Chapter 8

Roux and Y gastric bypass surgery, but not calorie restriction, reduces plasma branched chain amino acids in obese subjects independent of weight loss or the presence of type 2 diabetes mellitus

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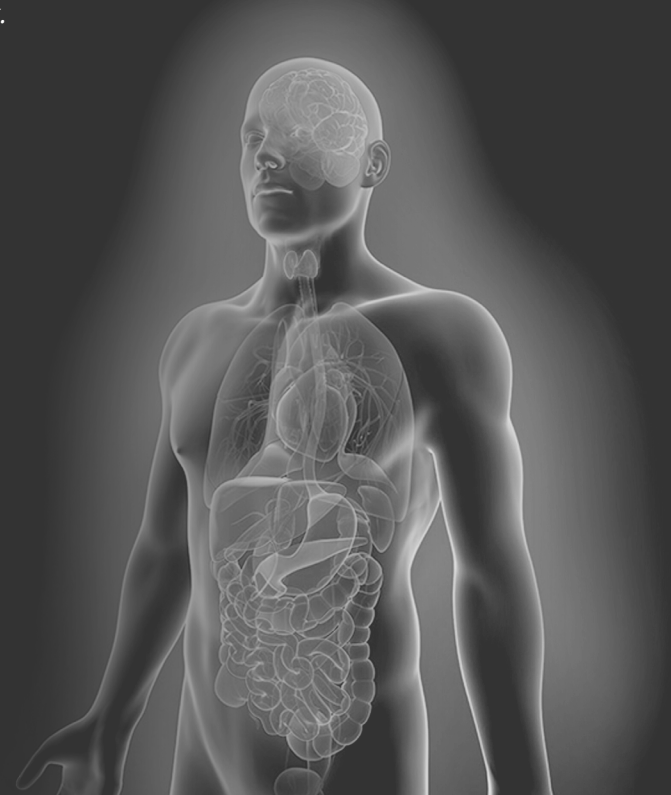
Submitted



ABSTRACT

Objective

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

Methods

We included 30 obese NGT, 32 obese T2DM and 12 Lean subjects. Obese subjects underwent either a restrictive procedure, (gastric banding (GB), a very-low-calorie-diet (VLCD)), or a restrictive/bypass procedure (Roux-and-Y Gastric Bypass (RYGB) surgery). Fasting blood samples were taken for determination of amine group containing metabolites 4 weeks before as well as 3 weeks and 3 months after the intervention. Dubcutaneous and omental adipose tissue samples were taken during surgery for transcriptome analysis using RNA sequencing.

Results

BCAA levels were higher in T2DM, but not NGT, as compared to Lean. Principal component analysis revealed a concise principal component (PC) consisting of all BCAAs, which showed a correlation with measures of insulin sensitivity and glucose tolerance. Expression of BCAA catabolic genes in white adipose tissue (WAT) was lower in T2DM as compared to NGT. Only at 3 weeks and 3 months after the RYGB procedure, circulating BCAA levels were reduced.

Conclusions

Our data confirm an association between deregulation of BCAA metabolism in plasma and WAT, and insulin resistance and glucose intolerance. Three weeks after RYGB, a significant decrease of BCAAs in both NGT as well as T2DM subjects was observed. After three 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 from weight loss or presence of T2DM, reduces BCAA levels in obese subjects.

INTRODUCTION

Obesity is strongly associated with glucose intolerance and insulin resistance, 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, branched chain amino acids (BCAA) 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 levels of circulating BCAAs as well as products of BCAA catabolism (3). Other studies confirmed that BCAA levels are elevated in obese individuals as compared to lean, and correlate with insulin resistance (3;5-7). Why circulating BCAA are elevated in obesity is unclear. Evidence has been provided for a role of white adipose tissue (WAT) BCAA metabolism in 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 compared the effects of similar amounts of weight loss induced by calorie restriction and RYGB, and found a decrease in circulating BCAA after RYGB. It was concluded that the decrease of BCAA could contribute to the better improvement in glucose homeostasis observed with the RYGB intervention (11). Another study showed that a similar amount of weight loss induced by either gastric banding (GB) or RYGB induced a comparable decrease in BCAA (12). This would argue against a primary role for BCAA 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-group containing metabolites. Moreover, we determined whether the expected changes in circulating amines are affected by the presence of T2DM in obese subjects. To gain insight in the underlying mechanisms, we analyzed the transcriptome by RNA-sequencing of omental and subcutaneous adipose tissue samples obtained at time of intervention. Furthermore, we compared the effects of calorie restriction and RYGB on BCAA levels in patients groups in the early phase of weight loss (three weeks after intervention) and three months after the intervention. Since the obese (diabetic and normal glucose tolerant) subjects lost exactly the same amount of weight 3 weeks after intervention, we suggest this may be a reliable comparison to test 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 (NGT) or T2DM (treated with oral medication only), eligible for dietary or surgical treatment were included. Age-matched, healthy females with normal BMI served as a control group for pre-intervention comparisons. The protocol *(ClinicalTrials.gov: NTC01167959)* 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 ≥10 hrs fasting overnight) within a month before, 3 weeks after, and 3 months after intervention. All anti-diabetic medications were discontinued 48 hours before study days. Anthropometric measurements were taken and bioelectric impedance analysis (Quadscan Bodystat® United Kindom) was performed. A canula was inserted into an antecubital vein and a fasting blood sample was taken. Blood was collected in a SST® Gel and Clot Activator tube (Becton and Dickinson) and a vacutainer on EDTA.

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 BV, Valkenswaard, The Netherlands), a high-protein, low-calorie meal replacement plan (13). Subjects were allowed 4-5 Prodimed sachets (for preparation of soups, shakes etc) 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 produce (800 ckal/day in total). Thereafter, a light evening meal was allowed on intermittent days (1000 ckal/day in total).

Assays

Glucose, insulin and HbA1c

Serum glucose, insulin and HbA1c 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 employing an Accq-tag derivatization strategy adapted from the protocol supplied by Waters (Etten-Leur, The Netherlands). 5 µL of each plasma sample was spiked with an internal standard solution, followed by deproteination by addition of MeOH. The supernatant was transferred to a deactivated autosampler vial (Waters) and dried under N_2. The residue was reconstituted in borate buffer (pH 8.5) with 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) reagent. After reaction, the vials were transferred to an autosampler tray and cooled to 10°C until the injection. 1.0 µL of the reaction mixture was injected into the UPLC-MS/MS 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-MS/MS using an Accq-Tag Ultra column (Waters). The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Selective Reaction Monitoring (SRM) using nominal mass resolution.

Acquired data were evaluated using Quanlynx software (Waters), by integration of assigned SRM peaks and normalization using proper internal standards. For analysis of amino acids their $^{13}C^{15}N$-labeled analogs were used (supplementary table 1) . For other amines, the closest-eluting internal standard was employed. Blank samples were used to correct for background, and in-house developed algorithms were applied using the pooled QC samples to compensate for shifts in the sensitivity of the mass spectrometer over different batches (15).

Next generation sequencing

Isolation of RNA

Next generation sequencing was performed on subcutaneous and omental adipose tissue samples taken at the time of surgery. Directly after taking the samples from the subcutaneous and omental adipose tissue compartments, samples were put in RNA later (Ambion®, Life Technologies, Bleiswijk, The Netherlands) and subsequently stored at -80°C. For isolation of total RNA the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) was used, following the instructions of the manufacturer. The quality of each RNA sample was assessed by lab-on-a-chip technology using the Agilent 2100 Bioanalyzer (Santa Clara, CA). All RNA samples had a RIN value >7.

RNA Deep sequencing

RNA (fifty µg) of the omental and subcutaneous adipose tissue samples obtained during bariatric surgery was used for RNA deep sequencing, performed at an Illumina Hiseq2000 at the Beijing Genomics Institute (Beijing, China).

Alignment and gene annotation

The quality of the raw data was assessed using FastQC, version: 0.9.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Thereafter, we aligned the reads to the Human reference genome build 19 (hg19, GRCh37) using GSNAP (16) with the novel splicing option (-N 1) enabled. To further convert the aligned data to a sorted BAM file we used SAMTools, version: 0.1.18 (17). For the quantification of the number of nucleotides that were mapped per exon, we used BEDTools, version: 2.13.2 (18) in conjunction with an in-house program (https://git.lumc.nl/lgtc-bioinformatics/ngs-misc/blob/master/src/hist2count.py) to obtain a histogram of coverage per exon and the associated count data. We retrieved gene annotation (RefSeq version v54) from the UCSC (http://genome.ucsc.edu/cgi-bin/hgTables?db=hg19, retrieved July 9, 2012).

Differential Gene expression

After summing the coverage values of all nucleotides in the exonic regions of the gene, differential expression analysis was done. We filtered out low abundant genes by retaining only genes expressed in 75% or more of the samples in the statistical analysis. To account for differences in number or reads per sample, count data were normalized with the TMM function from the edgeR package (19). With the voom function from the limma package data were log-transformed. Weights from the voom transformation were taken into subsequent linear models. A hierarchical linear model was fit with the voom transformed expression data as dependent variables and health status and tissue as the independent variables, using the lmFit function from the limma package. Multiple test correction of p-values was performed by using Benjamini-Hochberg false discovery rate. Entrez Gene identifiers were retrieved using the biomaRt package v2.12.0 in R. For this study we only looked at the expression of genes coding for enzymes that participate in the branched chain amino acid degradation pathway, which were selected from the KEGG database (http://www.genome.ad.jp/kegg/).

Data processing and statistics

All amino acid data were analyzed as normalized to internal standard (supplementary table 3 and 4). Data were log-transformed when appropriate. Differences between obese subjects and lean controls 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 fixed effects and the subject specific deviances modelled 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 PCs (supplementary table 2) were found with eigenvalues larger than 1, which explained 74,4% of the total variation. Varimax rotation was performed on the 8 PCs and factor loads with an absolute value larger than 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 Bonferroni's method. Mixed effects model analysis and PCA were performed in MATLAB (The MathWorks Inc., Natick, Massachusetts, USA) and the processing of RNA-seq data was performed in R (Version 2.15.1; R Development Core Team). Graphs were developed in Prism Graph Pad 5.

RESULTS

Baseline characteristics of subjects

Baseline subject characteristics are shown in table 1 (mean ± *SEM)*. All obese subjects and healthy controls were Caucasian females, with a mean age of 49.4 ± 0.6 yrs. Eighty percent of subjects were postmenopausal, percentages were comparable between groups. We included 32 female subjects with T2DM and 30 NGT obese females. Average diabetes duration was 3.8±0.7 years, and medication was comparable between groups (supplementary table 6). 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 from the GB group, one NGT and one 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 at the day of operation or start of the diet. Only Metformin treatment was continued if fasting blood glucose levels remained above 7 mmol/l after intervention (27% of subjects after RYGB versus 17 % of subjects after VLCD, p=ns). If subjects, at baseline, used medication for chronic conditions such as hypertension or hypercholesterolemia, this was continued throughout the whole time course of

Table 1 - Baseline characteristics of the study groups.

	NGT (27)	T2DM (27)	Controls (12)	NGT vs Lean	T2DM vs Lean	T2DM vs NGT
BMI (kg/m^2)	43.8 ± 0.6	42.0 ± 1.1	21.7 ± 0.5	**8.41E-24**	**5.62E-22**	0.119133
Weight (kg)	124.3 ± 2.3	117.2 ± 3.3	64.4 ± 2.1	**0.000130**	**0.000800**	0.059
HbA1c (mmol/mol)	5.5 ± 0.14 (36.1 ± 1.5)	6.7 ± 0.19 (49.6 ± 2.1)	5.1 ± 0.06 (31.9 ± 0.7)	0.156349	**3.71E-08**	**7.96E-08**
HOMA-IR	2.4 ± 0.4	4.6 ± 0.6	0.3 ± 0.0	0.004094*	**3.01E-08**	**0.000149**
FFA (mmol/L)	1.0 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.284697	0.016636*	0.086563
Fasting glucose (mmol/L)	5.0 ± 0.2	8.7 ± 0.3	4.7 ± 0.1	0.563268	**8.52E-13**	**4.51E-16**
Fasting insulin (mU/L)	10.5 ± 1.3	12.0 ± 1.5	1.6 ± 0.1	**0.000298**	**3.21E-05**	0.435538
Fasting triglycerides (mmol/L)	1.5 ± 0.2	1.8 ± 0.1	1.0 ± 0.1	0.03803*	**0.000018**	0.002954*

Values are presented as means ± SEM. Differences between subject groups (NGT vs. T2DM) and lean controls at baseline were compared with a mixed effects model. Statistical tests that were significant after Bonferroni correction for multiple testing (p-value <0,00238 = 0,05 divided by 21 tests) were marked in boldface. A trend (not significant after correction) was defined as p<0,05 and marked by *.

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 between Obese NGT, Obese DM and Lean

Baseline levels of the 29 detected metabolites containing an amine group are presented in supplementary table 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 as compared to Lean. Asparagine was also significantly lower in NGT obese subjects versus Lean. Glutamic acid was the sole amino acid significantly higher in obese T2DM versus NGT subjects.

Principal component and regression analysis at baseline

PCA at baseline revealed 8 PCs of correlated amino acids, as described in supplementary table 2. The first PC consisted of leucine, isoleucine, valine and aminoadipic acid, whose levels were strongly correlated to one another (supplementary figure 1) and were also

positively correlated to glucose, insulin, triglycerides, HOMA-ir and BMI at baseline (table 2). A second cluster 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= $2.4*10^{-6}$) were negatively correlated to triglyceride level, whereas trends towards a negative correlation were observed for BMI (glycine), HOMA (taurine, glycine, serine) and glucose (taurine, glycine, serine). When BMI was taken into account as covariate the correlations were no longer significant. A third cluster of correlating amino acids consisted of asparagine, histidine, tryptophan, methionine and threonine. In this PC, there was only a trend towards 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 as compared to lean controls (respectively PC1 p=$3.1*10^{-6}$, PC3 p=$9.8*10^{-5}$), whereas there was a trend in NGT (PC1 higher, PC3 lower) as compared to lean controls (supplementary table 5 / figure 1). Moreover, PC1, containing all BCAAs, correlated with HOMA-ir (r=0.64; p= $1.23*10^{-8}$), glucose (r=0.58, p=$4.58*10^{-7}$), insulin (r=0.56; p=$1.37*10^{-6}$), triglycerides (r=0.48; p=$7.17*10^{-5}$) and BMI (r=0.50, p=$2.14*10^{-5}$) (table 2). Of note, correlation of PC1 with HOMA-ir and glucose were still significant when BMI was included as covariate (resp r=0.52; p=$1.14*10^{-5}$ and r=0.51; p=$1.63*10^{-5}$), suggesting that BCAAs are BMI-independently associated with glucose and HOMA-ir.

Effect of intervention

BMI decreased significantly after all interventions (supplementary table 4). 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 as compared to GB and VLCD. There was a comparable effect of the VLCD and RYGB on glucose levels in T2DM subjects (supplementary table 4 and data shown elsewhere (13)).

Effect of intervention on individual amines between Obese NGT, Obese DM

No amino acids were affected by weight loss through gastric banding in NGT subjects. RYGB, however, in NGT subjects induced a decrease in leucine, valine, isoleucine and 2-aminoadipic-acid after three weeks (except for isoleucine) and after three months (supplementary table 4). A comparable significant decrease was observed after RYGB in T2DM subjects, and moreover, mixed model analysis showed a significant greater effect of RYGB as compared to GB in NGT subjects (leucine p=$2.3*10^{-5}$, valine p=$3.1*10^{-7}$) and of RYGB as compared to VLCD in T2DM subjects (leucine p=$3.4*10^{-5}$, valine p=$7.6*10^{-6}$).

Table 2 - Correlations of brached chain amino acids and linear regression of principal components at baseline with biochemical parameters.

	BMI		HOMA IR		Glucose		Insulin		Triglycerides	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
A. Brached chain amino acids										
Leucine	0.43	$4.59*10^{-04}$	0.59	**$2.76*10^{-07}$**	0.58	**$5.71*10^{-07}$**	0.49	**$3.33*10^{-05}$**	0.51	**$2.01*10^{-05}$**
Valine	0.53	**$6.20*10^{-06}$**	0.59	**$3.82*10^{-07}$**	0.55	**$2.11*10^{-06}$**	0.50	**$2.57*10^{-05}$**	0.47	**$9.91*10^{-05}$**
Isoleucine	0.50	**$2.81*10^{-05}$**	0.64	**$1.14*10^{-08}$**	0.52	**$9.56*10^{-06}$**	0.59	**$2.37*10^{-07}$**	0.35	$4.43*10^{-03}$
2-aminoadipic acid	0.36	$3.72*10^{-03}$	0.49	**$3.86*10^{-05}$**	0.45	**$1.79*10^{-04}$**	0.43	**$3.39*10^{-04}$**	0.41	$8.11*10^{-04}$
B. Principal components										
PC1	0.50	**$2.14*10^{-5}$**	0.64	**$1.23*10^{-8}$**	0.58	**$4.58*10^{-7}$**	0.56	**$1.37*10^{-6}$**	0.48	**$7.17*10^{-5}$**
PC2	-0.19	0.14	-0.27	0.03*	-0.29	0.02*	-0.21	0.10	-0.42	**0.000573**
PC3	-0.34	0.01*	-0.32	0.01*	-0.42	**0.000594**	-0.23	0.06	-0.278	0.03*

Correlations of branched chain amino acids (A) and of most important PCs (B) with biochemical parameters. The Bonferroni posthoc test was used to correct for multiple testing: (A) after correction a $p < 0.00034$ (=0,05 divided by 5 (biochemical parameters and by 29 amino acids) and (B) after correction a $p < 0.0033$ (=0,05 divided by 5, (biochemical parameters) and by 3, the number of PCs tested baseline, indicated by bold values, was considered statistically significant.

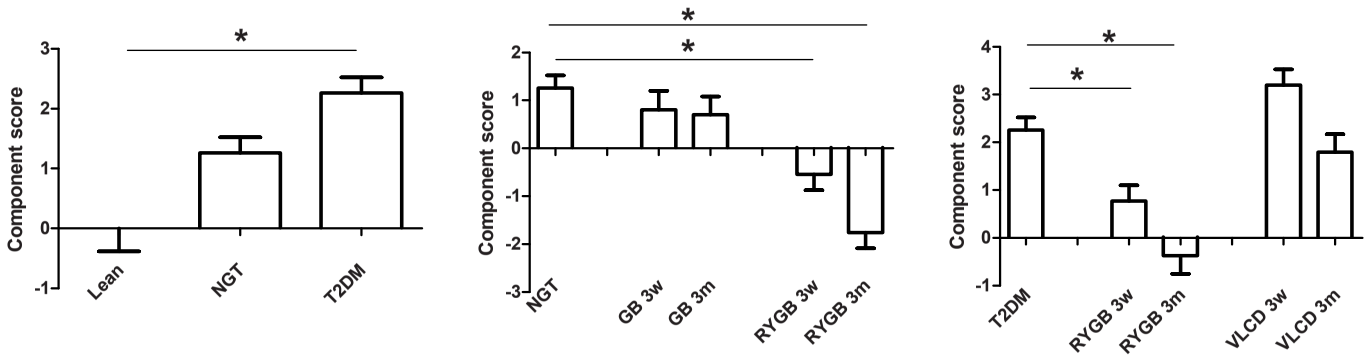


Figure 1. Bar graphs of effects of intervention on principal components.
Strengths of PC 1, BCAAs, at baseline and after the different interventions in NGT and T2DM subjects

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 three weeks and three months and glycine and serine showed a strong increase after three months.

Principal component and regression analysis after intervention

The mean score of PC1 strongly decreased after RYGB in both NGT and T2DM subjects after three weeks and three months (NGT $p= 7.0*10^{-7}$ and $1.7*10^{-14}$ respectively, T2DM $p=3.2*10^{-05}$ and $5.9*10^{-12}$ respectively), whereas there was a trend towards increased PC1 score three weeks after the VLCD and no effect of GB (supplementary table 5, figure 1). The mean score of PC3 increased 3 weeks and three months after RYGB.

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

Adipose tissue gene expression

A total of 44 genes, present in the "valine, leucine and isoleucine degradation" pathway (KEGG pathway nr 280) were expressed in the adipose tissue samples. Table 3 shows the gene expression levels of all these genes in NGT and T2DM subjects in both omental and subcutaneous adipose tissue at baseline. As compared to NGT subjects, expression levels of most of the genes of the BCAA degradation pathway were lower in T2DM subjects, with more pronounced differences in omental (32 of the 44 genes down-regulated) than in subcutaneous adipose tissue (27 of the 44 genes down-regulated). Testing for tissue-specific differences, we found that most genes were expressed differentially between omental and subcutaneous adipose tissue (table 6, last column) but that these effects were independent of disease (NGT/T2DM) status. We did not find any correlation between BCAA degradation pathway gene expression levels and serum levels of valine, leucine and isoleucine (data not shown).

Table 3 - Expression of genes in the branched chain amino acid degradation pathway in omental and subcutaneous adipose tissue.

gene		OMENTAL		SUBCUTANEOUS		NGT vs T2DM (OM)	NGT vs T2DM (SC)	OM min SC
		NGT	T2DM	NGT	T2DM			
ABAT	4-aminobutyrate aminotransferase	25.1 ± 1.3	22.9 ± 0.9	21.2 ± 1.5	19.3 ± 1.2	0.081	0.179	**1E-04**
ACAA1	acetyl-CoA acyltransferase 1	123.3 ± 3.6	117.1 ± 3.4	147.2 ± 4.3	149.9 ± 4.5	0.065	0.784	**2E-09**
ACAA2	acetyl-CoA acyltransferase 2	296.7 ± 8.5	254.9 ± 7.6	345.8 ± 12.1	363.4 ± 11.9	7E-04	0.682	**2E-09**
ACAD8	acyl-CoA dehydrogenase family. member 8	34.3 ± 1.2	29.3 ± 1.3	36.3 ± 1.0	32.9 ± 1.2	6E-04	0.012	0.003
ACADM	acyl-CoA dehydrogenase. C-4 to C-12 straight chain	128.5 ± 8.3	94.8 ± 4.7	132.4 ± 7.2	105.1 ± 5.4	**3E-04**	0.002	0.007
ACADS	acyl-CoA dehydrogenase. C-2 to C-3 short chain	147.5 ± 7.9	108.7 ± 5.8	176.3 ± 6.5	155.8 ± 6.3	**2E-04**	0.013	**4E-07**
ACADSB	acyl-CoA dehydrogenase. short/branched chain	95.1 ± 4.4	76.5 ± 2.5	103.2 ± 4.1	84.8 ± 2.8	**3E-04**	6E-04	**2E-04**
ACAT1	acetyl-CoA acetyltransferase 1	289.8 ± 12.5	222.9 ± 7.8	307.5 ± 10.7	262.1 ± 10.0	**2E-05**	0.002	**2E-04**
ACAT2	acetyl-CoA acetyltransferase 2	31.3 ± 1.0	31.0 ± 1.1	35.9 ± 1.4	38.3 ± 1.8	0.541	0.545	**3E-06**
ALDH1B1	aldehyde dehydrogenase 1 family. member B1	54.3 ± 2.6	56.4 ± 3.6	57.7 ± 2.6	55.4 ± 1.6	0.937	0.378	0.145
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	2548.1 ± 124.8	1884.7 ± 104.3	2720.6 ± 144.3	2117.0 ± 127.1	**1E-04**	0.001	0.004
ALDH3A2	aldehyde dehydrogenase 3 family. member A2	368.0 ± 14.0	339.6 ± 9.4	420.2 ± 17.9	388.2 ± 19.2	0.058	0.086	**2E-05**
ALDH6A1	aldehyde dehydrogenase 6 family. member A1	123.1 ± 8.4	77.2 ± 3.1	116.5 ± 6.6	79.8 ± 4.9	**1E-06**	**5E-05**	0.979
ALDH7A1	aldehyde dehydrogenase 7 family. member A1	86.8 ± 2.6	77.2 ± 2.7	90.0 ± 2.0	85.0 ± 2.9	0.004	0.035	0.001
ALDH9A1	aldehyde dehydrogenase 9 family. member A1	252.0 ± 14.9	206.3 ± 7.9	415.3 ± 20.2	346.5 ± 14.3	0.005	0.008	**1E-18**
AOX1	aldehyde oxidase 1	729.3 ± 63.0	825.3 ± 104.0	264.1 ± 21.2	245.1 ± 12.9	0.719	0.45	**4E-15**
AUH	AU RNA binding protein/enoyl-CoA hydratase	30.9 ± 1.3	27.0 ± 1.0	24.6 ± 1.1	22.1 ± 0.8	0.008	0.05	**3E-07**
BCAT1	branched chain amino-acid transaminase 1. cytosolic	93.1 ± 7.2	111.0 ± 6.4	72.2 ± 5.3	110.0 ± 15.2	0.051	0.006	0.021

Table 3 - Continued.

gene		OMENTAL		SUBCUTANEOUS		NGT vs T2DM (OM)	NGT vs T2DM (SC)	OM min SC
		NGT	T2DM	NGT	T2DM			
BCAT2	branched chain amino-acid transaminase 2, mitochondrial	43.4 ± 2.0	44.8 ± 2.4	37.4 ± 1.6	38.5 ± 1.5	0.996	0.992	0.002
BCKDHA	branched chain keto acid dehydrogenase E1, alpha polypeptide	118.8 ± 4.9	105.6 ± 2.4	104.1 ± 2.9	101.5 ± 3.3	0.005	0.192	0.008
BCKDHB	branched chain keto acid dehydrogenase E1, beta polypeptide	61.2 ± 4.0	46.1 ± 2.3	61.5 ± 3.1	51.5 ± 2.1	7E-04	0.006	0.018
DBT	dihydrolipoamide branched chain transacylase E2	73.9 ± 3.0	63.7 ± 2.1	71.7 ± 3.3	55.5 ± 1.9	0.004	**4E-05**	0.045
DLD	dihydrolipoamide dehydrogenase	193.7 ± 7.6	154.2 ± 5.9	202.5 ± 5.9	178.2 ± 5.7	**1E-04**	0.003	**4E-05**
ECHS1	enoyl CoA hydratase, short chain, 1, mitochondrial	355.5 ± 14.5	253.4 ± 10.0	407.8 ± 16.5	340.2 ± 16.5	**1E-06**	0.003	**4E-08**
EHHADH	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	92.9 ± 6.5	69.3 ± 4.6	109.5 ± 5.9	84.5 ± 4.8	0.005	0.002	**2E-05**
HADH	hydroxyacyl-CoA dehydrogenase	347.0 ± 20.0	237.6 ± 12.4	333.9 ± 18.5	249.8 ± 11.5	**8E-06**	**1E-04**	0.414
HADHA	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit	632.9 ± 23.0	580.1 ± 13.9	668.1 ± 14.1	702.1 ± 20.6	0.017	0.664	**5E-07**
HADHB	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, beta subunit	305.3 ± 10.9	256.5 ± 7.2	328.3 ± 11.4	307.6 ± 7.0	**1E-04**	0.071	**2E-07**
HIBADH	3-hydroxyisobutyrate dehydrogenase	172.1 ± 5.7	139.3 ± 5.0	177.4 ± 5.3	150.5 ± 5.6	**2E-05**	5E-04	0.004
HIBCH	3-hydroxyisobutyryl-CoA hydrolase	68.9 ± 4.0	55.1 ± 2.8	69.0 ± 4.2	57.0 ± 2.4	0.005	0.013	0.197
HMGCL	3-hydroxymethyl-3-methylglutaryl-CoA lyase	56.2 ± 1.7	50.7 ± 1.5	55.2 ± 1.5	49.9 ± 2.1	0.004	0.008	0.981
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	60.8 ± 2.1	58.3 ± 2.0	67.1 ± 2.3	68.3 ± 4.3	0.217	0.67	**3E-04**
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	1.3 ± 0.4	1.2 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.658	0.118	**2E-11**

gene		OMENTAL		SUBCUTANEOUS		NGT vs T2DM (OM)	NGT vs T2DM (SC)	OM min SC
		NGT	T2DM	NGT	T2DM			
HSD17B10	hydroxysteroid (17-beta) dehydrogenase 10	85.6 ± 3.0	75.7 ± 2.9	89.4 ± 2.9	82.5 ± 2.8	0.008	0.028	0.018
IL4I1	interleukin 4 induced 1	33.7 ± 2.1	35.7 ± 1.7	42.1 ± 2.0	47.6 ± 4.1	0.574	0.423	**4E-06**
IVD	isovaleryl-CoA dehydrogenase	141.2 ± 4.6	127.7 ± 5.0	129.5 ± 3.5	113.3 ± 3.3	0.005	9E-04	0.003
MCCC1	methylcrotonoyl-CoA carboxylase 1 (alpha)	140.4 ± 9.7	102.9 ± 3.1	147.7 ± 6.8	120.9 ± 5.2	**8E-05**	0.002	0.001
MCCC2	methylcrotonoyl-CoA carboxylase 2 (beta)	120.2 ± 4.6	105.6 ± 2.7	137.2 ± 3.2	115.1 ± 3.9	0.001	**5E-05**	**3E-06**
MCEE	methylmalonyl CoA epimerase	13.6 ± 0.8	11.5 ± 0.8	14.3 ± 0.7	12.2 ± 0.5	0.032	0.012	0.049
MUT	methylmalonyl CoA mutase	83.2 ± 3.4	65.9 ± 2.6	82.8 ± 3.0	69.6 ± 2.8	**6E-05**	4E-04	0.081
OXCT1	3-oxoacid CoA transferase 1	197.6 ± 8.5	142.2 ± 8.5	224.4 ± 10.9	173.5 ± 8.7	**2E-05**	4E-04	**4E-07**
OXCT2	3-oxoacid CoA transferase 2	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	0.626	0.334	0.674
PCCA	propionyl CoA carboxylase, alpha polypeptide	81.3 ± 3.7	63.7 ± 2.7	88.1 ± 3.8	73.4 ± 3.1	**2E-04**	0.005	**2E-05**
PCCB	propionyl CoA carboxylase, beta polypeptide	64.6 ± 3.2	50.3 ± 2.4	75.2 ± 2.4	68.2 ± 2.8	**1E-04**	0.023	**2E-07**

Gene expression levels are presented as means ± SEM, with as units total counts × 1000, with p-values presented in de thee collums at the right end of the table. Statistical tests that were significant after Bonferroni correction for multiple testing (*p-value <0,00038* = 0,05 divided by 44, the number of genes, and 3, the number of hypotheses) were marked in boldface. As the differential expression of most genes between omental and subcutaneous adipose tissue was independent of disease (NGT/T2DM) status, the table gives the difference between OM/SC tissue expression levels for all DM/NGT subjects as one group.

CONCLUSION

In this study, we directly determined the effects of VLCD, GB and RYGB intervention on circulating amino acids and related compounds in obese NGT and T2DM subjects, compared to lean subjects. At baseline, levels of BCAAs (leucine, valine and isoleucine) were significantly higher in obese T2DM subjects as compared to lean, while these levels in obese NGT 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 type 2 diabetes. PCA and regression analysis confirmed these findings, by showing a significant correlation of BCAA amino acids (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;20). 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 (20;21). Alternatively, down-regulation of catabolic enzymes in adipose or other tissues might be involved (8;9). We show that virtually all BCAA catabolic genes were down-regulated in omental and subcutaneous adipose tissue of obese T2DM subjects as compared to equally obese NGT subjects, 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 (BCAT) and the branched chain a-keto acid dehydrogenase (BCKD) in adipose tissue after weight loss (22). In another study, it was shown that expression of BCAA catabolic genes in adipose tissue correlated positively with insulin sensitivity (23).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 down regulation of BCAA degradation was more pronounced in omental adipose tissue as compared to subcutaneous adipose tissue, which is in agreement with a more pronounced role for omental adipose tissue in the control of metabolic health (10). Surprisingly, we did not find correlation between expression levels of genes involved in BCAA metabolism and BCAA serum levels. 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 as compared to 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-aminoadipic-acid). These effects were even more apparent three months after RYGB. At this 3 month time point, weight loss induced by GB or VLCD was significant, however still no effect on plasma BCAAs was detected. There was no correlation of PC1 with anthropometric or metabolic parameters (glucose, insulin, HOMA-IR and BMI) three months after RYGB, indicating that the decrease in BCAAs is predominantly caused by the bypass procedure of RYGB surgery and independent of the effect of weight loss 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 (11) but in contrast with other reports (12;20). 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 three months after the different interventions), we conclude that the decrease in BCAAs after RYGB is predominantly caused by the bypass procedure and not due to weight loss. Nevertheless, the fact that calorie restriction had a beneficial effect on glucose metabolism without affecting circulating BCAA concentrations, suggests that BCAAs do not play a decisive pathogenic role in obesity associated insulin resistance, or at least it suggests that that reversal of insulin resistance after calorie restriction of RYGB is BCAA independent.

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) in adipose tissue (22). To what extent the observed decline of circulating BCAA after the RYGB procedure is due to increased expression of BCAA catabolic genes remains to be determined.

Interestingly, aminoadipic acid, which is not a BCAA, clustered together with the BCAA's and showed a decrease after RYGB. Wang *et al.* (24) have shown that aminoadipic acid is a biomarker for diabetes risk and a potential modulator of glucose homeostasis. Aminoadipic acid is generated via lysine degradation and is involved in tryptophan metabolism. In the paper by Wang *et al.* no correlation was found between the BCAA's and aminoadipic acid, so they suggested that aminoadipic acid is involved in different

pathophysiological pathways than BCAAs. Whether the association of aminoadipic acid with the BCAAs in our study is due to the specifics of subjects and/or intervention remains to be investigated.

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 interpretation of the observed effects. However, longer follow up studies are needed to confirm whether the observed effects remain. In addition, due to the intensity of the protocol we were not able to perform hyperinsulinemic-euglycemic clamp studies to measure the extent of insulin resistance. Therefore we estimate insulin resistance 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 levels is comparable, it seems unlikely that diabetes medication was a major confounder.

In conclusion, we show that BCAA tend to be higher in obese NGT subjects and are significantly higher in T2DM subjects as compared to 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 immediately after RYGB is due to the bypass procedure, and 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, does not fit with the postulate that BCAA play a decisive role in the pathogenesis of obesity associated insulin resistance.

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Supplementary Table 1 - Amino acids and their internal standards.

Amino acid	Internal standard
leucine	Leu_C13N15
valine	Val_C13N15
isoleucine	Ile_C13N15
2-aminoadipic acid	Pro_C13N15
glutamic acid	Glu_C13N15
n1-methyhistidine	L-NT-methyl-d3-L-histidine
alpha-aminobutyric acid	L-2-aminobutyric acid-d6 acid
D3-aminoisobutyric acid	L-2-aminobutyric acid-d6 acid
kynurenine	Ile_C13N15
asparagine	Asn_C13N15
histidine	Asn_C13N15
tryptophan	Trp_C13N15
lysine	Lys_C13N15
methionine	Met_C13N15
threonine	Thr_C13N15
citrulline	Thr_C13N15
arginine	Arg_C13N15
glutamine	Gln_C13N15
ornithine	L-ornithine-3,3,4,4,5,5,-d6
taurine	Asn_C13N15
glycine	Gly_C13N15
serine	Ser_C13N15
sarcosine	Thr_C13N15
ethanolamine	Asp_C13N15
phenylalanine	Phe_C13N15
tyrosine	Tyr_C13N15
4-hydroxy-proline	Asn_C13N15
alanine	Ala_C13N15
proline	Pro_C13N15

Supplementary Table 2 - PCA revealed several clusters of related/co-regulated amino acids.

Principal component	Amino acids
1	***leucine, valine, isoleucine, L-2-aminoadipic acid***
2	***glycine, citrulline, arginine, glutamine, ornithine***
3	***asparagine, histidine, tryptophan, methionine, threonine***
4	*glutamic-acid, ethanolamine, phenylalanine*
5	*alpha-aminobutyricacid, 4-hydroxy-proline, proline*
6	*D3-aminoisobutyricacid, sarcosine*
7	*kynurenine, tyrosine*
8	*n1-methyhistidine, taurine*

Supplementary Table 3 - Baseline levels of amino acids and differences between Lean, Obese NGT and Obese T2DM subjects.

	Control	sem	NGT	sem	T2DM	sem	NGT vs. control	T2DM vs. control	T2DM vs. NGT
leucine	0.88	± 0.05	0.99	± 0.03	1.13	± 0.03	5.01E-02	**1.81E-05**	2.25E-03
valine	2.29	± 0.11	2.70	± 0.07	2.96	± 0.07	2.46E-03	**1.16E-06**	1.22E-02
isoleucine	0.78	± 0.05	0.95	± 0.03	1.05	± 0.03	6.37E-03	**2.70E-05**	4.83E-02
2-aminoadipicacid	4.46	± 0.39	5.02	± 0.26	5.96	± 0.26	2.35E-01	1.78E-03	1.29E-02
glutamicacid	0.34	± 0.05	0.35	± 0.04	0.55	± 0.04	9.35E-01	1.26E-03	**8.73E-05**
n1-methyhistidine	0.07	± 0.01	0.07	± 0.00	0.08	± 0.00	7.95E-01	5.71E-01	6.99E-01
alpha-aminobutyricacid	0.22	± 0.02	0.19	± 0.02	0.22	± 0.02	2.09E-01	9.54E-01	1.32E-01
D3-aminoisobutyricacid	24.84	± 3.66	23.44	± 2.49	17.91	± 2.49	7.53E-01	1.21E-01	1.20E-01
kynurenine	8.82	± 0.64	10.32	± 0.44	9.42	± 0.44	5.54E-02	4.40E-01	1.46E-01
asparagine	0.87	± 0.02	0.69	± 0.02	0.62	± 0.02	**6.23E-08**	**5.21E-13**	3.43E-03
histidine	0.19	± 0.01	0.17	± 0.00	0.16	± 0.00	4.60E-03	**5.24E-05**	1.00E-01
tryptophan	2.46	± 0.10	2.14	± 0.07	2.18	± 0.07	1.28E-02	3.22E-02	6.48E-01
lysine	8.21	± 0.33	8.22	± 0.23	8.38	± 0.23	9.77E-01	6.65E-01	6.11E-01
methionine	1.44	± 0.06	1.38	± 0.04	1.33	± 0.04	3.47E-01	1.20E-01	4.34E-01
threonine	2.24	± 0.14	2.19	± 0.09	1.98	± 0.09	7.53E-01	1.24E-01	1.24E-01
citrulline	0.26	± 0.02	0.20	± 0.01	0.22	± 0.01	1.50E-02	9.84E-02	3.11E-01
arginine	2.51	± 0.16	2.27	± 0.11	2.12	± 0.11	2.09E-01	4.09E-02	3.14E-01
glutamine	40.97	± 1.58	38.81	± 1.07	37.91	± 1.07	2.62E-01	1.13E-01	5.52E-01
ornithine	0.93	± 0.06	0.91	± 0.04	0.93	± 0.04	8.19E-01	9.88E-01	7.60E-01
taurine	1.03	± 0.06	0.88	± 0.04	0.76	± 0.04	4.65E-02	6.22E-04	5.71E-02
glycine	4.77	± 0.32	3.41	± 0.22	2.89	± 0.22	8.44E-04	**7.93E-06**	9.55E-02

Supplementary Table 3 - Continued.

	Control	sem	NGT	sem	T2DM	sem	NGT vs. control	T2DM vs. control	T2DM vs. NGT
serine	3.29	± 0.16	2.85	± 0.11	2.66	± 0.11	2.79E-02	1.91E-03	2.30E-01
sarcosine	33.97	± 2.45	26.96	± 1.66	32.83	± 1.66	1.96E-02	7.02E-01	1.40E-02
ethanolamine	1.11	± 0.05	1.26	± 0.03	1.18	± 0.03	1.18E-02	2.47E-01	8.14E-02
phenylalanine	0.75	± 0.03	0.82	± 0.02	0.82	± 0.02	6.02E-02	4.80E-02	9.00E-01
tyrosine	1.76	± 0.10	1.86	± 0.07	1.96	± 0.07	4.47E-01	1.19E-01	3.10E-01
4-hydroxy-proline	0.12	± 0.01	0.15	± 0.01	0.14	± 0.01	8.61E-02	1.80E-01	6.33E-01
alanine	2.65	± 0.17	2.96	± 0.11	3.29	± 0.11	1.31E-01	2.03E-03	4.17E-02
proline	6.39	± 0.48	7.70	± 0.33	8.58	± 0.33	2.74E-02	3.02E-04	6.09E-02

Values are presented as means ± SEM. Differences between subject groups (NGT vs. T2DM) and lean controls at baseline were compared with Mixed model. The Bonferroni posthoc test was used to correct for multiple testing: after correction a $p < 0{,}000157$ (= p <0,05 divided by 29, the number of parameters tested, and by 11, the numbers of hypotheses tested at baseline and after the different interventions), indicated by bold values, was considered statistically significant.

Supplementary Table 4a - Levels of amino acids in Obese NGT subjects at baseline versus the ffects of GB or RYGB.

	NGT	NGT-GB 3w		NGT-GB 3m		NGT-RYGB 3w		NGT-RYGB 3m	
	mean sem	mean sem	p-value	mean sem	p-value	mean sem	p-value	mean sem	p-value
BMI (kg*m^{-1})	43.77 ± 0.79	41.10 ± 0.85	**7.7E-09**	39.02 ± 0.82	**0.0E+00**	40.48 ± 0.85	**2.8E-15**	36.63 ± 0.82	**0.0E+00**
Weight (kg)	124.3 ± 2.3	113.1 ± 4.2	1.2E-03	106.0 ± 4.2	**1.6E-08**	119.4 ± 2.6	**2.8E-14**	108.0 ± 2.7	**3.8E-17**
Weight loss (%)		4.80 ± 0.8		10.2 ± 1.0		6.9 ± 0.8		15.7 ± 1.1	
HOMAIR	2.42 ± 0.36	1.50 ± 0.54	1.1E-01	2.38 ± 0.45	9.4E-01	1.93 ± 0.54	3.2E-01	1.17 ± 0.45	1.2E-02
Glucose (mmol/L)	5.01 ± 0.25	4.90 ± 0.37	7.9E-01	5.19 ± 0.31	6.4E-01	4.82 ± 0.37	5.9E-01	4.87 ± 0.31	6.8E-01
Insulin (mU/mL)	10.53 ± 1.22	6.45 ± 1.82	3.3E-02	9.61 ± 1.53	6.3E-01	9.07 ± 1.82	3.7E-01	5.20 ± 1.53	1.4E-03
leucine	0.99 ± 0.03	0.97 ± 0.05	5.9E-01	0.96 ± 0.04	5.3E-01	0.81 ± 0.05	**4.2E-05**	0.71 ± 0.04	**7.6E-10**
valine	2.70 ± 0.07	2.43 ± 0.11	2.7E-02	2.53 ± 0.09	1.5E-01	2.02 ± 0.11	**4.7E-10**	1.80 ± 0.09	**4.0E-15**
isoleucine	0.95 ± 0.03	0.97 ± 0.05	7.9E-01	0.88 ± 0.04	1.7E-01	0.89 ± 0.05	1.7E-01	0.73 ± 0.04	**2.9E-06**
2-aminoadipic acid	5.02 ± 0.26	4.49 ± 0.39	1.8E-01	4.37 ± 0.33	8.5E-02	3.27 ± 0.37	**5.2E-07**	2.80 ± 0.33	**6.4E-10**
glutamic acid	0.35 ± 0.04	0.32 ± 0.06	6.4E-01	0.30 ± 0.05	4.5E-01	0.35 ± 0.05	9.2E-01	0.26 ± 0.05	1.3E-01
n1-methyhistidine	0.07 ± 0.00	0.07 ± 0.01	7.9E-01	0.07 ± 0.01	6.7E-01	0.08 ± 0.01	6.4E-01	0.07 ± 0.01	4.8E-01
alpha-aminobutyric acid	0.19 ± 0.02	0.29 ± 0.02	3.4E-04	0.23 ± 0.02	1.3E-01	0.27 ± 0.02	3.2E-04	0.20 ± 0.02	4.8E-01
D3-aminoisobutyric acid	23.44 ± 2.49	30.03 ± 3.51	4.9E-02	24.77 ± 3.01	6.8E-01	34.76 ± 3.39	**8.5E-05**	33.81 ± 3.01	3.0E-04
kynurenine	10.32 ± 0.44	8.59 ± 0.61	3.5E-03	9.54 ± 0.53	1.7E-01	7.25 ± 0.59	**5.6E-09**	7.07 ± 0.53	**9.6E-10**
asparagine	0.69 ± 0.02	0.64 ± 0.03	8.3E-02	0.68 ± 0.02	5.5E-01	0.61 ± 0.02	4.8E-04	0.58 ± 0.02	**5.8E-06**
histidine	0.17 ± 0.00	0.15 ± 0.01	3.2E-03	0.17 ± 0.00	7.8E-01	0.14 ± 0.00	**9.3E-08**	0.15 ± 0.00	1.7E-04
tryptophan	2.14 ± 0.07	1.90 ± 0.10	1.2E-02	2.03 ± 0.09	2.3E-01	1.57 ± 0.10	**6.0E-11**	1.46 ± 0.09	**3.3E-14**
lysine	8.22 ± 0.23	7.96 ± 0.33	4.4E-01	8.23 ± 0.28	9.7E-01	7.70 ± 0.32	6.9E-02	6.92 ± 0.28	**1.2E-05**
methionine	1.38 ± 0.04	1.26 ± 0.06	5.7E-02	1.28 ± 0.05	1.1E-01	1.27 ± 0.06	3.4E-02	1.15 ± 0.05	**2.5E-05**
threonine	2.19 ± 0.09	2.34 ± 0.13	1.7E-01	2.01 ± 0.11	1.0E-01	1.96 ± 0.12	1.7E-02	1.73 ± 0.11	**3.2E-06**

Supplementary Table 4a - Continued.

	NGT	NGT-GB 3w		NGT-GB 3m		NGT-RYGB 3w		NGT-RYGB 3m	
	mean sem	mean sem	p-value	mean sem	p-value	mean sem	p-value	mean sem	p-value
citrulline	0.20 ± 0.01	0.16 ± 0.02	7.3E-03	0.20 ± 0.01	6.8E-01	0.17 ± 0.02	4.9E-03	0.20 ± 0.01	5.0E-01
arginine	2.27 ± 0.11	1.93 ± 0.16	4.3E-02	2.35 ± 0.14	6.5E-01	1.97 ± 0.16	3.8E-02	1.99 ± 0.14	4.7E-02
glutamine	38.81 ± 1.07	40.60 ± 1.50	2.0E-01	41.81 ± 1.29	2.8E-02	40.14 ± 1.45	2.6E-01	39.99 ± 1.29	3.2E-01
ornithine	0.91 ± 0.04	0.83 ± 0.05	9.8E-02	0.89 ± 0.05	6.9E-01	0.66 ± 0.05	**2.7E-09**	0.68 ± 0.05	**4.2E-08**
taurine	0.88 ± 0.04	0.88 ± 0.06	9.6E-01	0.89 ± 0.05	7.3E-01	0.91 ± 0.05	5.1E-01	0.92 ± 0.05	3.0E-01
glycine	3.41 ± 0.22	3.96 ± 0.26	3.1E-03	3.84 ± 0.24	1.6E-02	3.87 ± 0.25	2.8E-03	4.18 ± 0.24	**1.5E-06**
serine	2.85 ± 0.11	3.28 ± 0.15	3.5E-03	3.29 ± 0.13	1.8E-03	3.18 ± 0.15	7.0E-03	3.34 ± 0.13	**8.6E-05**
sarcosine	26.96 ± 1.66	25.68 ± 2.48	6.1E-01	22.91 ± 2.08	9.7E-02	20.34 ± 2.39	2.3E-03	21.45 ± 2.08	1.1E-02
ethanolamine	1.26 ± 0.03	1.21 ± 0.05	2.9E-01	1.23 ± 0.04	5.2E-01	1.22 ± 0.05	3.5E-01	1.10 ± 0.04	1.9E-04
phenylalanine	0.82 ± 0.02	0.74 ± 0.03	4.5E-03	0.82 ± 0.02	9.0E-01	0.67 ± 0.03	**1.9E-08**	0.59 ± 0.02	**1.4E-15**
tyrosine	1.86 ± 0.07	1.57 ± 0.09	6.8E-04	1.63 ± 0.08	4.3E-03	1.36 ± 0.09	**5.4E-11**	1.20 ± 0.08	**3.3E-16**
4-hydroxy-proline	0.15 ± 0.01	0.13 ± 0.02	1.7E-01	0.13 ± 0.01	1.6E-01	0.10 ± 0.01	9.8E-04	0.12 ± 0.01	2.5E-02
alanine	2.96 ± 0.11	2.47 ± 0.17	6.0E-03	2.35 ± 0.14	3.8E-04	2.57 ± 0.16	9.5E-03	2.25 ± 0.14	**4.6E-06**
proline	7.70 ± 0.33	6.83 ± 0.48	6.2E-02	6.79 ± 0.40	4.3E-02	5.71 ± 0.46	**1.5E-06**	5.64 ± 0.40	**6.8E-07**

Supplementary Table 4b - Levels of amino acids in Obese T2DM subjects at baseline versus the effects of RYGB and VLCD.

	T2DM	T2DM-RYGB 3w		T2DM-RYGB 3m		T2DM-VLCD 3w		T2DM-VLCD 3m	
	mean sem	mean sem	p-value	mean sem	p-value	mean sem	p-value	mean sem	p-value
BMI ($kg*m^{-1}$)	42.03 ± 0.79	38.95 ± 0.82	**2.4E-13**	34.67 ± 0.84	**0.0E+00**	39.44 ± 0.82	**5.8E-09**	36.26 ± 0.85	**0.0E+00**
Weight (kg)	117.2 ± 3.3	112.5 ± 3.9	**2.4E-10**	1.9.0 ± 3.5	**1.1E-16**	1.5.0 ± 5.1	**1.4E-07**	97.2 ± 3.1	**2.9^{E}-11**
Weight loss (%)		7.3 ± 0.5		16.9 ± 0.6		6.0 ± 0.4		14.6 ± 1.0	
HOMAIR	4.63 ± 0.36	3.42 ± 0.47	1.8E-02	1.85 ± 0.52	**2.0E-07**	1.09 ± 0.47	**2.6E-09**	1.26 ± 0.59	**2.4E-07**
Glucose (mmol/L)	8.74 ± 0.25	6.68 ± 0.32	**3.9E-08**	5.72 ± 0.36	**3.1E-14**	5.33 ± 0.32	**4.7E-15**	6.00 ± 0.37	**2.2E-10**
Insulin (mU/mL)	12.00 ± 1.22	11.37 ± 1.58	7.1E-01	7.35 ± 1.75	6.2E-03	4.30 ± 1.58	**4.8E-05**	4.22 ± 1.98	2.3E-04
leucine	1.13 ± 0.03	0.95 ± 0.04	**3.9E-05**	0.84 ± 0.05	**4.9E-10**	1.28 ± 0.04	2.6E-03	1.09 ± 0.05	4.1E-01
valine	2.96 ± 0.07	2.29 ± 0.09	**5.3E-10**	2.10 ± 0.11	**3.4E-14**	3.12 ± 0.09	1.6E-01	2.74 ± 0.11	4.9E-02
isoleucine	1.05 ± 0.03	1.03 ± 0.04	7.0E-01	0.87 ± 0.05	**1.2E-04**	1.24 ± 0.04	2.9E-04	0.99 ± 0.05	2.8E-01
2-aminoadipic acid	5.96 ± 0.26	4.56 ± 0.33	**4.4E-05**	3.65 ± 0.37	**1.8E-10**	6.45 ± 0.33	1.9E-01	5.80 ± 0.37	6.7E-01
glutamic acid	0.55 ± 0.04	0.52 ± 0.05	6.4E-01	0.39 ± 0.05	3.6E-03	0.46 ± 0.05	1.4E-01	0.41 ± 0.05	3.1E-02
n1-methyhistidine	0.08 ± 0.00	0.09 ± 0.01	1.3E-02	0.09 ± 0.01	1.8E-02	0.06 ± 0.01	3.1E-03	0.08 ± 0.01	6.9E-01
alpha-aminobutyric acid	0.22 ± 0.02	0.31 ± 0.02	**1.1E-04**	0.21 ± 0.02	7.3E-01	0.53 ± 0.02	**0.0E+00**	0.35 ± 0.02	**2.5E-06**
D3-aminoisobutyric acid	17.91 ± 2.49	28.92 ± 3.01	**1.3E-04**	32.20 ± 3.39	**1.1E-06**	37.80 ± 3.01	**7.1E-09**	26.23 ± 3.39	1.0E-02
kynurenine	9.42 ± 0.44	7.35 ± 0.53	**4.4E-05**	7.64 ± 0.59	3.9E-04	7.17 ± 0.53	**9.8E-05**	8.30 ± 0.59	4.8E-02
asparagine	0.62 ± 0.02	0.58 ± 0.02	1.3E-01	0.59 ± 0.02	2.6E-01	0.62 ± 0.02	9.4E-01	0.67 ± 0.02	3.2E-02
histidine	0.16 ± 0.00	0.15 ± 0.00	6.2E-03	0.16 ± 0.00	4.0E-01	0.15 ± 0.00	2.9E-03	0.16 ± 0.00	5.9E-01
tryptophan	2.18 ± 0.07	1.68 ± 0.09	**2.2E-09**	1.55 ± 0.10	**7.0E-13**	1.60 ± 0.09	**2.0E-09**	1.92 ± 0.10	4.0E-03
lysine	8.38 ± 0.23	8.04 ± 0.28	2.2E-01	7.88 ± 0.32	8.0E-02	8.16 ± 0.28	4.9E-01	9.04 ± 0.32	4.2E-02
methionine	1.33 ± 0.04	1.26 ± 0.05	1.5E-01	1.15 ± 0.06	6.5E-04	1.25 ± 0.05	1.5E-01	1.27 ± 0.06	2.7E-01
threonine	1.98 ± 0.09	1.82 ± 0.11	9.8E-02	1.72 ± 0.12	6.3E-03	1.82 ± 0.11	1.5E-01	1.96 ± 0.12	8.9E-01
citrulline	0.22 ± 0.01	0.19 ± 0.01	1.1E-02	0.21 ± 0.02	3.5E-01	0.18 ± 0.01	4.4E-03	0.17 ± 0.02	**1.4E-04**

Supplementary Table 4b - Continued.

	T2DM	T2DM-RYGB 3w		T2DM-RYGB 3m		T2DM-VLCD 3w		T2DM-VLCD 3m	
	mean sem	mean sem	p-value	mean sem	p-value	mean sem	p-value	mean sem	p-value
arginine	2.12 ± 0.11	2.08 ± 0.14	7.9E-01	2.14 ± 0.16	8.8E-01	1.75 ± 0.14	2.6E-02	2.28 ± 0.16	3.4E-01
glutamine	37.91 ± 1.07	43.12 ± 1.29	**2.2E-05**	46.30 ± 1.45	**1.1E-10**	36.67 ± 1.29	3.6E-01	41.35 ± 1.45	1.2E-02
ornithine	0.93 ± 0.04	0.77 ± 0.05	**1.1E-04**	0.87 ± 0.05	1.2E-01	0.75 ± 0.05	**1.4E-04**	0.80 ± 0.05	5.7E-03
taurine	0.76 ± 0.04	0.83 ± 0.05	1.1E-01	0.86 ± 0.05	1.8E-02	0.59 ± 0.05	3.0E-04	0.76 ± 0.05	9.8E-01
glycine	2.89 ± 0.22	3.37 ± 0.24	1.8E-03	4.05 ± 0.25	**8.2E-12**	2.98 ± 0.24	5.9E-01	3.48 ± 0.25	9.3E-04
serine	2.66 ± 0.11	3.10 ± 0.13	3.8E-04	3.41 ± 0.15	**9.3E-09**	2.95 ± 0.13	4.0E-02	3.37 ± 0.15	**1.3E-06**
sarcosine	32.83 ± 1.66	25.59 ± 2.08	8.9E-04	25.43 ± 2.39	7.0E-04	20.80 ± 2.08	**2.3E-06**	25.56 ± 2.39	3.2E-03
ethanolamine	1.18 ± 0.03	1.24 ± 0.04	9.4E-02	1.10 ± 0.05	5.3E-02	1.05 ± 0.04	9.0E-03	1.06 ± 0.05	1.4E-02
phenylalanine	0.82 ± 0.02	0.72 ± 0.02	**3.3E-05**	0.64 ± 0.03	**3.4E-11**	0.72 ± 0.02	4.7E-04	0.76 ± 0.03	2.3E-02
tyrosine	1.96 ± 0.07	1.55 ± 0.08	**2.2E-08**	1.40 ± 0.09	**4.2E-13**	1.51 ± 0.08	**6.0E-08**	1.60 ± 0.09	**1.3E-05**
4-hydroxy-proline	0.14 ± 0.01	0.11 ± 0.01	1.2E-02	0.15 ± 0.01	6.8E-01	0.08 ± 0.01	**9.2E-05**	0.14 ± 0.01	6.1E-01
alanine	3.29 ± 0.11	2.76 ± 0.14	4.6E-04	2.72 ± 0.16	2.0E-04	2.08 ± 0.14	**5.9E-11**	2.52 ± 0.16	**1.1E-05**
proline	8.58 ± 0.33	7.01 ± 0.40	**1.0E-04**	7.82 ± 0.46	5.3E-02	6.79 ± 0.40	**1.1E-04**	7.17 ± 0.46	2.1E-03

Supplementary Table 5 - Factor scores of main principal components at baseline and after intervention.

Baseline						
	Control	**NGT**	**T2DM**	**NGT vs control**	**T2DM vs control**	**T2DM vs. NGT**
PC1	0.0 ± 0.38	1.26 ± 0.25	2.26 ± 0.26	0.007*	3.177E-06	0.008*
PC2	0.0 ± 0.47	-1.15 ± 0.32	-1.43 ± 0.32	0.048*	0.015*	0.544
PC3	0.0 ± 0.42	-1.30 ± 0.28	-2.05 ± 0.29	0.012*	9.771E-05	0.067

NGT after intervention									
	Baseline	**NGT GB 3w**	**p-value**	**NGT GB 3m**	**p-value**	**NGT RYGB 3 w**	**p-value**	**NGT RYGB 3 w**	**p-value**
PC1	1.26 ± 0.25	0.81 ± 0.39	0.26	0.70 ± 0.38	0.16	-0.54 ± 0.33	7.0E-07	-1.75 ± 0.33	1.71E-14
PC2	-1.15 ± 0.32	-1.72 ± 0.44	0.15	-0.72 ± 0.38	0.25	-1.98 ± 0.38	0.01	-1.72 ± 0.38	0.08
PC3	-1.30 ± 0.28	-2.20 ± 0.43	0.05	-1.78 ± 0.36	0.27	-3.18 ± 0.36	2.12E-06	-3.73 ± 0.36	2.68E-09

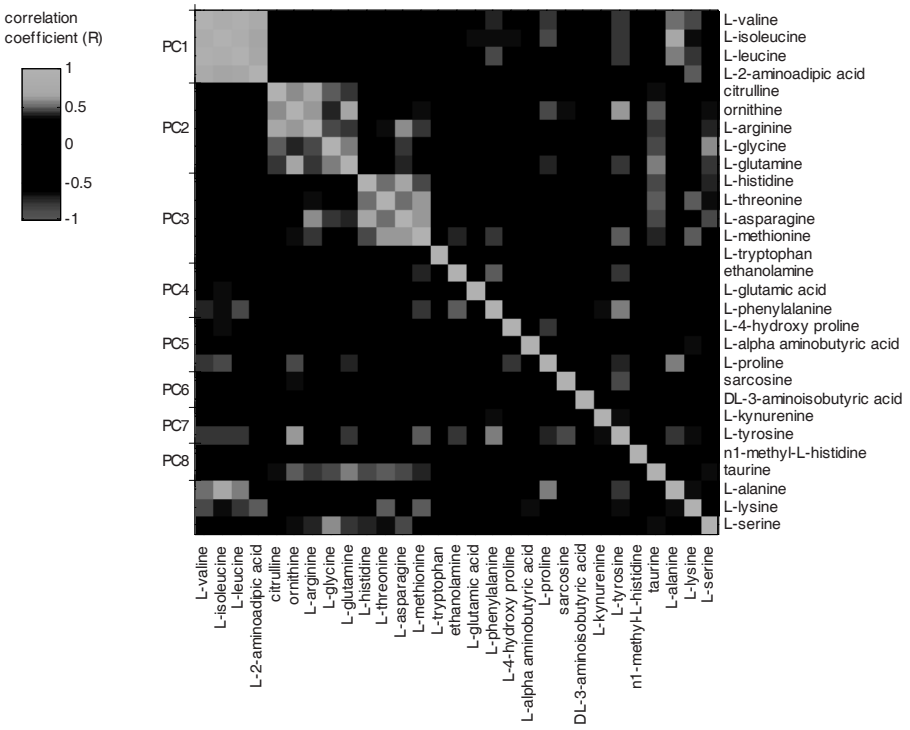
DM after intervention									
	Baseline	**T2DM RYGB 3w**	**p-value**	**T2DM RYGB 3m**	**p-value**	**T2DM VLCD 3 w**	**p-value**	**T2DM VLCD 3m**	**p-value**
PC1	2.26 ± 0.26	0.77 ± 0.33	3.18E-05	-0.37 ± 0.32	5.89E-12	3.20 ± 0.38	0.02	1.80 ±0.38	0.23
PC2	-1.43 ± 0.32	-1.43 ± 0.38	0.991	-0.57 ± 0.38	0.01	-2.49 ± 0.42	0.01	-1.72 ± 0.42	0.43
PC3	-2.05 ± 0.29	-3.25 ± 0.36	0.002	-3.44 ± 0.36	0.0003	-3.15 ± 0.41	0.01	-2.05 ± 0.41	0.99

Values are presented as means ± SEM. Differences between subject groups (NGT vs. T2DM) and the effects of intervention were compared with a mixed effects model. The Bonferroni posthoc test was used to correct for multiple testing: after correction a $p < 0{,}0015$ (= p <0,05 divided by 3, the number of PCs, and by 11, the numbers of hypotheses tested at baseline and after the different interventions), indicated by bold values, was considered statistically significant.

Supplementary Table 6 - Pre-intervention medication use in the study groups.

	NGT subjects RYGB (n=16)/GB (n=11)	T2DM subjects RYGB (n=15)/VLCD (n=12)
Metformin	0/0	13/10
Sulfonyl-urea derivatives	0/0	6/5
beta-Blockers	2/2	3/3
ACE-inhibitors	2/1	3/2
Diuretics	2/2	4/3

Values signify the numbers of patients using the indicated medication in the respective groups.



Supplementary Figure 1 - Correlation matrix of metabolite levels and associated principal components.