



# Roux-en-Y Gastric Bypass Surgery, but Not Calorie Restriction, Reduces Plasma Branched-Chain Amino Acids in Obese Women Independent of Weight Loss or the Presence of Type 2 Diabetes

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## OBJECTIVE

Obesity and type 2 diabetes mellitus (T2DM) have been associated with increased levels of circulating branched-chain amino acids (BCAAs) that may be involved in the pathogenesis of insulin resistance. However, weight loss has not been consistently associated with the reduction of BCAA levels.

## RESEARCH DESIGN AND METHODS

We included 30 obese normal glucose-tolerant (NGT) subjects, 32 obese subjects with T2DM, and 12 lean female subjects. Obese subjects underwent either a restrictive procedure (gastric banding [GB], a very low-calorie diet [VLCD]), or a restrictive/bypass procedure (Roux-en-Y gastric bypass [RYGB] surgery). Fasting blood samples were taken for the determination of amine group containing metabolites 4 weeks before, as well as 3 weeks and 3 months after the intervention.

## RESULTS

BCAA levels were higher in T2DM subjects, but not in NGT subjects, compared with lean subjects. Principal component (PC) analysis revealed a concise PC consisting of all BCAAs, which showed a correlation with measures of insulin sensitivity and glucose tolerance. Only after the RYGB procedure, and at both 3 weeks and 3 months, were circulating BCAA levels reduced.

## CONCLUSIONS

Our data confirm an association between deregulation of BCAA metabolism in plasma and insulin resistance and glucose intolerance. Three weeks after undergoing RYGB surgery, a significant decrease in BCAAs in both NGT as well as T2DM subjects was observed. After 3 months, despite inducing significant weight loss, neither GB nor VLCD induced a reduction in BCAA levels. Our results indicate that the bypass procedure of RYGB surgery, independent of weight loss or the presence of T2DM, reduces BCAA levels in obese subjects.

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Although at baseline, reduced plasma branched-chain amino acid (BCAA) levels are associated with glucose intolerance, improvement of glucose tolerance does not necessarily lead to a reduction of plasma BCAA levels.

Obesity is strongly associated with glucose intolerance and insulin resistance (IR), which are important risk factors for the development of type 2 diabetes mellitus (T2DM) (1). Disturbances in numerous pathways have been suggested to be responsible for the association between obesity and T2DM (2). Recently, BCAAs were suggested to play a role in the association between obesity and T2DM (3,4). Comprehensive metabolic profiling of obese versus lean human subjects revealed a BCAA metabolic signature, marked by increased circulating levels of BCAAs as well as products of BCAA catabolism (3). Other studies (3,5–7) confirmed that BCAA levels are elevated in obese individuals compared with lean individuals, and correlate with IR. Why levels of circulating BCAAs are elevated in obesity is unclear. Evidence has been provided for a role of white adipose tissue BCAA metabolism in the modulation of circulating BCAA levels (8,9).

Roux-en-Y gastric bypass (RYGB) surgery effectively improves glycemic control in obesity and T2DM, possibly through mechanisms independent of weight loss (10). One study (11) compared the effects of similar amounts of weight loss induced by calorie restriction and RYGB, and found a decrease in circulating BCAAs after RYGB. It was concluded that the decrease of BCAAs could contribute to the better improvement in glucose homeostasis observed with the RYGB intervention (11). Another study (12) showed that a similar amount of weight loss induced by either gastric banding (GB) or RYGB induced a comparable decrease in BCAAs. This would argue against a primary role for BCAAs in the RYGB-associated improvement of glycemic control.

In the current study, we directly compared the effects of a very low-calorie diet (VLCD) or GB with the effect of RYGB on BCAAs and other amine groups containing metabolites. Moreover, we determined whether the expected changes in circulating amine levels are affected by the presence of T2DM in obese subjects. Furthermore, we

compared the effects of calorie restriction and RYGB on BCAA levels in patient groups in the early phase of weight loss (3 weeks after intervention) and 3 months after the intervention. Since the obese subjects (diabetic and normal glucose-tolerant [NGT] subjects) had lost exactly the same amount of weight 3 weeks after the intervention, we suggest that this may be a reliable comparison with testing the weight loss-independent metabolic effects of RYGB.

## RESEARCH DESIGN AND METHODS

### Subjects

#### *Subjects and Study Design*

The research design and methods have been described in detail elsewhere (13). In short, obese females, with normal fasting glucose levels (NGT) or T2DM (treated with oral medication only), who were eligible for dietary or surgical treatment were included in the study. Age-matched, healthy females with normal BMI served as a control group for preintervention comparisons. The protocol (clinical trial reg. no. NCT01167959) was approved by the medical ethics committee of the Leiden University Medical Center, and all subjects provided written informed consent before participation.

Subjects were studied (after  $\geq 10$  h fasting overnight) within a month before, 3 weeks after, and 3 months after the intervention. All antidiabetes medications were discontinued 48 h before the study. Anthropometric measurements were made, and bioelectric impedance analysis (QuadScan; Bodystat, Cronkbourne Douglas, Isle of Man, U.K.) was performed. A cannula was inserted into an antecubital vein and a fasting blood sample was taken. Blood was collected in an SST Tube with gel and clot activator and a Vacutainer on EDTA (Becton Dickinson).

#### *Interventions*

Standard operating procedures were followed for GB and RYGB, and patients were prescribed a staged meal plan after surgery (13). Patients were prescribed a clear liquid diet for the first 4–5 days after surgery. For the first 3 months after surgery, a staged meal progression was prescribed, containing liquids and ground or pureed protein sources and vegetables. T2DM subjects undergoing dietary intervention (VLCD) were prescribed commercially

available Prodimed (Prodimed Benelux B.V., Valkenswaard, the Netherlands), a high-protein, low-calorie meal replacement plan (13). Subjects were allowed four to five Prodimed sachets (e.g., for the preparation of soups, shakes) a day and an additional choice of selected vegetables (600 kcal/day in total) during the first 3 weeks. Up to 2 months, patients were allowed to expand their intake with vegetable and light dairy products (800 kcal/day in total). Thereafter, a light evening meal was allowed on intermittent days (1,000 kcal/day in total).

### Assays

#### *Glucose, Insulin, and HbA<sub>1c</sub>*

Serum glucose, insulin, and HbA<sub>1c</sub> were measured as described elsewhere (13).

#### *Amino Acids*

Amine measurements were performed based on methods described previously by Noga et al. (14). The amine platform covers amino acids and biogenic amines using an AccQ-Tag derivatization strategy adapted from the protocol supplied by Waters (Etten-Leur, the Netherlands). Five microliters of each plasma sample was spiked with an internal standard solution, followed by deproteination by the addition of MeOH. The supernatant was transferred to a deactivated autosampler vial (Waters) and dried under N<sub>2</sub>. The residue was reconstituted in borate buffer (pH 8.5) with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate reagent. After reaction, the vials were transferred to an autosampler tray and cooled to 10°C until the injection. One microliter of the reaction mixture was injected into the ultra-performance liquid chromatography (UPLC)–tandem mass spectrometry system.

An ACQUITY UPLC system with autosampler (Waters) was coupled online with a Xevo tandem quadrupole mass spectrometer (Waters) operated using MassLynx data acquisition software (version 4.1; Waters). The samples were analyzed by UPLC–tandem mass spectrometry using an AccQ-Tag Ultra column (Waters). The Xevo tandem quadrupole spectrometer was used in the positive-ion electrospray mode, and all analytes were monitored in selective reaction monitoring using nominal mass resolution.

Acquired data were evaluated using QuanLynx software (Waters) by integration of assigned selective reaction

monitoring peaks and by normalization using proper internal standards. For the analysis of amino acids, their  $^{13}\text{C}^{15}\text{N}$ -labeled analogs were used (Supplementary Table 1). For other amines, the closest eluting internal standard was used. Blank samples were used to correct for background, and algorithms developed in-house were applied using the pooled quality control samples to compensate for shifts in the sensitivity of the mass spectrometer over different batches (15).

### Data Processing and Statistics

All amino acid data were analyzed as normalized to the internal standard (Supplementary Tables 2 and 3). Data were log transformed when appropriate. Differences between obese subjects and lean control subjects at baseline and the effects of the different interventions within each group and between intervention groups were calculated with a mixed-effects model, with the patient groups and diabetes as the fixed effects and the subject-specific deviances modeled with random intercepts.

Principal component analysis (PCA) was performed on the correlation matrix for metabolite levels at baseline to extract groups of metabolites that strongly covaried. Eight principal components (PCs) (Supplementary Table 4) were found with eigenvalues  $>1$ , which explained 74.4% of the total variation. Varimax rotation was performed on the eight PCs, and factor loads with an absolute value  $>0.3$  were retained to obtain interpretable components. Subsequently, PC scores before and after

intervention were calculated and analyzed with the same mixed model as described above.

A  $P$  value  $<0.05$  was considered statistically significant for a single test. For multiple tests, a trend was defined as a  $P$  value  $<0.05$ , and the level of statistical significance was determined using the Bonferroni method. Mixed-effects model analysis and PCA were performed in MATLAB (MathWorks, Natick, MA), and the processing of RNA sequencing data was performed in R (version 2.15.1; R Development Core Team). Graphs were developed in Prism GraphPad version 5.

## RESULTS

### Baseline Characteristics of Subjects

Baseline subject characteristics are shown in Table 1 (mean  $\pm$  SEM). All obese subjects and healthy control subjects were Caucasian females, with a mean age of  $49.4 \pm 0.6$  years. Eighty percent of subjects were postmenopausal, and percentages were comparable between groups. We included 32 female subjects with T2DM and 30 NGT obese females. The average duration of diabetes was  $3.8 \pm 0.7$  years, and medication was comparable between groups (Supplementary Table 5). Eight subjects dropped out during the course of the study because they were not able to comply with the VLCD ( $n = 2$ ), because of logistic issues ( $n = 3$ , one subject from the GB group, one subject from the NGT group, and one subject with T2DM from the RYGB group), and because of mild postoperative complications ( $n = 3$ ) associated with the RYGB procedure.

According to protocol, all diabetic subjects discontinued their glucose-regulatory medication on the day of the operation or at the start of the diet. Only metformin treatment was continued if fasting blood glucose levels remained  $>7$  mmol/L after intervention (27% of subjects after RYGB vs. 17% of subjects after VLCD,  $P = \text{NS}$ ). If subjects, at baseline, used medication for chronic conditions such as hypertension or hypercholesterolemia, this was continued throughout the whole time course of the study. None of the subjects reported any problems adhering to the VLCD or the prescribed meal plan during the 3-month time course of the study.

### Serum Data

#### Baseline Comparison of Amine Levels Among Obese NGT, Obese T2DM, and Lean Subjects

Baseline levels of the 29 detected metabolites containing an amine group are presented in Supplementary Tables 2 and 3. Levels of the BCAAs leucine, valine, and isoleucine were significantly higher, whereas levels of asparagine, histidine, and glycine were lower in T2DM obese subjects compared with lean subjects. Asparagine was also significantly lower in NGT obese subjects compared with lean subjects. Glutamic acid was the sole amino acid that was significantly higher in obese T2DM compared with NGT subjects.

#### PCA and Regression Analysis at Baseline

PCA at baseline revealed eight PCs of correlated amino acids, as described in Supplementary Table 4. The first PC (PC1) consisted of leucine, isoleucine, valine,

**Table 1—Baseline characteristics of the study groups**

	NGT ( $n = 27$ )	T2DM ( $n = 27$ )	Control subjects ( $n = 12$ )	$P$ value		
				NGT vs. lean	T2DM vs. lean	T2DM vs. NGT
BMI, $\text{kg}/\text{m}^2$	$43.8 \pm 0.6$	$42.0 \pm 1.1$	$21.7 \pm 0.5$	<i>8.41E-24</i>	<i>5.62E-22</i>	0.119133
Weight, kg	$124.3 \pm 2.3$	$117.2 \pm 3.3$	$64.4 \pm 2.1$	<i>0.000130</i>	<i>0.000800</i>	0.059
HbA <sub>1c</sub> , % (mmol/mol)	$5.5 \pm 0.14$ ( $36.1 \pm 1.5$ )	$6.7 \pm 0.19$ ( $49.6 \pm 2.1$ )	$5.1 \pm 0.06$ ( $31.9 \pm 0.7$ )	0.156349	<i>3.71E-08</i>	<i>7.96E-08</i>
HOMA-IR	$2.4 \pm 0.4$	$4.6 \pm 0.6$	$0.3 \pm 0.0$	0.004094*	<i>3.01E-08</i>	<i>0.000149</i>
FFA, mmol/L	$1.0 \pm 0.1$	$1.2 \pm 0.1$	$0.9 \pm 0.1$	0.284697	<i>0.016636*</i>	0.086563
Fasting glucose, mmol/L	$5.0 \pm 0.2$	$8.7 \pm 0.3$	$4.7 \pm 0.1$	0.563268	<i>8.52E-13</i>	<i>4.51E-16</i>
Fasting insulin, mU/L	$10.5 \pm 1.3$	$12.0 \pm 1.5$	$1.6 \pm 0.1$	<i>0.000298</i>	<i>3.21E-05</i>	0.435538
Fasting triglycerides, mmol/L	$1.5 \pm 0.2$	$1.8 \pm 0.1$	$1.0 \pm 0.1$	0.03803*	<i>0.000018</i>	<i>0.002954*</i>

Values are presented as mean  $\pm$  SEM. Differences between subject groups (NGT vs. T2DM) and lean control subjects at baseline were compared with a mixed-effects model. Statistical tests that were significant after Bonferroni correction for multiple testing ( $P < 0.00238 = 0.05$  divided by 21 tests) are shown in italics. FFA, free fatty acid. \*A trend (not significant after correction) was defined as  $P < 0.05$ .

and amino adipic acid, the levels of which were strongly correlated to one another (Supplementary Fig. 1) and were also positively correlated to levels of glucose, insulin, and triglycerides; HOMA-IR; and BMI at baseline (Table 2). A second cluster (PC2) of correlating amino acids consisted of glycine, citrulline, arginine, glutamine, taurine, and ornithine. Of this PC, taurine ( $r = -0.52$ ,  $P = 0.00001$ ), serine ( $r = -0.50$ ,  $P = 0.00002$ ), and glycine ( $r = -0.55$ ,  $P = 2.4 \times 10^{-6}$ ) were negatively correlated to triglyceride level, whereas trends toward a negative correlation were observed for BMI (glycine), HOMA-IR (taurine, glycine, and serine), and glucose (taurine, glycine, and serine). When BMI was taken into account as a covariate, the correlations were no longer significant. A third cluster (PC3) of correlating amino acids consisted of asparagine, histidine, tryptophan, methionine, and threonine. In this PC, there was only a trend toward a negative correlation between BMI and asparagine, histidine, and tryptophan.

Of the most important PCs, PC1 scores were higher and PC3 scores were lower in T2DM subjects compared with lean control subjects (PC1  $P = 3.1 \times 10^{-6}$ , PC3  $P = 9.8 \times 10^{-5}$ ), whereas there was a trend in NGT subjects (PC1 higher, PC3 lower) compared with lean control subjects (Supplementary Table 6 and Fig. 1). Moreover, PC1, containing all BCAAs, correlated with HOMA-IR ( $r = 0.64$ ;  $P = 1.23 \times 10^{-8}$ ), glucose ( $r = 0.58$ ,  $P = 4.58 \times 10^{-7}$ ), insulin ( $r = 0.56$ ;  $P = 1.37 \times 10^{-6}$ ), triglycerides ( $r = 0.48$ ;  $P = 7.17 \times 10^{-5}$ ), and BMI ( $r = 0.50$ ,  $P = 2.14 \times 10^{-5}$ ) (Table 2). Of note, the correlations of PC1 with HOMA-IR and glucose were still significant when BMI

was included as a covariate ( $r = 0.52$ ;  $P = 1.14 \times 10^{-5}$  and  $r = 0.51$ ;  $P = 1.63 \times 10^{-5}$ , respectively), suggesting that BCAAs are associated with glucose and HOMA-IR, independent of BMI.

#### Effect of Intervention

BMI decreased significantly after all interventions (Supplementary Table 3). There were no differences between the groups as to the decrease in BMI after 3 weeks; however, RYGB induced a larger decrease in BMI after 3 months in NGT and T2DM subjects compared with GB and VLCD. There was a comparable effect of the VLCD and RYGB on glucose levels in T2DM subjects (Supplementary Table 3; data shown elsewhere [13]).

#### Effect of Intervention on Individual Amines Between Obese NGT and Obese T2DM

No amino acids were affected by weight loss through GB in NGT subjects. In NGT subjects, however, RYGB induced a decrease in leucine, valine, isoleucine, and 2-amino adipic acid levels after 3 weeks (not significant for isoleucine) and after 3 months (Supplementary Table 3). A comparable significant decrease was observed after RYGB in T2DM subjects, and, moreover, mixed-model analysis showed a significantly greater effect of RYGB compared with GB in NGT subjects (leucine  $P = 2.3 \times 10^{-5}$ , valine  $P = 3.1 \times 10^{-7}$ ) and of RYGB compared with VLCD in T2DM subjects (leucine  $P = 3.4 \times 10^{-5}$ , valine  $P = 7.6 \times 10^{-6}$ ). Several other amino acids were affected by RYGB, in contrast to no effect after GB or VLCD; specifically, kynurenine, tryptophan, phenylalanine, and tyrosine decreased after 3 weeks and 3 months, and

glycine and serine showed a strong increase after 3 months.

#### PCA and Regression Analysis After Intervention

The mean score of PC1 strongly decreased after RYGB in both NGT and T2DM subjects after 3 weeks and 3 months (NGT  $P = 7.0 \times 10^{-7}$  and  $1.7 \times 10^{-14}$ , respectively; T2DM  $P = 3.2 \times 10^{-5}$  and  $5.9 \times 10^{-12}$ , respectively), whereas there was a trend toward increased PC1 score 3 weeks after the VLCD, and no effect of GB (Supplementary Table 6 and Fig. 1). The mean score of PC3 increased 3 weeks and 3 months after RYGB.

Regression analysis was performed in three groups at 3 weeks and 3 months after intervention, as follows: 1) T2DM and NGT subjects after RYGB; 2) T2DM subjects after VLCD; and 3) NGT subjects after GB. This revealed significant correlations among several amino acids; however, no correlations between individual amino acids and biochemical parameters (glucose, insulin, triglycerides, HOMA-IR, and BMI) were found. Levels of leucine, isoleucine, valine, and amino adipic acid were strongly correlated to one another 3 weeks after RYGB, and this correlation further increased 3 months after RYGB, whereas no effect of VLCD was seen.

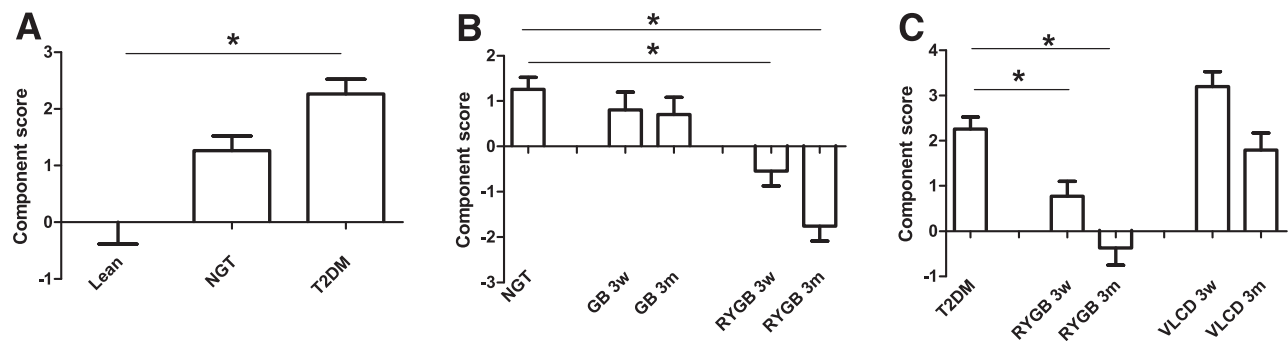
#### CONCLUSIONS

In this study, we directly determined the effects of VLCD, GB, and RYGB intervention on levels of circulating amino acids and related compounds in obese NGT and T2DM subjects, compared with lean subjects. At baseline, levels of BCAAs (leucine, valine, and isoleucine) were significantly higher in obese

**Table 2—Correlations of BCAAs and linear regression of PCs at baseline with biochemical parameters**

	BMI		HOMA-IR		Glucose		Insulin		Triglycerides	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
<b>BCAAs</b>										
Leucine	0.43	$4.59 \times 10^{-4}$	0.59	<b><math>2.76 \times 10^{-7}</math></b>	0.58	<b><math>5.71 \times 10^{-7}</math></b>	0.49	<b><math>3.33 \times 10^{-5}</math></b>	0.51	<b><math>2.01 \times 10^{-5}</math></b>
Valine	0.53	<b><math>6.20 \times 10^{-6}</math></b>	0.59	<b><math>3.82 \times 10^{-7}</math></b>	0.55	<b><math>2.11 \times 10^{-6}</math></b>	0.50	<b><math>2.57 \times 10^{-5}</math></b>	0.47	<b><math>9.91 \times 10^{-5}</math></b>
Isoleucine	0.50	<b><math>2.81 \times 10^{-5}</math></b>	0.64	<b><math>1.14 \times 10^{-8}</math></b>	0.52	<b><math>9.56 \times 10^{-6}</math></b>	0.59	<b><math>2.37 \times 10^{-7}</math></b>	0.35	$4.43 \times 10^{-3}$
2-Amino adipic acid	0.36	$3.72 \times 10^{-3}$	0.49	<b><math>3.86 \times 10^{-5}</math></b>	0.45	<b><math>1.79 \times 10^{-4}</math></b>	0.43	<b><math>3.39 \times 10^{-4}</math></b>	0.41	$8.11 \times 10^{-4}$
<b>PCs</b>										
PC1	0.50	<b><math>2.14 \times 10^{-5}</math></b>	0.64	<b><math>1.23 \times 10^{-8}</math></b>	0.58	<b><math>4.58 \times 10^{-7}</math></b>	0.56	<b><math>1.37 \times 10^{-6}</math></b>	0.48	<b><math>7.17 \times 10^{-5}</math></b>
PC2	-0.19	0.14	-0.27	0.03	-0.29	0.02	-0.21	0.10	-0.42	<b>0.000573</b>
PC3	-0.34	0.01	-0.32	0.01	-0.42	<b>0.000594</b>	-0.23	0.06	-0.278	0.03

Correlations of BCAAs and of most important PCs with biochemical parameters. The Bonferroni post hoc test was used to correct for multiple testing. For BCAAs, after correction,  $P < 0.00034$  (0.05 divided by 5 [biochemical parameters and by 29 amino acids]), indicated by bold values, was considered statistically significant. For PCs, after correction,  $P < 0.0033$  (0.05 divided by 5 [biochemical parameters] and by 3 [the number of PCs tested baseline]), indicated by bold values, was considered statistically significant.



**Figure 1**—Bar graphs of the effects of intervention on PCs. Strengths of PC1 and BCAAs, at baseline and after the different interventions in NGT and T2DM subjects, are shown. \*Significant difference between groups at baseline (A), NGT subjects after RYGB (B), and T2DM subjects after RYGB (C).

T2DM subjects compared with lean subjects, while these levels in obese NGT subjects tended to be intermediate between those of lean and T2DM subjects. This suggests that circulating BCAA levels gradually increase in obesity, exacerbating even further in T2DM. PCA and regression analysis confirmed these findings by showing a significant correlation of BCAA (individually and clustered in a PC) with metabolic parameters such as glucose, insulin, and HOMA-IR, independently of BMI. Our results on BCAA levels and the correlation of BCAAs with measures of insulin sensitivity in obese subjects are in line with earlier reported findings (3,16). The fact that we do not find a significant increase of BCAAs in obese NGT versus lean subjects is likely due to the relative low number of subjects in the comparison.

The mechanism underlying increased levels of circulating BCAAs in obesity and T2DM is unknown. Increased protein consumption containing these essential amino acids may raise their plasma levels, but some data suggest that protein intake and circulating BCAA levels are not necessarily correlated (16,17). Alternatively, downregulation of catabolic enzymes in adipose or other tissues might be involved (8,9). In a parallel study, we analyzed the transcriptome in visceral and subcutaneous adipose tissue samples by RNA sequencing in the cohort of the current study (18). The adipose tissue samples were obtained at the time of the bariatric surgery; thus, at baseline. We used KEGG pathway over-representation analyses and network-based approaches to identify gene sets that distinguish NGT from T2DM obese individuals. The

top dysregulated pathway when comparing visceral adipose tissue from obese women with and without T2DM was the BCAA catabolism pathway (18). Indeed, we showed that virtually all BCAA catabolic genes were downregulated both in visceral and subcutaneous adipose tissue of obese T2DM subjects compared with equally obese NGT subjects (for the expression of the genes in the BCAA degradation pathway in adipose tissues of NGT and T2DM subjects, see Supplementary Table 7). Thus, in these women the adipose tissue BCAA degradation pathway was downregulated, while simultaneously their circulating BCAA levels were increased. These results are in line with a study showing that a substantial decrease in circulating BCAA levels occurred in parallel with an increase in two main catabolic enzymes of the BCAA degradation pathway, the branched-chain amino acid transferase and the branched-chain  $\alpha$ -ketoacid dehydrogenase in adipose tissue after weight loss (19). In another study (20), it was shown that the expression of BCAA catabolic genes in adipose tissue correlated positively with insulin sensitivity. Thus, our and previous studies suggest that adipose tissue may play an important role in the increased circulating BCAA levels in T2DM. Interestingly, in our study, the downregulation of BCAA degradation genes was more pronounced in omental adipose tissue compared with subcutaneous adipose tissue (18) (Supplementary Table 7), which is in agreement with a more pronounced role for omental adipose tissue in the control of metabolic health (9). Surprisingly, we did not find correlation between expression levels of genes involved in BCAA metabolism and BCAA

serum levels (data not shown). However, this is likely due to power issues caused by our relatively small subject group.

Three weeks after the interventions, when minimal weight loss had occurred, the RYGB procedure had markedly different effects compared with GB and VLCD (i.e., a reduction of individual BCAAs and a marked decrease in the score of PC1 [leucine, valine, isoleucine, and L-2-amino adipic acid]). These effects were even more apparent 3 months after RYGB. At this 3-month time point, weight loss induced by GB or VLCD was significant; however, still no effect on plasma BCAA levels was detected. There was no correlation of PC1 with anthropometric or metabolic parameters (levels of glucose and insulin, HOMA-IR, and BMI) 3 months after RYGB, indicating that the decrease in BCAAs is predominantly caused by the bypass procedure of RYGB surgery and is independent of the effect of weight loss per se, which is seen after the restrictive procedures.

Our observation that weight loss per se by VLCD or GB does not result in lower BCAA levels is in line with some reports (11) but is in contrast with other reports (12,16). It is possible that specific subject characteristics are responsible for these contrasting results. However, by performing a direct comparison in matched groups of obese subjects (in T2DM subjects, weight loss was comparable 3 months after the different interventions), we conclude that the decrease in BCAA levels after RYGB is predominantly caused by the bypass procedure and is not due to weight loss. Nevertheless, the fact that calorie restriction had a beneficial effect on glucose metabolism without affecting

circulating BCAA concentrations, contrary to the association between BCAA levels in plasma and glucose intolerance at baseline, suggests that for the reversal of IR after calorie restriction or RYGB, reduction of BCAA levels is not required. We suggest that calorie restriction and relative starvation are possibly the most important contributors to the fast improvement of glucose tolerance after RYGB (13).

It is unclear through which mechanisms the RYGB could cause the observed decrease in circulating BCAA levels. Both the dramatically altered food digestion and absorption brought about by the bypass, and an increase in BCAA catabolic gene expression could play a role. Indeed, it was previously reported that the RYGB procedure promotes BCAA catabolic gene expression (BCATm [human mitochondrial branched-chain aminotransferase]) in adipose tissue (19). To what extent the observed decline of circulating BCAAs after the RYGB procedure is due to increased expression of BCAA catabolic genes remains to be determined.

Interestingly, amino adipic acid, which is not a BCAA, clustered together with the BCAAs and showed a decrease after RYGB. Wang et al. (21) have shown that amino adipic acid is a biomarker for diabetes risk and a potential modulator of glucose homeostasis. Amino adipic acid is generated via lysine degradation and is involved in tryptophan metabolism. In the study by Wang et al. (21), no correlation was found between the BCAAs and amino adipic acid, so they suggested that amino adipic acid is involved in different pathophysiological pathways than BCAAs. Whether the association of amino adipic acid with the BCAAs in our study is due to the specifics of subjects and/or intervention remains to be investigated.

The limitations of the current study include a relatively short-term follow-up to dissociate the effect of the intervention from the effect of weight loss. A longer follow-up period was expected to cause more differences in weight loss, and thus to complicate the interpretation of the observed effects. However, longer follow-up studies are needed to confirm whether the observed effects remain. In addition, because of the intensity of the protocol we were not able to perform hyperinsulinemic-euglycemic

clamp studies to measure the extent of IR. Therefore, we estimate IR by HOMA-IR. Furthermore, formally, we cannot rule out a confounding effect of metformin use in our T2DM groups. However, as metformin was used in a similar proportion of subjects in both T2DM groups, the effect would have been equal in both groups. Since the effect of RYGB on BCAA levels in NGT and T2DM subjects is comparable, it seems unlikely that diabetes medication was a major confounder.

In conclusion, we show that BCAA levels tend to be higher in obese NGT subjects and are significantly higher in T2DM subjects compared with lean subjects. This may at least be partly caused by decreased expression of BCAA catabolic genes in white adipose tissue. Our data show that the reduction of BCAA levels immediately after RYGB is due to the bypass procedure and is independent of weight loss. The fact that calorie restriction had a similar effect on insulin sensitivity and glucose tolerance without affecting plasma BCAA concentrations, however, suggests that reduction in BCAA levels is not necessary for the improvement in obesity-associated IR.

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**Author Contributions.** M.A.L. researched the data and wrote the manuscript. J.B.V.K., H.K.D., J.F.J.L., G.-J.v.O., I.M.J., B.V.R., B.A.V.W., D.J.S., F.V.D., A.D., A.H., R.V., and T.H. researched the data. V.v.H. researched the data and edited the manuscript. P.A.C.'t H. edited the manuscript. J.W.A.S. reviewed and edited the manuscript. H.P. and K.W.v.D. contributed to the discussion and reviewed and edited the manuscript. M.A.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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