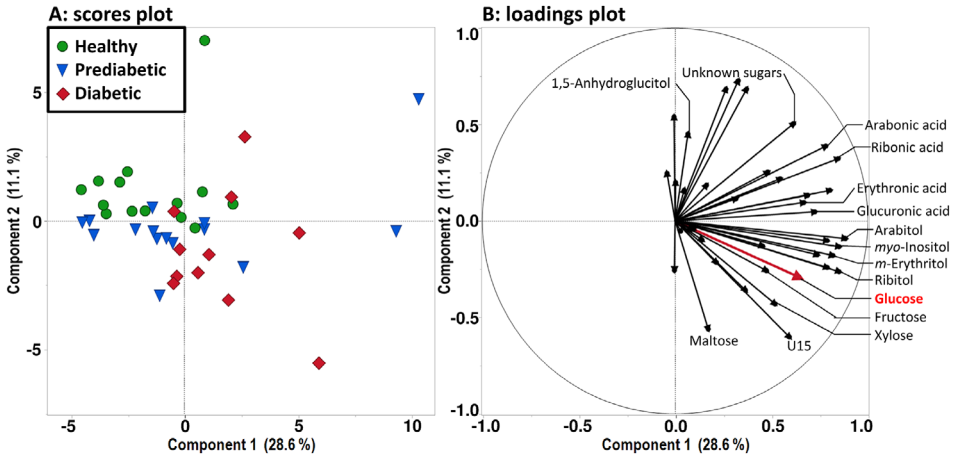

Figure 4. Separation of healthy ($n = 15$), prediabetic ($n = 15$), and diabetic ($n = 11$) volunteers based on fasting plasma sugar profile using a principal component analysis (PCA). A) scores plot; B) loadings plot; glucose marked as bold, red arrow.

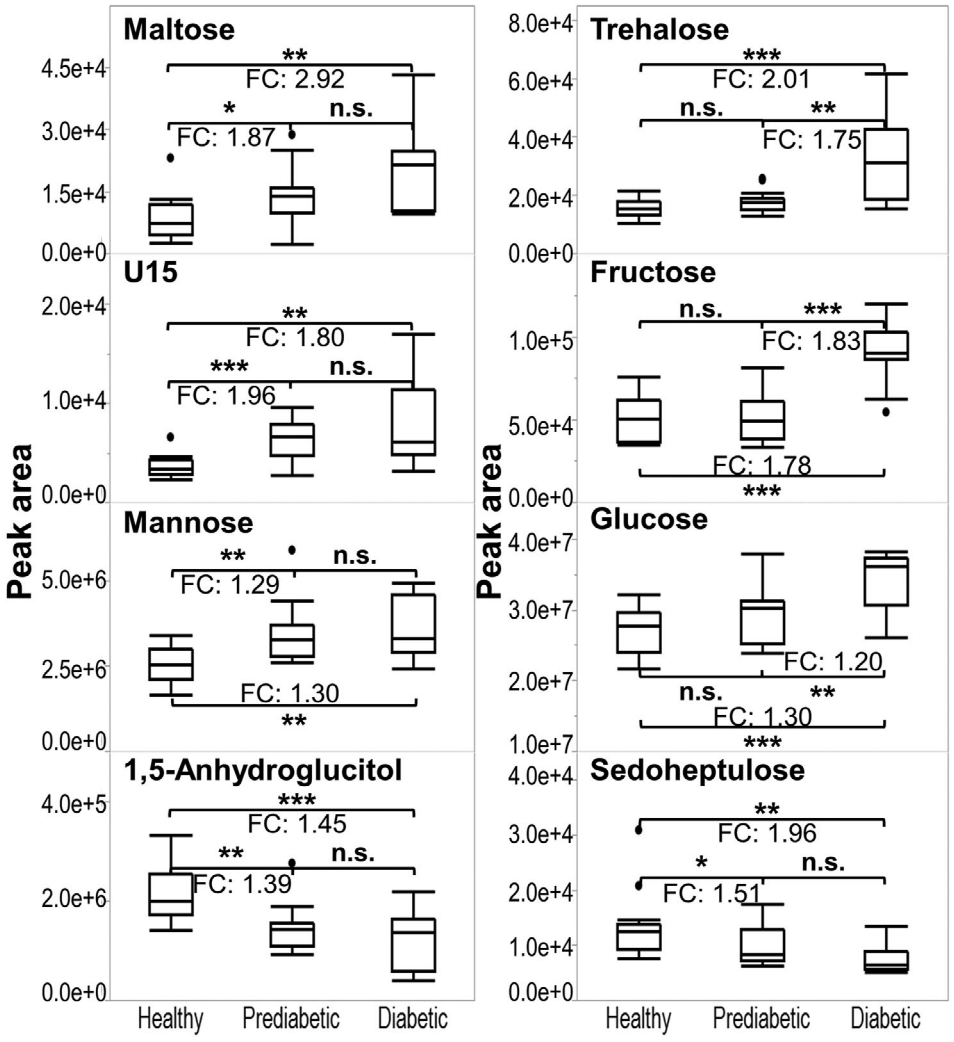

Figure 5. Box plots of peak areas for sugar compounds identified as significantly distinguishing between healthy ($n = 15$), prediabetic ($n = 15$), and diabetic ($n = 11$) volunteers based on fasting plasma using the response screening workflow. *$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.001$; n.s., not significant. FC, median fold change. Only one of the two detected derivatives is shown in the case of maltose, fructose, glucose, and sedoheptulose. In the case of glucose and 1,5-anhydroglucitol, the 2nd ANOVA was a normal one-way ANOVA and the post-hoc test was a Tukey-HSD, while all other sugar compounds were subjected to a Wilcoxon test as the 2nd ANOVA and a Steel–Dwass as post-hoc test.

Table 3. Results of the correlation analysis and unpaired hypothesis tests for verification of potential relevant sugar compounds using fasting plasma of a selected subgroup of the KarMeN cohort.

Sugar compounds	Correlation				*t*-test[a]					
	Age		BMI		Age		BMI		Sex	
	Spearman's rho	*p*-value	Spearman's rho	p-value	*p*-value	Median fold change	*p*-value	Median fold change	*p*-value	Median fold change
Threonic acid	−0.286	0.029	−0.297	0.024	**0.008**	**−1.25**	0.023[c]	−1.15	0.283	−1.07
Sedoheptulose[b]	−0.229	0.084	−0.144	0.282	**<0.001**	**−1.57**	0.417	−1.08	**0.018**	**1.33**
U07	−0.102	0.448	−0.066	0.624	0.232	−1.08	0.303	1.01	0.269	−1.02
1,5-Anhydroglucitol	−0.074	0.580	0.070	0.601	0.604	1.03	0.546	1.02	**<0.001**[c]	**1.42**
Trehalose	−0.038	0.778	0.238	0.072	0.605	−1.11	0.634	1.14	0.077	1.25
scyllo-Inositol	−0.002	0.987	−0.172	0.196	0.430	−1.17	0.133	−1.34	0.831	1.04
U18	−0.001	0.992	−0.044	0.744	0.572	−1.02	0.836	−1.00	0.827[2]	1.06
Sorbitol	0.043	0.746	0.026	0.849	0.660	−1.02	0.517	−1.05	0.686	−1.10
Maltose[b]	0.055	0.682	0.173	0.193	0.976	−1.05	0.150	1.09	0.944	−1.04
Fructose[b),d]	0.058	0.667	0.092	0.492	0.433	1.03	0.549	1.06	0.926	1.01
U16	0.075	0.578	**0.315**	**0.016**	0.171	−1.05	0.170	1.13	0.054	1.10
Glucose[b]	0.121	0.368	0.128	0.340	0.572	1.01	0.290	−1.00	0.233	1.01
U01	0.188	0.158	0.120	0.369	0.812	1.01	0.917	1.02	0.133	−1.11
Ribitol[d]	0.245	0.064	−0.068	0.610	0.060	1.12	0.247	−1.03	0.017	−1.14
U15[d]	0.252	0.057	0.264	0.045	0.137	1.32	0.164	1.40	0.343	1.26
Psicose[d]	0.267	0.043	0.270	0.041	0.389	1.02	0.130	1.15	0.016	1.15
Arabinose	0.292	0.026	0.283	0.031	0.226	1.07	0.808	1.09	0.349	1.05
Mannose	0.297	0.024	0.161	0.229	0.086	1.17	0.178	1.12	0.050	1.04
Xylose[d]	**0.327**	**0.012**	0.246	0.063	0.327	1.04	0.101	1.05	0.437	1.03
U17	**0.374**	**0.004**	0.099	0.461	0.123	1.24	0.701	1.06	0.891	1.00
Threitol[d]	**0.415**	**0.001**	**0.302**	**0.021**	0.078	1.10	0.070	1.08	0.828	−1.05
Erythronic acid	**0.441**	**<0.001**	**0.467**	**<0.001**	0.239	−1.00	0.002	1.14	0.185	1.05
meso-Erythritol[d]	**0.477**	**<0.001**	**0.443**	**<0.001**	0.021	1.11	0.012	1.16	0.815	−1.01
Glucuronic acid[b]	**0.547**	**<0.001**	**0.418**	**0.001**	0.059	1.18	0.016	1.13	0.058	1.08

[a] For the *t*-test the following groups were defined for comparison: age: 21–30 years ($n = 10$) against 50–72 years ($n = 23$); BMI: 19–23.9 kg m^{-2} against 24–30 kg m^{-2} (each $n = 29$) and sex: male ($n = 28$) against female ($n = 30$). Significant values ($p < 0.05$) with rho > $|0.3|$ or with median fold changes higher $|1.25|$ are highlighted in bold; [b] For readability only one of two detected derivatives is listed; [c] In these cases a Welch's *t*-test was applied due to unequal variances; [d] *t*-test was a non-parametric Wilcoxon test, while for the other sugar compounds normal one-way ANOVA was used (depending on normality and homogeneity of variances).

differences between groups, fasting plasma samples selected from the KarMeN cohort were analyzed for comparison. Spearman correlation coefficients as well as results for hypothesis tests with variables such as age, BMI, and sex covering ranges similar to those of the volunteers in the present study are shown in **Table 3**. They revealed a significant correlation with age for xylose and the unknown sugar U17, in the case of threitol, erythronic acid, *meso*-erythritol, and glucuronic acid for both age and BMI, and in the case of unknown sugar U16 for BMI (Figure S4, Supporting Information File 1). Significant differences in the hypothesis testing were found for threonic acid regarding age, sedoheptulose regarding age and sex, and for 1,5-anhydroglucitol regarding sex (Figures S5 and S6, Supporting Information File 1). Thus, for some sugar compounds (xylose, threonic acid, threitol, *meso*-erythritol, glucuronic acid, sedoheptulose, and 1,5-anhydroglucitol) an extended general model accounting for the confounders, as suggested in Supporting Information File 2, would be more suitable and especially should be taken into consideration for subsequent studies. Since all other sugar compounds were not significantly affected by these variables (age, BMI, and sex) or had weak correlations (rho < $|0.3|$) or median fold changes below $|1.25|$, it is likely that these variables (age, BMI, and sex) are not confounding results for these sugar compounds and the simpler model applied in this pilot study (Supporting Information File 3) should be sufficient.

3.5. Analysis of the Ingested OGTT Test Drink and Further Commercial OGTT Solutions

To ascertain whether some of the significant sugar compounds listed in Table 2 might be occurring or also increase in plasma due to their presence in the test drink used for the OGTT, this test drink was analyzed using the same method as for the plasma sugar profiling. U18, one of the two sugar compounds showing a distinct time-dependent increase after the OGTT (Figure 3) was detected in the test drink. Furthermore, we found large amounts of tagatose, an epimer of fructose. Other sugar compounds were also detectable in the test drink, but in comparatively small amounts relative to glucose and maltose (Table 2).

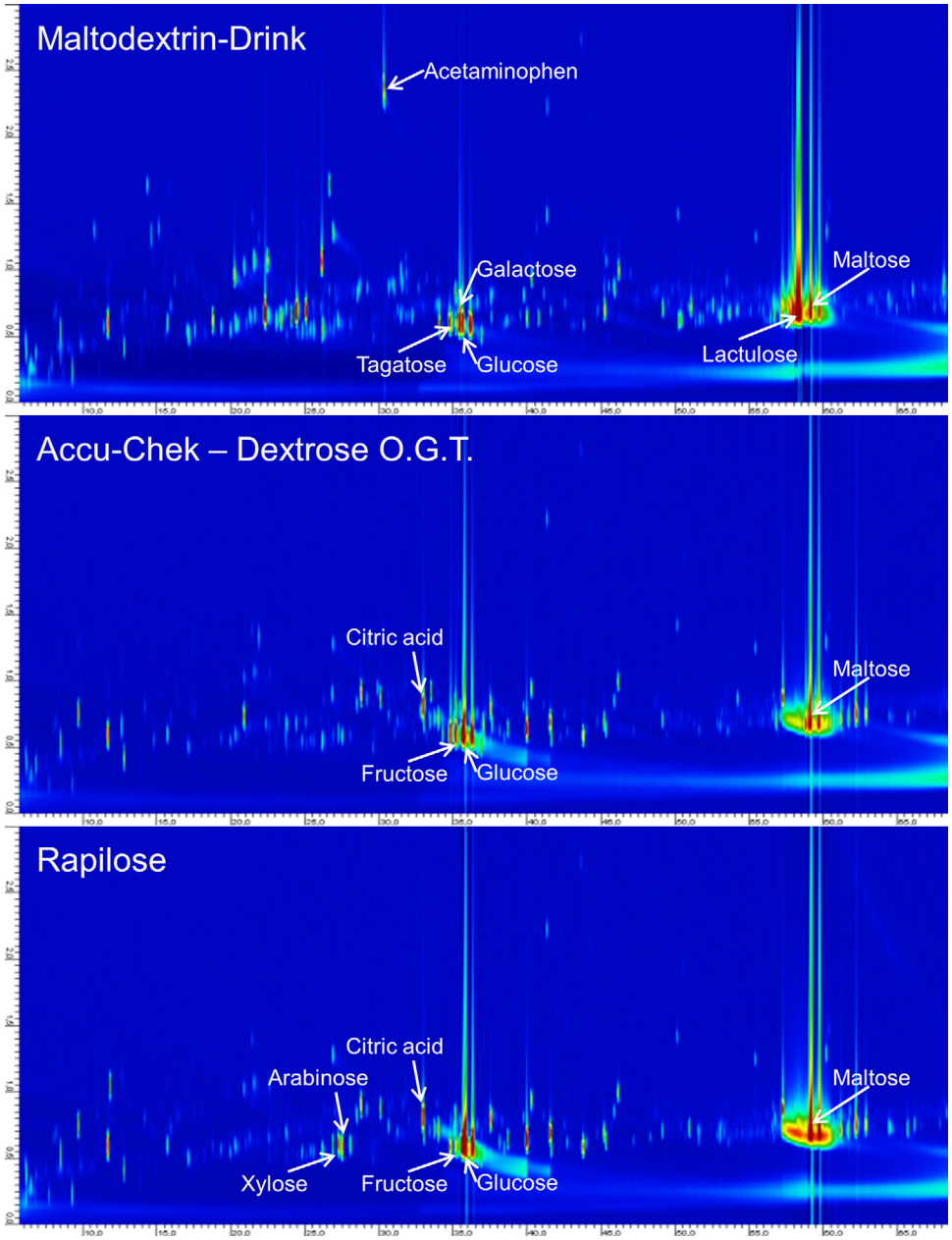

Figure 6. GC × GC-MS chromatograms from the start to 68 min of the maltodextrin-drink, and two commercial OGTT solutions. In the maltodextrin the additionally added acetaminophen and lactulose are highlighted. Some of the major differences between the OGTT solutions were also identified. The part of the chromatogram from 68 to 80 min was excluded due to many artifacts arising in this region.

Additionally, a general comparison of compounds occurring in the test drink applied in this study and further commercially available OGTT solutions was performed using GC × GC-MS measurements to assess whether the occurrence of other sugars and non-sugar compounds in OGTT solutions is common. Chromatograms of the GC × GC-MS measurements clearly show that the test drink and commercial OGTT solutions contained additional compounds to glucose, such as maltose, galactose, fructose, tagatose, and many more, even non-sugar compounds such as citric acid (**Figure 6**). In the case of the test drink from this study, one can also see the added acetaminophen and lactulose.

4. Discussion

4.1. Plasma Sugar Profile

Around 40 different sugar compounds, 27 known and 13 unknown, were reliably and reproducibly detected in plasma. Among the known mono- and disaccharides, polyols and sugar acids, we also detected a variety of rare sugar compounds such as psicose, trehalose, or xylonic acid. Interestingly, amounts of disaccharides such as sucrose, lactose, maltose, trehalose, and lactulose found in plasma were similar to other monosaccharides

(except glucose), challenging the dogma of complete hydrolysis of disaccharides by mucosal disaccharidases prior to absorption of monomers. Transfer of disaccharides into systemic circulation was already described decades ago,[40,41] but has recently received new attention since passive permeation of disaccharides such as lactulose across the epithelium can serve as a measure of gut permeability.[42] We here demonstrate the appearance of lactulose from the test drink (assessment of oral-cecal transit time) in peripheral blood. We also detected lactose, which in urine has been described as candidate marker for dairy consumption[19,43] and similarly, sucrose in urine was proposed as a marker for total dietary sugar intake.[44] The origin of the other detected disaccharides is currently a mystery. They may come from the diet, or they might be generated endogenously, for example by degradation of heteroglycans, or as by-product of other metabolic processes.

4.2. Sugar Compounds and Diabetes

The fasting plasma sugar profile already allowed a separation of healthy and diabetic volunteers, with prediabetic volunteers in between. The loadings plot revealed that other sugar compounds are just as relevant for this separation as the "key separator" glucose. Although glucose is a good and easily assessable clinical biomarker for both diabetic and prediabetic states, a metabolite profile may enable simpler detection of disease subtypes, for example to better predict complications or optimize preventive measures.[1] In addition to known marker compounds, such as 1,5-anhydroglucitol,[45] mannose,[46,47] and fructose,[12,48] other sugar compounds such as maltose,[11] trehalose, sedoheptulose, and an unknown sugar-like compound (U15) were found to significantly distinguish between healthy, prediabetic, and diabetic volunteers based on their fasting plasma levels. They thus represent promising biomarker candidates for IR and type 2 diabetes worth to be further explored in population studies. Such markers are important in the light of the ever-increasing incidence of diabetes mellitus type 2, as tools for diagnosing and preventing the disease as early as possible.[2,10,11,49]

1,5-anhydroglucitol is used as an indicator of short-term glycemic control in type 2 diabetes.[45] When the tubular load of glucose is high, 1,5-anhydroglucitol is rapidly excreted in urine due to competition for reabsorption via the renal brush-border membrane glucose transporters.[45] However, 1,5-anhydroglucitol plasma levels differed in males and females in the KarMeN cohort, suggesting that different threshold levels for diagnostic purposes may be needed.[50] Other sugar compounds such as xylose, threitol, sedoheptulose, *meso*-erythritol, erythronic acid, threonic acid, glucuronic acid, and two unknown sugars (U16, U17) were also associated with age, BMI, or sex in the KarMeN plasma sample set, and therefore, should be interpreted with caution when conducting subsequent studies. Recently, sedoheptulose was reported to decrease with age in urine sample data from the same cohort,[51] which suggests that differences found between healthy, prediabetic, and diabetic volunteers are mainly due to differences in age. The correlation of *meso*-erythritol with BMI is interesting with regard to a recent publication where erythritol was described as associated with weight gain in young adults.[52]

Other confounders could be the diet on the day before the intervention or more generally the habitual diets with respect to high or low sugar intake or consumption of sugar substitutes (by prediabetic and diabetic volunteers) such as sorbitol, mannitol, or erythritol. However, overnight fasting (12 h) minimizes the influence of the diet on the day before due to the fast clearance and excretion of the very polar sugar compounds. For those sugar compounds whose plasma levels changed during the OGTT, the effects of previous consumption can in essence be ruled out.

The disaccharide trehalose was found in higher levels in fasting plasma of diabetic volunteers, and plasma levels also changed differently during the OGTT. Although the changes mimic those of glucose, with a transient peak at 30 min and a decline at 90 min in healthy, and a steady increase over time in prediabetic and diabetic individuals, it cannot be confirmed that these changes are similarly affected by insulin and IR as known for glucose. To our knowledge, such effects on plasma trehalose have not been reported before, but an increased activity of plasma trehalase was described in diabetic states [53,54] and single nucleotide polymorphisms in the trehalase gene were recently found to be associated with type 2 diabetes.[55] Additionally, in a study on diabetic retinopathy, trehalose was detectable in most diabetic, but not in non-diabetic participants.[23] However, neither the origin of trehalose in plasma, nor the cause of the differences found between healthy and diabetic individuals are currently known.

Trehalose could not be detected in the test drink, and thus its appearance and time-profile in plasma argues for an endogenous origin. That maltose as a degradation product of maltodextrin provided in the test drink becomes detectable in plasma is in line with a significant permeation of intact disaccharides across the intestinal epithelium. What makes the findings on the disaccharides even more interesting is that their change over time in plasma during the OGTT is similar to the pattern of glucose, suggesting similar underlying insulin-dependent processes in clearance. This also holds true for mannose, fructose, xylose, sorbitol, *scyllo*-inositol, *meso*-erythritol, threitol, ribitol, arabinose, and two of the unknown sugar-like compounds (U07, U15). An endogenous production from glucose cannot be confirmed for these compounds as a result of the detection of trace quantities of fructose, sorbitol, xylose, sedoheptulose, and arabinose in the test drink itself. Nonetheless, the differences over time based on health status suggest underlying insulin-dependent processes and therefore these sugar compounds might be interesting for further exploration as biomarkers of IR and type 2 diabetes.

A comparison of the test drink applied in this study and further commercial OGTT solutions depicts clearly that further sugar and non-sugar compounds in addition to glucose can commonly be found in such test solutions. The additional sugar compounds likely arise from the commercial maltodextrin or glucose syrup used for production of OGTT solutions. This urges for care when interpreting postprandial changes of metabolites in plasma when using such test agents, as the occurrence of additional compounds might influence the metabolism of the compounds of interest and an endogenous production of additionally found compounds cannot be assumed. Therefore, a good characterization of the test products is an important step in any intervention study. More intensive work for the harmonization of the preparation of different OGTT solutions is desirable for comparable results in OGTT's.

Xylose has to our knowledge not been described as a metabolite that changes during the OGTT in volunteers with IR or diabetes. However, a recent analysis described different plasma levels in obese and lean individuals.[15] Xylose can be produced in the pentose phosphate pathway (PPP), which frequently shows impaired activity in diabetic states.[23] Although we detected sedoheptulose in plasma, further intermediates of the PPP such as ribulose and xylulose were only found in traces, and thus could not prove that PPP per se is altered. Similarly, arabinose could be a by-product of an epimerase reaction in PPP, but its production in human metabolism has not been confirmed.

Mannose is produced from glucose via fructose-6-phosphate, and has previously been identified as a diabetes metabolite marker.[11,20,56] Fasting mannose levels correlate with fasting plasma glucose, and mannose seems to be only minimally influenced by meals.[46] Early in vitro studies[57] demonstrated that mannose shares the uptake system of glucose in muscle, likely via Glut4, which is insulin-dependent. Consequently, mannose has a diagnostic marker quality, especially in the case of borderline diabetes,[46,47] and our data with the pronounced difference in mannose levels after the OGTT across the groups also demonstrates this special quality.

Two conspicuous sugar compounds, an unknown sugar-like compound U18 (possibly a C4 sugar acid) and psicose, showed a uniform strong increase after the OGTT, dependent on health status for U18 and independently of health status for psicose (U18 by 18.5-fold and psicose by sevenfold; Figure 3). Whereas U18 was detectable in the test drink, psicose was not, but we found high amounts of tagatose, which like psicose is an epimer of fructose. It may well be that tagatose was completely converted to psicose in human metabolism, explaining the steep postprandial increase in plasma psicose. Alternatively, psicose may also be produced endogenously from glucose via fructose in glycolysis by an epimerase in response to the glucose load. We also showed that fructose in plasma increases upon the glucose load, which requires that a fraction of the cytosolic fructose-phosphate pool is dephosphorylated, resulting in efflux of free fructose via a Glut-transporter system. Thus, it appears plausible that psicose as a fructose-epimer is generated from glucose in cells. Further research on the origin of psicose will be especially interesting in light of its proposed anti-hyperglycemic and anti-hyperlipidemic effects.[58–61]

Major limitations of our pilot study are the limited sample size, and the unbalanced design of the study, especially with respect to sex and age. A complex general modelling would have been able to adjust for these confounders. However, due to the limited sample size, a general model would result in overfitting. Therefore, to address the limitation of the unbalanced design as best as possible and generally assess the influence of age, BMI, and sex on sugar compounds of interest, an independent, external study was taken into account. Overall, our study can be viewed as a pilot study, which led to highly interesting and unexpected results about the influence of the diabetic state on the sugar profile after an OGTT. In subsequent studies with a larger sample size and a more balanced design, a general modelling accounting for confounders, interactions, random slope and intercept will be possible. Moreover, with a larger sample size, multivariate statistical analyses could additionally be conducted to focus on the different relationships between the sugar compounds.

5. Concluding Remarks

The 40 different sugar and sugar-like compounds we detected in human plasma were almost all recently identified by us as normal constituents of human urine.[19] This is an important finding with respect to large cohort studies, where often only plasma samples are available; meaning plasma sugar compounds are similar to 24 h urine samples, although concentrations in plasma are generally lower. In volunteers with IR or type 2 diabetes numerous sugar compounds show characteristic plasma profiles that mimic those of glucose, with rapid clearance during the OGTT in healthy individuals, but not in an IR or diabetic state. This suggests that, like glucose, many more sugar compounds are subject to insulin-dependent removal from plasma after absorption or when produced endogenously. Although we could not confirm that compounds such as psicose, trehalose, mannose, and others are indeed produced from glucose in metabolic pathways during the OGTT, for some this appears likely, suggesting an avenue for additional research, preferentially using stable-isotope labeled glucose. However, we demonstrated that the source of glucose for the OGTT, here in form of maltodextrin, can contain other sugars and additional compounds. We found this also holds true for commercial OGTT solutions, highlighting the need for good characterization of the test product and care in interpreting postprandial metabolite changes.

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

S.E.K., H.D. designed the research project; H.D., T.S., Y.-M.L., A.S.H. developed the MIPROMET concept and design; C.I.M. conducted the analytical experiment; C.I.M., B.E., C.H.W., P.G.F. analyzed the data and performed statistical analysis; C.I.M. wrote the initial draft of the manuscript; C.H.W., S.E.K., H.D., P.G.F. critically reviewed and contributed to the manuscript;

C.I.M., H.D., S.E.K. had primary responsibility for final content. All authors read and approved the final version of the manuscript.

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