METABOLOMIC MARKER ERYTHRITOL ASSOCIATED WITH CENTRAL ADIPOSITY GAIN IN YOUNG ADULTS AND SYNTHESIZED AS A PENTOSE-PHOSPHATE-PATHWAY BY-PRODUCT

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completed the targeted and non-targeted metabolomic assays; JPT, LK, XD performed blood incubation experiments and analyzed the data; PAC, KCH, KH, LSB and JPT collected, analyzed and interpreted *in vivo* dried blood spot experiments; PAC, KCH, PJS, KH, JPT, CJ, and LK analyzed and interpreted all data; KCH, JPT, KH, LSB, LK and PAC drafted the manuscript, and all coauthors edited and approved the manuscript.

ABSTRACT

Metabolomic markers associated with incident central adiposity gain were investigated in young adults. In a nine-month prospective study of freshmen (N=264), blood samples and anthropometry measurements were collected in the first 3 days on campus and at the end of the year. Plasma from individuals was pooled by phenotype (incident central adiposity, stable adiposity, baseline hemoglobinA1c (HbA1c)>5.05%, HbA1c<4.92%) and assayed using GC-MS, chromatograms were analyzed using the MetaboliteDetector software, and normalized metabolite levels were compared using Welch's t-test. Assays were repeated using freshly prepared pools, and statistically significant metabolites were quantified in a targeted GC-MS approach. Isotope tracer studies were performed to determine if the potential marker was an endogenous human metabolite in men and in whole blood.

Participants with incident central adiposity gain had statistically significantly higher blood erythritol ($p<0.001$, FDR = 0.0435), and the targeted assay revealed a 15-fold (95% CI 13.27, 16.25) higher blood erythritol compared to participants with stable adiposity. Participants with baseline HbA1c>5.05% had 21-fold (95% CI 19.84, 21.41) higher blood erythritol compared to participants with lower HbA1c ($p<0.001$, FDR = 0.00016).

Erythritol was shown to be synthesized endogenously from glucose *via* the pentose phosphate pathway in stable isotope-assisted *ex vivo* blood incubation experiments, and through *in vivo* conversion of erythritol to erythronate in stable isotope-assisted dried blood spot experiments. We found evidence for endogenous erythritol synthesis from glucose by the pentose phosphate pathway. Therefore, endogenous production of erythritol from glucose may contribute to the erythritol—obesity association observed in young adults.

SIGNIFICANCE STATEMENT

The prevention of weight gain in adulthood is a public health challenge, particularly given the difficulty of losing weight. Data on freshmen were collected at the beginning and end of the academic year, and baseline blood samples were studied to find markers of incident weight gain. A metabolite, erythritol, was elevated at the beginning of the year in freshmen who went on to gain weight, fat and abdominal fat compared to those with stable weight. Erythritol is an artificial sweetener and prior studies claimed no endogenous synthesis. We report a novel previously unrecognized metabolism of glucose to erythritol, and, given the erythritol—weight gain association, research is needed to understand whether and how this pathway contributes to weight gain risk.

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Introduction

In fall 2015, an estimated 3.3 million high school graduates enrolled in postsecondary education as first-time college freshmen (1), and the transition to a residential college environment is associated with weight gain. About 75% of the population experiences weight gain during this transition (2, 3), but there have been few efforts to identify biomarkers of risk that could guide prevention efforts. A study (4) in monozygotic twins discordant for BMI reported divergence at about age 18, corresponding to a time in life when environment shifts, and further underscoring the importance of young adulthood in the lifetime trajectory of adiposity and as a window of opportunity for prevention (5).

Observational studies of young adults focus on behavioral/environmental risk factors for adiposity gain, with few studies reporting biological markers in relation to either cross-sectional and/or longitudinal changes in adiposity or body weight. A recent overview of intervention studies to prevent weight gain in young adults (6) identified 37 studies; the majority assessed diet, physical activity and behaviors, only 10 studies directly measured changes in weight, body mass index (BMI; weight, kg/height, m^2) and/or body composition, and none of the studies measured biological markers. Existing prediction or risk scores integrate across the various domains contributing to weight gain risk, including demographic, anthropometric, behavioral, psychological, diet-related, and physical activity (7), but do not incorporate biological markers.

Metabolomics approaches, which measure the final downstream products of the complex interactions among genetic, environmental, and pharmacologic influences, have been used to study obesity as a phenotype, but few studies have investigated metabolite profiles that are predictive of risk of future weight gain in young adults who are not currently overweight or obese. A recent study (8) investigated the metabolic signature associated with adiposity and

adiposity change in participants aged 16 to 39 years. This study focused on a pre-specified set of 67 metabolites, including lipoproteins, inflammatory markers, fatty acids and glycolysis precursors. Branched chain amino acids, lipoprotein-related metabolites, and glycolysis-related metabolites were all positively associated with change in BMI. Studies in older adults also identified the branched chain amino acid (BCAA)-related signature, and reported higher leucine/isoleucine and valine in obese compared to non-obese individuals (4, 9-11). Furthermore, experimental research using metabolomic profiling identified causal effects of weight gain on multiple blood metabolites, including elevation of BCAAs, very low density lipoprotein (VLDL) (8, 10, 12), triacylglycerol (10), C-reactive protein (8, 10), and insulin-like growth factor (10). However, the role of antecedent metabolomic markers in weight gain and the human adiposity phenotype is not established.

We investigated the relation of the metabolome to incident adiposity gain in young adults over the first year of college. A non-targeted GC-MS approach was used to identify novel metabolites associated with incident adiposity gain, and the endogenous synthesis of a novel biomarker associated wtih obesity was investigated by stable isotope assisted metabolomics approaches. Finally, *in vivo* and *ex vivo* stable-isotope assisted metabolomics approaches were used to investigate whether the risk biomarker originated from diet and/or through endogenous synthesis.

Methods

Study Sample

The study used data from a recently completed longitudinal cohort study of college freshmen residing on-campus during their freshman year (2011-12) at a university in the Northeastern United States. Study participants were selected through stratified random sampling

of the incoming freshman class to recruit equal numbers of males and females, and to represent the characteristics of the full incoming class. Participants were aged 18-19 years, about 50% were female, and at the study baseline about 10.5% were overweight (BMI > 25). The study was approved by the Cornell University Institutional Review Board, and all participants provided informed consent.

Collection of Participant Data

Data were collected during participant study visits and included anthropometric measurements (height, weight, waist and hip circumferences) using standardized methods and plasma for metabolomics (non-fasting), which was collected within the participants' first three days on-campus and at the end of the academic year (mean follow-up time 35 weeks, SD 1.5). Adiposity, measured via dual energy X-ray absorptiometry (DXA, Hologic Inc., Bedford, MA), was assessed within the first two weeks and at the end of the academic year.

Blood was collected into EDTA vacutainers (BD, Franklin Lakes, New Jersey), stored at 4°C for 30-90 minutes, centrifuged at relative centrifugal force of 1300 x g. for 10 minutes, divided into 3-4 cryovials, and transferred to -80°C freezers for storage. Hemoglobin A1c (HbA1c), a marker of usual glycemia and long-term glycemic control, was measured in whole blood using the Dimension Xpand Plus Integrated Chemistry System (Siemens, Germany).

Phenotypes related to cardiometabolic risk groups were defined as follows: 1) incident central adiposity gain defined by changes in all three indicators: weight increase >0.5kg, DXA truncal adiposity increase >200g, and waist circumference increase >0.5cm (N = 66); 2) stable adiposity defined by minimal changes in the same 3 markers of adiposity namely body weight, DXA truncal adiposity and waist circumference (N = 16); 3) HbA1c in the top 25% of baseline distribution (HbA1c >5.05%, or ~ 32 nmol/mol; N = 21), and 4) HbA1c in the bottom 10% of

baseline distribution (HbA1c <4.92%, or ~ 30 nmol/mol; N = 7). Within each phenotype group, individual participant blood samples were combined to form a pooled sample for metabolomics analysis, and triplicate aliquots of each pool were assayed. To investigate dose-response associations, sub-pools were created and assayed in triplicate. For the HbA1c groups, sub-pools were defined by the median of the distribution of individual values (two sub-pools for each HbA1c group). The incident central adiposity gain group was divided into 3 subgroups based on the degree of central adiposity gain (for 3 sub-pools, see supplemental methods). Plasma aliquots were express-shipped on dry ice for metabolomics assays conducted at the Luxembourg Centre for Systems Biomedicine, University of Luxembourg.

Data Collection: Metabolomics

Metabolite extraction and chemical derivatization was followed by gas chromatography coupled to mass spectrophotometry (GC-MS) to yield high-dimensional data that were analyzed by the MetaboliteDetector software (13) and subsequently statistically evaluated by R (release 3.1.2). All samples were assayed in triplicate to provide estimates of technical variation. Prior to statistical analysis, metabolite levels were normalized by reference pools (every metabolite level divided by the mean signal of the same metabolite level in a pooled sample of two measured pools chronologically closest to the measured sample). Firstly, a non-targeted metabolomics assay of 305 metabolites was conducted. Secondly, an optimized assay of 107 metabolites was designed based on the first set of findings, and starting over with freshly prepared pools, the four original groups and the sub-pools were assayed. Signals that persisted were considered as validated. Thirdly, a targeted and quantitative GC-MS analysis was conducted on the subset of metabolites of interest including fructose, leucine, isoleucine, valine, lactic acid and erythritol. For this purpose, external standard solution series were produced containing the metabolites of

interest.

Statistical Analysis

In the non-targeted GC-MS analysis, multiple testing was accounted for by calculating false discovery rate (FDR) Q-values (14); the threshold of FDR = 0.2 was set *a priori* for statistical significance. The metabolome at study baseline in participants with incident central adiposity gain was compared to the metabolome of participants with stable adiposity. The metabolome of participants with higher HbA1c (HbA1c >5.05%) at the study baseline was compared to the metabolome of participants with lower HbA1c (HbA1c <4.9%). Welch's t-test was used to test for differences in the mean metabolites between groups, and the nominal p-value was adjusted for multiple comparisons to yield the FDR. The FDR Q-values were computed separately for the comparison of the weight groups, and for the comparison of the glycemia groups. Discovery and quantitative assay results were analyzed using the same statistical methods.

Human and Cell Studies of Erythritol Metabolism

Blood Incubation Experiments

To investigate erythritol metabolism in humans, an *ex vivo* stable-isotope assisted blood incubation experiment was performed on 5 healthy male volunteers. Before blood collection, basal glucose levels were determined with a commercially available glucometer (ForaCare, Diamond Mini). From each volunteer, 1 ml whole blood was collected *via* finger prick into K2EDTA coated vacutainers (BD, Franklin Lakes, New Jersey) and aliquotted in 300 µl portions. Each aliquot was supplemented with either a 1M U^{13}C-glucose, 6-$^{13}C_1$-glucose, 1,2-$^{13}C_2$-glucose or 3,4-$^{13}C_2$-glucose (Cambridge Isotopes; CLM-1396-1, CLM-2717-PK, CLM-504, CLM-6750-MPT-PK) solution to a final total glucose (labeled + unlabeled) concentration of 15

mM. The volume of the 1 M tracer solution added to the blood depended on the blood glucose concentration measured before and was adjusted to reach a final concentration of 15mM glucose in all samples. After spiking the tracer, the blood samples were incubated at 37 °C under continuous shaking on an orbital shaker at 600 rpm (Eppendorf Thermomixer).

Whole blood samples were collected 5 min prior to the addition of the tracers, and 1, 15, 30, 60 and 120 min after addition of the tracers. For metabolite extraction, each sample was extracted in triplicate by mixing 10 µl of whole blood with 90 µl of ice-cold extraction fluid (MeOH/water 8+1) containing 5 µg/ml pentanedioic-d6 acid (C/D/N Isotopes Inc, D-5227) as an internal standard. After mixing at 1400 rpm on an orbital shaker (Eppendorf Thermomixer) for 5 min at 4°C, and subsequent centrifugation at 21.000 x g and 4°C for 10 minutes, 70 µl supernatant was transferred into a GC glass vial with micro insert (5-250 µl) and dried in a refrigerated rotary vacuum evaporator (Labconco) at -4 °C for 3 hours prior to GC-MS measurement.

Dried Blood Spot Analysis

To evaluate erythritol synthesis from glucose *in vivo*, 2g $U^{13}C$-Glucose (Cambridge Isotopes, CLM-1396-MPT D-GLUCOSE (U-13C6, 99%)) was administered orally to 3 healthy male donors and dried blood samples were collected via finger prick at T0, and 5, 15, 30, 45, 60, 90, 120 and 180 minutes *post* ingestion on dried blood spot cards (Sigma-Aldrich, Whatman protein saver cards, Z699519-100EA). To analyze the impact of erythritol intake on human glucose metabolism *in vivo*, 50 g commercially available erythritol (Erythrit, Natur Total, Charge: 435FET8B77) was ingested 2 minutes before the 45 minute time point. The DBS cards were allowed to dry at room temperature for 3h, transferred to zip lock foil bags containing a desiccant and stored at 4 °C until metabolite extraction.

The exact blood sampling times and blood glucose concentrations *post* ingestion of $U^{13}C$-glucose were monitored by an in-house Android application and glucometers (ForaCare, Diamond Mini). For each card (representing one time point), dried blood metabolites were extracted in triplicate. For each replicate, 2 punch-outs of 3 mm diameter were added to 80 µl ice-cold extraction fluid (MeOH/water 8+1) containing 1 µg/ml $U^{13}C$-ribitol as an internal standard (Omicron Biochemicals Inc, ALD-062), vortexed at 4 °C for 10 min, 1400 rpm and centrifuged at 4 °C for 10 min, 16000 x *g*; 60 µl supernatant was transferred into GC glass vials and dried in a refrigerated vacuum concentrator (Labconco) overnight at -4 °C.

In addition, to analyze whether erythritol is oxidized to erythronate *in vivo,* erythritol and erythronate levels were quantified by standard addition. 25µl of defined standard solutions were pipetted onto each spot of the dried blood spot cards and extracted in accordance with the extraction protocol used for dried blood spots.

GC-MS Measurements

Automated sample derivatization was performed using a GERSTEL multi-purpose sampler (Muehlheim an der Ruhr, Germany). Dried samples were dissolved in 15 µl pyridine, containing 20 mg/ml methoxyamine hydrochloride, at 40 °C for 90 min under shaking. After adding 15 µl N-methyl-N-trimethylsilyl-triflouroacetamide (MSTFA) samples were incubated at 40 °C for 30 min under continuous shaking.

GC-MS analysis was performed by using an Agilent 7890A GC coupled to an Agilent 5975C inert XL Mass Selective Detector (Agilent Technologies, Germany). A sample volume of 1 µl was injected into a Split/Splitless inlet in split mode (10:1) at 270 °C. The gas chromatograph was equipped with a 30 m DB-35MS (I.D. 250 µm, film 0.25 µm) capillary column + 5 m DuraGuard capillary in front of the analytical column (Agilent J&W GC Column). Helium was

used as carrier gas with a constant flow rate of 1.2 ml/min. The GC oven temperature was held at 90 °C for 1 min and increased to 320 °C at 15 °C/min, then held at that temperature for 8 min. The total run time for each sample was 24.333 min. The transfer line temperature was set constantly to 280 °C. The MSD was operating under electron ionization at 70 eV. The MS source was held at 230 °C and the quadrupole at 150 °C. Full scan mass spectra were acquired from m/z 70 to m/z 800. For precise absolute quantification, GC-MS measurements of the derivatives of interest were additionally performed in selected ion monitoring mode (Supplemental Methods).

Deconvolution of mass spectra, peak picking, integration, and retention index calibration were performed using the MetaboliteDetector software. Compounds were identified using an in-house mass spectra library. The following deconvolution settings were applied: Peak threshold: 5; Minimum peak height: 5; Bins per scan: 10; Deconvolution width: 5 scans; No baseline adjustment; Minimum 15 peaks per spectrum; No minimum required base peak intensity. Retention index calibration was based on an C10–C40 even n-alkane mixture.

Results

Sample Description

In the longitudinal study of 264 freshmen members of the Class of 2015, 65% (N=172) of participants had data available at both the beginning and the end of the academic year. In those with two data points, 75% gained weight (>0.5 kg) over the year; on average, the weight gain was a 3.6% increase in body weight.

There were no participants with medical history of diabetes, other cardiometabolic disease, or insulin use. Sixty-six participants had incident central adiposity gain; these participants increased 4.0 kg (SD 2.0) in weight, 3.9 cm (SD 2.0) in waist circumference, and 2.6% (SD 1.5) in DXA-

derived truncal adiposity. In contrast, 16 participants were stable on the adiposity indicators, with an average weight change of 0.6 kg (SD 1.1), waist circumference change of 0.4 cm (SD 1.7), and DXA-derived truncal adiposity change of -0.1% (SD 0.5).

Given the sample was young and healthy, the HbA1c levels were within normal limits (4.0-6.0% for ages ≥ 18 years). Thus, a distributional approach was used to define two phenotype groups for comparisons: the top 25% of the baseline HbA1c distribution (N=21, HbA1c>5.05%; mean 5.66% [SD 0.18]) and the bottom 10% (N=7, HbA1c<4.92%; mean 4.80% [SD 0.084]).

At study baseline, the phenotype groups were similar in DXA-derived adiposity indicators and anthropometry measurements (**Table 1**). The higher HbA1c group and the group with incident central adiposity gain weighed slightly more than the other groups at the baseline. At study baseline, each of the phenotype groups was near the population median on weight-for-age (range 45^{th}-52^{nd} percentile), slightly above the population median on height-for-age (52^{nd}-60^{th} percentiles), and, thus, on average below the population median on BMI-for-age (38^{th}-48^{th} percentiles).

Metabolites Predictive of Incident Central Adiposity Gain Phenotype

Five metabolites differed between the stable adiposity and the incident central adiposity gain groups at a nominal $p<0.05$ (Table 2). The difference in one metabolite, meso-erythritol (nominal p-value = 0.0004; FDR = 0.0435), reached the FDR threshold for statistical significance, and the concentration of meso-erythritol was 13.4-fold greater in the baseline pooled blood aliquots from participants with incident central adiposity gain compared to pooled blood aliquots from participants who maintained a stable adiposity phenotype. The differences between the phenotype pools for fructose and an unidentified metabolite were near the significance threshold after adjusting for multiple comparisons (both nominal p-values = 0.006, FDR = 0.23).

These, and several other candidate metabolites were analyzed further in a targeted GC-MS approach with absolute quantification of erythritol, fructose, lactic acid and 3 branched-chain amino acids (Supplemental table 1). The signal for erythritol was confirmed in a targeted GC-MS approach with absolute quantification of erythritol; the concentration of erythritol was 14.7-fold greater (95% CI 13.27, 16.25) in incident central adiposity gain pooled samples compared to pooled samples of participants with stable adiposity (60.8 (SE 3.1) vs. 4.1 (SE 0.0) μmol/L, p< 0.0001). The concentration of fructose was 2.2-fold greater in the baseline pooled blood aliquots from participants with incident central adiposity gain compared to the stable adiposity group (46.2 (SE 0.9) vs. 27.8 (SE 1.6), p=0.022). The concentrations of leucine, isoleucine and valine were higher in the incident central adiposity gain phenotype group, but these findings did not reach statistical significance (p-values=0.76, 0.64 and 0.55, respectively).

Finally, the phenotype of incident central adiposity gain was refined to investigate dose-response patterns for the metabolites of interest. The concentration of meso-erythritol varied by tertiles of the central adiposity score (Supplemental figure 2); the highest meso-erythritol concentration was in the lowest central adiposity change sub-group (N = 26), and the pattern indicated lower meso-erythritol with greater central adiposity change. There was no difference in the concentration of meso-erythritol between the stable adiposity phenotype sub-groups.

Metabolite Profile Associated with Higher Glycemia Phenotype

The metabolomic profile between phenotype groups defined by higher versus lower glycemia was compared using HbA1c concentrations at the study baseline (Table 2), and 9 metabolites had nominal p-values < 0.05. The group with higher glycemia had a 22-fold greater concentration of meso-erythritol (nominal p-value < 0.0001; FDR = 0.0002) and about half the concentration of fructose (nominal p-value = 0.0006; FDR = 0.0302) compared to the lower glycemia phenotype.

Valine and leucine concentration differences reached the nominal p-value threshold, but not the FDR threshold, and isoleucine concentration differences were consistent in direction though not statistically significant (p-value = 0.0620, FDR = 0. 5346~~6155~~; Supplemental Table 4~~; data not shown~~).

Based on these findings, six metabolites were further analyzed in a targeted GC-MS approach with absolute quantification of erythritol, fructose, lactic acid, valine, leucine and isoleucine. Meso-erythritol concentration was 20.6-fold greater (95% CI 19.84, 21.41) in the higher glycemia group (105.6 (SE 1.4) vs. 5.1 (SE 0.1) µmol/L, p= 0.0024). Fructose and lactic acid had significantly lower concentrations in the higher glycemia group (nominal p-values 0.0444 and 0.0088, respectively; supplemental table 2). The concentrations of the branched chain amino acids were higher in the higher glycemia group, but findings did not reach the statistical significance threshold.

In a further analysis of the dose-response, both glycemia phenotype groups were split at the median and the quantitative assay was repeated in the four resulting pools (supplemental figure 2). Meso-erythritol concentrations were about the same in the two halves of the lower glycemia group, but in the higher glycemia subgroup, meso-erythritol was higher in the top 12.5% of the baseline HbA1c distribution (HbA1c ≥ 5.64 %).

Erythritol Is Synthesized From Glucose In Vivo and *In Vitro*

Given that erythritol was associated with subsequent gain in central adiposity gain and with higher HbA1c (measured concurrent with the metabolite), further experiments were conducted to assess whether erythritol interacted with metabolic pathways, whether it affected glucose metabolism, and whether there was evidence for endogenous production of erythritol. *In vivo* stable isotope-assisted dried blood spot studies in 3 male volunteers showed evidence that

erythritol is synthesized from glucose in red blood cells after ingestion of U^{13}C-Glucose (supplemental figure 3~~data not shown~~). However, due to low erythritol signals and consequently low labeled erythritol enrichment patterns, this pathway was validated in an *ex vivo* blood incubation setup. Erythronate was shown to be produced from erythritol, and there was no evidence that glucose metabolism was perturbed after ingestion of 50 g erythritol. An immediate increase in erythritol blood concentrations was observed, followed by an increase of erythronate blood concentrations (Figure 1). Erythritol was oxidized to erythrose, which was fleeting, then further oxidized to erythronate. Overall, although a large portion of consumed erythritol was assumed to be excreted in urine, about 5-10% was oxidized through the pathway to erythronate.

To analyze if erythritol can be synthesized from glucose by human metabolism, a stable-isotope based labeling strategy was employed in human blood cells. In case of an endogenous synthesis capacity of erythritol in human (blood) cells, the production of isotopically enriched erythritol after supplementation of a U^{13}C glucose tracer was expected. To test this hypothesis, whole blood was incubated with U^{13}C-glucose and polar metabolites sampled after 120 min of incubation and mass isotopomer distributions (MIDs) of erythritol were determined.

A significant increase of fully labeled erythritol was observed after 120 min compared to the time point before adding the U^{13}C-Glucose tracer (-5 min) (Figure 2). In contrast to these changes, no significant changes could be observed after incubation with unlabeled U^{12}C-Glucose. These results highlight that human metabolism provides all the biochemical capabilities required to synthesize endogenous erythritol from glucose.

Erythritol Is Synthesized From Glucose Via the Pentose Phosphate Pathway

The biochemical pathway for the conversion of glucose into erythritol was investigated. Erythritol is the reduced form of the monosaccharide erythrose, which in its phosphorylated form is an intermediate of the reductive pentose phosphate pathway (PPP). For this reason, it was

proposed that glucose is phosphorylated by hexokinase and then oxidized to gluconate-6-phosphate in the oxidative branch of the PPP. After oxidative decarboxylation by gluconate-6-phosphate dehydrogenase, ribulose-5-phosphate is produced. During this step, the first carbon atom of glucose is lost as CO_2. Further conversions by transketolase and transaldolase in the reductive part of the PPP eventually produce erythrose-4-phosphate (E4P) (Figure 3A). It is well known that these reactions take place in almost all human cell types including erythrocytes (15). The next step is either the reduction of E4P to erythritol-4-phosphate followed by the action of a phosphatase to release erythritol or first the removal of the phosphate to release erythrose which is then reduced to erythritol. To validate this assumption, the labeling experiments were repeated in whole blood, but with three ^{13}C glucose tracer labeled at specific positions (Fig. 3A). In conclusion, these experiments validated that human blood cells convert glucose into erythritol and based on the positional labeling experiments most likely *via* E4P and thus the PPP (Fig. 3B).

Discussion

We conducted a metabolomics study to identify a metabolic profile predictive of adverse changes in body habitus, specifically incident gain in central adiposity, which is a phenotype with known cardiometabolic risk (16, 17). We found consistent differences for erythritol and fructose over several analytic steps that indicated overall positive associations between those metabolites and incident central adiposity gain in young adults. We also found the concentration of erythritol was higher in the group with higher glycemia compared to lower glycemia at study baseline. Conversely, fructose and lactic acid had lower blood concentrations in the higher glycemia group. The concentrations of branched chain amino acids (isoleucine, leucine, and valine) did not differ between the central adiposity phenotype groups, but these metabolites had higher concentrations in the higher glycemia phenotype group. Branched chain amino acids

reportedly perturb insulin signaling (18) and experimental feeding of branched chain amino acids, with and without concurrent high fat diet, contributes to insulin resistance in animal models (10).

Overall, the human and cell studies of erythritol support novel and heretofore unrecognized metabolism of glucose to erythritol. These findings are in contrast to previously published studies that reported erythritol is not metabolized in humans (19). Hiele et al. (19)) showed that ingestion of 25g ^{13}C-labelled glucose and lactitol led to altered human metabolism; in contrast, they reported that after ingestion of 25g ^{13}C -labelled erythritol, no alterations of the human metabolism could be observed and erythritol was not further metabolized. We showed that there is metabolism of erythritol to erythronate.

In the examination of subgroups of the incident central adiposity gain group, we compared the concentration of erythritol across three levels of central adiposity gain, defined by the degree of change. We observed the greatest concentration of erythritol in the subgroup with the least degree of central adiposity change. These data provide some evidence of a stepwise relationship between erythritol and central adiposity gain; the inverse association may indicate that erythritol is associated with overall risk of increase in central adiposity, but not with the magnitude of central adiposity gain. Replication of this study is needed to verify the direction and magnitude of this association. Also, future research characterizing specific foods or food groups that contribute to exogenous erythritol exposure, or variation in human endogenous erythritol synthesis, could improve the understanding of erythritol exposure, and lead to a better understanding of factors contributing to endogenous erythritol concentration, increased adiposity, or both.

Erythritol is a sugar alcohol that occurs naturally in a variety of foods (e.g., pear,

watermelon), is 60-80% as sweet as sucrose, and is an approved low-calorie sweetener food additive (20, 21). U.S. survey data estimates that the typical intake of erythritol is about 1 g/day (22). Erythritol is a 4 carbon polyol and up to 90% of the ingested quantity is rapidly absorbed through the gut lumen (19), with the unabsorbed fraction possibly (23) subject to fermentation by gut microbes (20, 22). Clinical feeding studies of 0.8-1g erythritol/kg body weight show ~90% of the dose is excreted (urine) in 24-48 hours (20). In animal models, plasma erythritol levels peak about 30-60 minutes post-ingestion, with 99% disappearance from the plasma within 24 hours (24). Erythritol exists endogenously in human tissues, with plasma levels approximately 9.8 μmol/L (25), and, although a prior study claimed that endogenous production of erythritol was null (19), we demonstrated that erythritol is endogenously synthesized by human metabolism *in vivo*. After ingestion of a $U^{13}C$ glucose tracer isotopically enriched erythritol was detected in the blood of the donor. We hypothesized that at least some of the erythritol production takes place in blood cells because, after transport by the enterocytes, these cells are the first that are in contact with the tracer, and the finding that these primary human blood cells can synthesize detectable amounts of isotopic labeled erythritol after incubation with stable isotope tracers supported this hypothesis. Due to the determined mass isotopomer distributions, erythritol is most probably synthesized from E4P, an intermediate of the PPP.

While no previous studies are directly comparable to this study, a few studies of young adult weight gain include biomarkers or look for predictive metabolomic profiles. Wurtz et al. (8) investigated 82 pre-specified metabolites to understand the causal effect of BMI and of adiposity change on the metabolome in a large consortium study of 16 to 39 year olds. They identified causal associations of BMI change with changes in lipid-related, inflammation-related and branched chain amino acid metabolites. Prior research also found an association of artificial

sweeteners with both metabolic dysfunction (26, 27) and with weight gain (28-30) but these studies were based on consumption, and the conclusions are likely to be affected by both confounding and reverse causality (persons already gaining weight choose to consume artificially sweetened beverages to prevent further weight gain). The biological mechanisms for the artificial sweetener—weight gain association is proposed to relate to gut microbiota metabolism (31) . Several recent studies of erythritol loading reported no effect on glucose (32, 33) consistent with our findings. A study in patients with diabetes reported that erythritol and enrichment of another pentose phosphate pathway biomarker (ribose) were increased in patients with diabetic retinopathy, and suggested that polyol pathway flux may yield biomarkers of clinical risk in diabetes(34).

We also found a higher baseline concentration of plasma fructose was associated with the incident central adiposity gain phenotype. Fructose is a normal component of the human diet and is naturally concentrated in sweet fruits, although fructose also is extracted from beets, cane, sucrose and concentrated as high-fructose corn syrup for use as a sweetener. The possible causal link between fructose intake and human weight gain is controversial (35) and we could not identify any published studies that used metabolomics to study the association of fructose with subsequent weight gain. We also found higher lactic acid concentrations associated with the incident central adiposity gain phenotype, and this finding may be driven by the fructose finding given evidence from isotopic tracer studies of fructose, which show that approximately one-fourth of ingested fructose is converted to lactic acid within hours (36) .

Previous studies used metabolomic approaches to construct metabolic ‘fingerprints’ associated with obesity, and repeatedly show higher concentrations of branched chain amino acids associated with obesity (8, 37, 38) across all age ranges and with risk for insulin resistance

in adolescents (37). In our study, concentrations of branched chain amino acids were higher in the higher glycemia phenotype group, supporting previous studies that implicate branched chain amino acids in long-term glucose economy. No differences in concentration were found in the incident central adiposity gain versus stable adiposity phenotype groups suggesting that increased branched chain amino acids in circulation are not associated with subsequent adiposity gain.

A limitation of this work is the use of pools of plasma for all comparisons because it is possible that a subset of the individuals within a pool could drive the overall levels of metabolites measured in the pool. A pooling approach was adopted because it offered a cost-effective strategy to investigate the relation between the metabolome and complex phenotypes in an understudied population. In addition, pooling is advantageous to reduce overall variation when the biological variation between individual samples is greater than the technical variation between replicate assays from the same pool (39). In this study, we had information on technical variation (pool replicates), but we had no information on biological variability among individual samples. The FDR-corrected statistical tests presented herein are not as robust as tests that account for both biological and technical variation (39). However, the consistency of the erythritol results in both the central adiposity and usual glycemia comparisons lend strength to the findings, and the cluster of metabolites has biological plausibility.

This pool-based study delivers two unique results about metabolite abundance related to a one-time measurement of glycemia and longitudinal increase in central adiposity. While the two pools within a comparison are mutually exclusive, there is minor overlap across two of the comparisons (12 of the 66 participants with central adiposity gain are also in the higher baseline glycemia group). Strengths of this work include a large sample representative of University

freshmen, longitudinal data to define the phenotype using both anthropometry and gold-standard DXA methods, and the use of rigorous protocols for all data collection.

In conclusion, we found a positive association between circulating levels of erythritol at the study baseline and the incidence of central adiposity gain in non-obese adults aged 18 years of age studied over 9 months. Moreover, we demonstrated for the first time that erythritol is constitutively synthesized in humans from glucose, most probably *via* E4P, which is an intermediate of the PPP. Further research is needed to understand the meaning of these findings, including the interplay between variation in endogenous erythritol synthesis and exogenous exposure to erythritol-containing foods. Further research to confirm these findings in a new cohort, and to identify metabolic profiles that predict changes in adiposity in early adulthood present a unique opportunity to identify novel targets for prevention, which are very important given the well-known difficulty of losing weight once it is gained.

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