




A systems biology approach to understand gut microbiota and host metabolism in morbid obesity: design of the BARIA Longitudinal Cohort Study

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Abstract. Van Olden CC, de Van Laar AW, Meijnikman AS, Aydin O, Van Olst N, Hoozemans JB, De Brauw LM, Bruin SC, Acherman YIZ, Verheij J, Pyykkö JE, Hagedoorn M, Sanderman R, Bosma NC, Tremaroli V, Lundqvist A, Olofsson LE, Herrema H, Lappa D, Nielsen J, Schwartz T, Groen AK, Nieuwdorp M, Bäckhed F, Gerdes VEA (Amsterdam UMC, Amsterdam; Spaarne Gasthuis, Hoofddorp; Amsterdam UMC, Amsterdam; Groningen UMC, Groningen, The Netherlands; University of Gothenburg, Goteborg; Chalmers University of Technology, Gothenburg, Sweden; University of Copenhagen, Kobenhavn, Denmark; Sahlgrenska University Hospital, Gothenburg, Sweden). A systems biology approach to understand gut microbiota and host metabolism in morbid obesity: design of the BARIA Longitudinal Cohort Study (Original). *J Intern Med* 2021; **289**: 340–354. <https://doi.org/10.1111/joim.13157>

Introduction. Prevalence of obesity and associated diseases, including type 2 diabetes mellitus, dyslipidaemia and non-alcoholic fatty liver disease (NAFLD), are increasing. Underlying mechanisms, especially in humans, are unclear. Bariatric surgery provides the unique opportunity to obtain biopsies and portal vein blood-samples.

Methods. The BARIA Study aims to assess how microbiota and their metabolites affect transcription in key tissues and clinical outcome in obese subjects and how baseline anthropometric and

metabolic characteristics determine weight loss and glucose homeostasis after bariatric surgery. We phenotype patients undergoing bariatric surgery (predominantly laparoscopic Roux-en-Y gastric bypass), before weight loss, with biometrics, dietary and psychological questionnaires, mixed meal test (MMT) and collect fecal-samples and intra-operative biopsies from liver, adipose tissues and jejunum. We aim to include 1500 patients. A subset (approximately 25%) will undergo intra-operative portal vein blood-sampling. Fecal-samples are analyzed with shotgun metagenomics and targeted metabolomics, fasted and postprandial plasma-samples are subjected to metabolomics, and RNA is extracted from the tissues for RNAseq-analyses. Data will be integrated using state-of-the-art neuronal networks and metabolic modeling. Patient follow-up will be ten years.

Results. Preoperative MMT of 170 patients were analysed and clear differences were observed in glucose homeostasis between individuals. Repeated MMT in 10 patients showed satisfactory intra-individual reproducibility, with differences in plasma glucose, insulin and triglycerides within 20% of the mean difference.

Conclusion. The BARIA study can add more understanding in how gut-microbiota affect metabolism, especially with regard to obesity, glucose metabolism and NAFLD. Identification of key factors may provide diagnostic and therapeutic leads to control the obesity-associated disease epidemic.

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Keywords: obesity, bariatric surgery, gut microbiota, metabolites, insulin resistance.

Introduction

Obesity is on the rise. At the current pace, more than one billion adults will be obese by 2030 [1]. An increase in obesity-associated diseases will follow in its wake, including type 2 diabetes mellitus (T2DM), dyslipidaemia, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease. However, it has been challenging to identify underlying molecular mechanisms contributing to cardiometabolic diseases, in part because T2DM has several subclasses [2]. Several pathways have been suggested to contribute to obesity and impaired glucose control, such as the immune system and gut microbiota [3–5]. They include short-chain fatty acids, bile acids, amino acids-derived metabolites, neural pathways and lymphoid cells. Interestingly, these have also been shown to be involved in glucose metabolism and the development of NAFLD, which illustrates the interconnectivity of cardiometabolic diseases. Moreover, a chronic low-grade inflammation can be measured in individuals with obesity, possibly caused by a disturbance in the intestinal microbiota composition. Faecal microbiota transplantation (FMT) from human subjects to mice transferred adiposity phenotype suggesting that, in mice, the microbiota may be a contributing factor [6]. In humans, the effect of FMT is less significant, yet insulin sensitivity can improve for a short whilst in individuals with metabolic syndrome after infusion of intestinal microbiota from lean donors [7].

The relative contribution of different organs (liver, adipose tissue and gut) to whole body metabolism as well as immunological tone on weight loss in relation to improvement of insulin sensitivity is not known. Neither are the mechanisms that trigger the innate and adaptive (intestinal) immune system by altered intestinal microbiota, or their effects on metabolism.

Most interventions aimed at losing weight in individuals with morbid obesity have little effect, except for bariatric surgery [8]. Bariatric surgery is also the most effective intervention to reduce obesity-related morbidity and mortality [9]. In this regard, one of the most common and well-studied bariatric procedures is laparoscopic Roux-en-Y gastric bypass (LRYGB). The increased insulin sensitivity found

shortly after LRYGB, even before significant weight loss is obtained, suggests immediate systemic changes in metabolism upon surgery, which are long standing, as even ten years after surgery beneficial effects on glucose metabolism, lipids and blood pressure can be seen [10, 11].

Although being an important treatment for over forty years, the mechanisms behind the beneficial effect of bariatric surgery have been elusive. They may include bile flow alteration, reduction of gastric size, anatomical rearrangement and altered flow of nutrients, vagal manipulation and enteric gut hormone modulation [12]. Although some studies have demonstrated that intestinal microbiota are altered after bariatric surgery as well, the prospective value of (baseline) intestinal microbiota composition and the relation with the (diet derived) metabolites that these bacteria produce has never been investigated at a larger scale [13, 14].

Significant differences in the response to bariatric surgery can be observed, both in weight loss, obesity-related morbidities and psychological factors, including self-esteem, risk of addiction and quality of life [15–18]. Despite some methodological limitations, psychological studies have shown improvements in psychopathology, eating disorders, depressive symptoms, body image and social functioning after bariatric surgery [19]. Systems biology models can provide an advanced reconstruction of individuals' metabolism at different organ levels in patients with morbid obesity. They could provide a valuable tool in predicting individuals' outcomes of bariatric surgery and hereby develop a personalized medicine approach for this disease. First steps in utilizing this technique to study altered metabolism in obesity-related diseases have produced interesting results [20–23].

We aim to perform a systems biology approach, as schematically depicted in Fig. 1, identifying gut microbial, immunological and metabolic markers in a large and well phenotyped bariatric surgery cohort (BARIA study) to identify signalling pathways that can affect metabolic circuits in humans. Our study aims to identify novel pathways in the pathogenesis of obesity, T2DM and NAFLD, taking the gut–brain axis into account as well, which may be targets for drug development. Finally, we will follow the

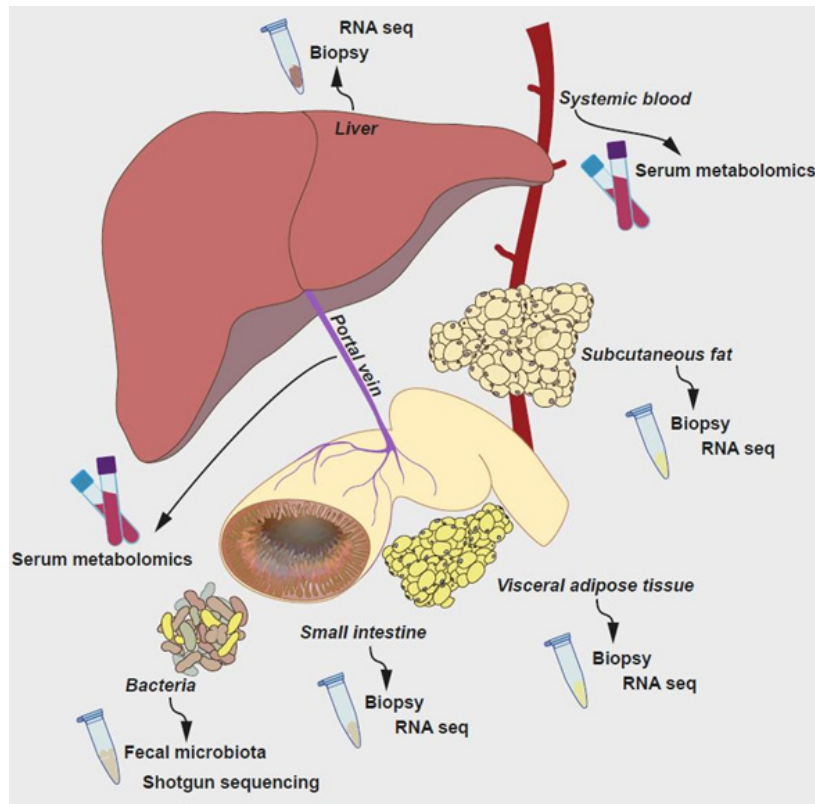


Fig. 1 A systems biology approach, identifying gut microbial, immunological and metabolic markers in a large and well-phenotyped bariatric surgery cohort: the BARIA study.

patients prospectively in an attempt to identify mechanisms affecting the surgical outcome.

Methods

Study design

We include subjects that are patients with morbid obesity scheduled for bariatric surgery. From September 2016 until the end of 2018, the study was performed at the former MC Slotervaart (Amsterdam) and is now continued, after closure of that hospital, by the same surgical group and research team at the Spaarne Gasthuis hospital (Hoofddorp) in the Netherlands. The study protocols were approved by the Ethical Review Board of the Academic Medical Center, Amsterdam, (approval code: NL55755.018.15), and all patients that have been (and will be) included provided informed consent. Preoperative screening, surgery and follow-up are performed following institutional procedure protocols. All patients are screened preoperatively by a bariatric surgeon, an internist, a dietician and a

psychologist. We aim to include predominantly LRYGB procedures. In a shared decision-making process, surgeon and patient decide for the bariatric procedure type: LRYGB, laparoscopic omega-loop gastric bypass (LOGB) or laparoscopic sleeve gastrectomy (LSG), which, in our bariatric surgery centre, has resulted in more than 90% LRYGB of all surgeries in the past ten years. All LRYGB procedures are standardized, with approximated measurements of 4 × 8 cm gastric pouch, 50 cm biliopancreatic limb, 150 cm alimentary limb [24]. The LOGB is made with a longer gastric pouch and a longer biliopancreatic limb of approximately 200 cm. The LSG is calibrated with a 34 Charrière bougie with the staple line starting at approximately 2 cm from the pylorus.

Study population

Patients are screened at the outpatient clinic (MC Slotervaart hospital, Spaarne Gasthuis hospital) after being approved for bariatric surgery. Screening started in September 2016. We aim to include 1500

patients. Subjects are considered eligible for participation if they meet following criteria:

Inclusion

- Male and female patients scheduled for primary bariatric surgery recruited from an experienced Dutch bariatric surgery clinic.
- Body mass index (BMI) $\geq 40 \text{ kg m}^{-2}$, or: BMI $\geq 35 \text{ kg m}^{-2}$ with obesity-related comorbidity.
- Recent history of supervised attempts to lose weight.
- Age 18 to 65 years.
- Ability to provide informed consent.

Exclusion

- Primary lipid disorder.
- Known genetic basis for insulin resistance or glucose intolerance.
- Psychiatric conditions.
- Coagulation disorders (patient reported or prolonged prothrombin time or activated partial thromboplastin time).
- Uncontrolled hypertension (blood pressure $>150/95 \text{ mmHg}$).
- Renal insufficiency (creatinine $>150 \mu\text{mol L}^{-1}$).
- Excessive alcohol intake (>14 units/week, patient reported).
- Pregnancy, breastfeeding.

Outcome measures

For the characterization of subjects before surgery, we have chosen variables that are linked to obesity and obesity-associated diseases. For clinical follow-up, we chose variables that can be tested minimally invasive (only venepuncture) and which can be easily reproduced, at low cost, without extensive training in a Western hospital. The reason for this is twofold. First, we aimed to minimize the demand of our study subjects. Secondly, our results need to be reproducible and applicable in other settings without the need for major investments in equipment or logistics. That way our project can benefit the greatest number of people whilst still remain ambitious in aiming to discover new mechanisms.

The included patients undergo the repetitive measurements detailed in Table 1. For the physicians and researchers, we made a standard operating procedure. The psychological measures were assessed with Dutch versions of validated questionnaires, presented in Table 2. Tissue biopsies are obtained during operation of three adipose

tissue compartments: subcutaneous (from one of the laparoscopic incisions in the upper abdomen), greater omentum and visceral fat (omental appendices of the transverse colon); from the diaphragmatic surface of segment three or five of the liver; and from the jejunum at the site of the jejunojejunostomy, approximately 50 cm from the Treitz ligament. The jejunum biopsy cannot be obtained during LOGB or LSG, as those operation techniques, unlike LRYGB, do not involve a jejunojejunostomy. Blood sample of the portal vein is taken at the beginning of the surgery, only if considered safe by the surgeon, mainly depending on the amount of fatty tissue surrounding the hepatoduodenal ligament. Biopsies are assessed for histology (paraffin embedded), gene regulation (RNA-sequencing) and protein expression (immunoblotting). NAFLD status is determined in histology of liver biopsies and individually scored by members of Dutch Liver Pathology Panel, after training sessions, whilst difficult or borderline cases are discussed during panel meetings for consensus. SAF scores are determined, separately assessing steatosis (S), activity (A, the sum of hepatocyte ballooning and lobular inflammation), and fibrosis (F) [25]. From the beginning of 2019, we added routine preoperative ultrasound of the gallbladder. Hollow needle subcutaneous fat aspirate biopsy under local anaesthesia (peri-umbilical region) is optional at follow-up. Of note, the tissues collected during surgery comprise tissue that is thought to play a crucial role in glucose metabolism and can be biopsied with minimal risk to the patient being small intestine, adipose tissue and liver samples. We assess all liver biopsies for NAFLD/NASH, as it is the gold standard for diagnosing liver disease.

Plasma metabolites are studied in portal vein blood (fasted) and in both fasted and two hours after mixed meal test (MMT) peripheral blood samples. Intestinal immunological cells are looked for in GALT tissue (Peyer's patches), visceral and subcutaneous adipose tissue, liver in relation to inflammation gene expression (IL-1 β , IL-6, IL-8, IL-18, CXCR2 TNF- α and TLR 1, 2, 4, 5 and 6 and IRX 3 and 5 and RNA-sequencing) and in specific innate lymphoid cells (ILC), macrophages, T/B-cells and dendritic cells and peripheral blood. Immunological parameters assessed in small-intestinal tissue and adipose tissue were selected for those that are linked in literature to have an effect on glucose metabolism and with which we have experience in the analysis. Morning faecal samples obtained at several time points will be analysed by shotgun

Table 1. Overview of visits and measurements. *BARIA longitudinal cohort study*

Visit	Type of measurement	Specific values	Biological samples stored in biobank
Baseline	Demographic	Age, sex, medical history, medication use,	
1 year		history of obesity, history of smoking and	
2 years		alcohol, education level, employment status,	
5 years*		anticonception use, physical activity	
10 years*	Biometric	height, weight, waist and hip circumference,	
		temperature, blood pressure, pulse, non-	
		invasive haemodynamics (stroke volume,	
		cardiac output, systemic vascular	
		resistance), bioelectrical impedance	
		measurement, electrocardiogram	
	Blood	Haemoglobin, CRP, leucocytes, platelets,	Stored plasma and DNA
		HbA1c, glucose, electrolytes, kidney function,	samples (−80°C)
		lipid profile, iron, hepatic enzymes, thyroid	
		profile, plasma metabolites	
	Mixed meal test	Glucose, insulin, triglycerides	Stored plasma samples
			(−80°C)
	Dietary questionnaire	Satiety (visual analogue scale)[42], dietary	
		intake last 3 days prior to 24 h faeces	
		collection	
	Psychological questionnaire	See Table 2.	
	Morning faecal samples	Gut microbiota composition and faecal	Stored samples (−80°C)
	24 h faeces	metabolites (scfa), bile acids and caloric	
		bomb	
	Gingival swab	Oral microbiota	Stored samples (−80°C)
	Urine	Albumin and creatinine, metabolites	Stored samples (−80°C)
Primary operation	Liver biopsy	Snap frozen (liquid N ₂) and formaldehyde	Stored samples (−80°C) and
			paraffin
Re-surgery	Subcutaneous adipose tissue	Snap frozen (liquid N ₂) and formaldehyde	Stored samples (−80°C) and
			paraffin
	Visceral adipose tissue	Snap frozen (liquid N ₂) and formaldehyde	Stored samples (−80°C) and
			paraffin
	Omental adipose tissue	Snap frozen (liquid N ₂) and formaldehyde	Stored samples (−80°C) and
			paraffin
	Portal vein blood (subset)	Plasma metabolites and proteomics	Stored plasma samples
			(−80°C)
	Small intestine biopsy (LRYGB only)	Snap frozen (liquid N ₂) and formaldehyde	Stored samples (−80°C) and
			paraffin

Table 1 (Continued)

Visit	Type of measurement	Specific values	Biological samples stored in biobank
6 weeks	Biometric	Weight, waist and hip circumference, blood pressure and pulse	
6 months	Blood	Haemoglobin, CRP, leucocytes, platelets, HbA1c, glucose, electrolytes, kidney function, lipid profile, iron, hepatic enzymes, thyroid profile, plasma metabolites	Stored plasma samples (−80°C)
2 weeks	Morning faeces	Gut microbiota composition and faecal metabolites (scfa)	Stored samples (−80°C)
6 weeks			
6 months	Urine	Albumin and creatinine, metabolites	Stored samples (−80°C)

*At 5 and 10 years, no mixed meal test will be performed.

sequencing (NovaSeq). Buffycoat samples of peripheral blood are taken at baseline for genomic DNA analyses. Cardiac output and peripheral resistance are assessed using the Nexfin system, measuring blood pressure beat-to-beat with a small cuff around the index finger [26].

In the case of a non-acute operation more than one month after primary surgery, for example for laparoscopic cholecystectomy, new liver and adipose tissue biopsies can be obtained, as well as gallbladder and bile from cholecystectomy patients. Gallbladder tissue will be assessed for bile acid composition, histology, gene expression (RNA-sequencing) and protein expression.

The two-hour seven-sample oral MMT, as described by Dalla Man *et al.*, is repeated several times over 2 years follow-up [27]. It consists of two Nutridrink compact 125 mL (Nutricia®), containing 23.3 grams fat, 74.3 grams carbohydrates (of which 38.5 grams sugar) and 24.0 grams protein. The patients receive this meal after fasting for a minimum of nine hours. Time point zero is the moment the patient fully consumed the meal. Blood samples are drawn via intravenous line at baseline, 10, 20, 30, 60, 90 and 120 min and analysed for insulin sensitivity / insulin resistance, plasma metabolites and bile acids.

Data handling and analysis

Data are collected on data collecting forms and entered after validation in a computer system for subsequent tabulation and statistical analysis. All research and medical data are kept strictly

confidential and registered under a unique study code. Only the researchers that are involved in this study are able to see the data and to identify a participant. Study material will be stored for a period of 20 years after study completion. Data from the first approximately 100 patients are analysed to check data quality and logistics (first data-freeze). A first interim analysis will be performed on data of the first approximately 300 patients, and the primary analysis will be performed on data of 500 patients (second and third data-freeze). We intend to continue inclusions till 1500 for additional analyses and validation of primary findings. The data are analysed using a range of different techniques, including being used as input for metabolic modelling and for phenotyping the patients using machine learning algorithms.

Study integrity, monitoring, safety

The BARIA study is conducted according to the principles of the Declaration of Helsinki (October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). All adverse events reported by the patients or observed by the investigator or staff will be recorded. All adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow-up may require additional tests or medical procedures as indicated.

Validation of the mixed meal test

Next to the elaborate analysis of data focussing on the aims of the BARIA study, we used the results of

Table 2. Psychological questionnaires. BARIA longitudinal cohort study

Questionnaire	No
Sociodemographic information: place of birth patient, father, mother; number of children; marital status; education; occupation.	7
Quality of life (WHO HIV QOL)	2
Change in life	1
Professional support	5
Self-management after Bariatric surgery (BSSQ)	8
TFEQ- hunger scale	9
Center for epidemiology studies depression scale revised (CES-D)	20
Impact of weight on quality of life (IWQOL-Lite)	31
Body image scale	10
De Jong-Gierveld loneliness scale	11
Social participation scale	3
SCI exercise self-efficacy	10
Stanford exercise behaviour	6
Weight efficacy lifestyle questionnaire (WEL-Q)	20
G-food craving questionnaire-trait (FCQ-T)	21
Quality of relationship and relationship ladder	2
Experience in close relationships scale (ECRR-SF)	16
Social support (SSQSR)	12
Social support and diet	10
Social support and exercise	13
Personality NEO-FFI (neuroticism and conscientiousness subscales)	12+12
Self-compassion scale short form	12
Rosenberg self-esteem questionnaire	10
Chronotype working day	8
Chronotype free day	8

the preoperative MMT of the patients included and operated in the first two years of the study to validate the reproducibility of the MMT-stimulated postprandial glucose, triglycerides and insulin curves. We therefore stratified these results by classifications of glycaemic control as formulated in the American Diabetes Association (ADA) criteria: normoglycemia (fasting glucose (FG) <100 mg dL⁻¹; <5.6 mmol L⁻¹), impaired FG (100–125 mg dL⁻¹; 5.6–6.9 mmol L⁻¹) and / or increased haemoglobin A1c (5.7–6.4%; 39–47 mmol mol⁻¹) and diabetes

mellitus (FG ≥ 126 mg dL⁻¹; ≥ 7.0 mmol L⁻¹) [28]. Of all measurements during MMT in these patients, there were 2.1% missing values for glucose, 5.5% for insulin and 1.8% for triglycerides. We repeated the preoperative MMT after one week in ten randomly selected patients. Of all repeat measurements of those ten patients, there were 2.9% missing values for glucose, 5.7% for insulin and none for triglycerides. For validating the MMT, imputation of predictive mean matching was performed for all missing values.

Results

Inclusion of patients in the BARIA study began in September 2016. During the first two years of the BARIA study, portal vein sampling was performed in 32% of the surgeries. Types of procedure were 94% LRYGB, 6% LOGB and no LSG. No serious adverse events occurred. Baseline characteristics and MMT results of the first 170 patients included in this two-year period are presented in Table 3. MMT curves of ten patients assigned to the category diabetes mellitus were excluded because of insulin use. Results of the preoperative MMT of the remaining 160 patients are presented in Fig. 2. Individuals with different classifications of glycaemic control showed markedly different profiles for MMT-stimulated plasma insulin, glucose and triglycerides. Triglycerides were clearly higher at baseline and all following time points in patients with IFG, with or without increased Hba1c. HOMA2-IR and HOMA2-B values and correlations with postprandial glucose and insulin curves are presented in Fig. 3. The HOMA2-IR and HOMA2-B values showed a good correlation with the AUC postprandial insulin, but not with the AUC postprandial glucose.

Results of the ten patients that underwent repeated (1-week interval) preoperative MMT are presented in Fig. 4. We found a good coefficient of variance (figure 4, blue lines) with a mean average of difference between two MMT measurements of 6.3% for area under the curve (AUC) postprandial glucose, 13.9% for AUC postprandial insulin and 7.4% for AUC postprandial triglycerides, whilst most of the differences between the two measurements were well within the 20% range of the average mean difference underscoring reasonably good intraindividual reproducibility.

Discussion

The BARIA cohort study will generate a large phenomic database on the systems biology of

subjects with morbid obesity, both before and after bariatric surgery. Advanced data science, including application of machine learning and artificial neural networks data analysis is used to select microbiome-produced metabolites and identify their receptors in target tissue. It will be the first large bariatric cohort study to include portal vein blood sampling in a considerable subset of patients for untargeted metabolites, which, when also studying peripheral metabolites, will enable to study the gradient of metabolites filtered by the liver. We aim to include 1500 patients undergoing primary laparoscopic bariatric surgery (gastric bypass or sleeve gastrectomy). Before surgery, they are subjected to MMT, blood and faecal sampling, and questionnaires, including psychology and VAS lists taken at the start of the MMT in all patients at all time-points to minimize variation. During surgery, biopsies are obtained from three fat depots, jejunum, liver and samples from portal

and peripheral venous blood. Thereafter, further sampling (MMT, blood and faecal samples) is performed. In the event of another surgery (revisory surgery, cholecystectomy) further biopsies can be obtained, which is included in the ethical protocol. We process tissues for RNA-sequencing, analyse intestinal microbiota and perform untargeted (postprandial) plasma metabolomics on both fasting and postprandial (MMT) plasma samples. These metabolites will be investigated further *in vitro* and *in vivo* to determine causality and identify receptors. After the primary analysis, the generated database will also allow for additional secondary analyses.

The bariatric patient scheduled for primary bariatric surgery is an interesting model for several reasons. All patients suffer from morbid obesity and generally expect to undergo examinations, measurements and interviews both prior to surgery

Table 3. Baseline characteristics and results of mixed meal test in 170 participants in the first two years of inclusion in the BARIA longitudinal cohort study, stratified by glycaemic classification, as formulated in the American Diabetes Association criteria: normoglycemic (Healthy), impaired fasting glucose (IFG), increased haemoglobin A1c (IHbA1c), combination of IFG and IHbA1c (Comb) and type 2 diabetes mellitus (T2DM). Categorical variables are displayed as absolute numbers (percentage), continuous variables as means (SD).

	Healthy	IFG	IHbA1c	Comb	T2DM
<i>n</i>	57	21	19	26	47
Age (years)	41.4 (11.1)	46.8 (11.7)	44.6 (9.5)	49.2 (9.2)	49.5 (10.2)
Sex (female)	45 (78.9)	20 (95.2)	17 (89.5)	16 (61.5)	31 (66.0)
BMI	39.5 (3.9)	39.4 (3.1)	40.6 (7.1)	40.6 (3.6)	39.2 (4.5)
Hypertension	8 (14.0)	5 (23.8)	3 (15.8)	8 (30.8)	25 (53.2)
Systolic BP (mmHg)	129.5 (16.6)	130.6 (13.6)	134.2 (15.8)	133.2 (12.0)	132.1 (13.7)
Diastolic BP (mmHg)	80.1 (11.3)	80.5 (8.2)	78.1 (13.2)	84.0 (7.9)	82.6 (9.4)
Insulin use					10 (21.3)
Glucose (mmol L ⁻¹)	5.1 (0.4)	5.9 (0.2)	5.2 (0.2)	6.1 (0.4)	7.4 (1.5)
Insulin (pmol L ⁻¹)	84.8 (48.0)	89.4 (46.5)	79.2 (37.2)	111.2 (46.9)	180.2 (222.5)
HbA1c (%)	5.31 (0.23)	5.41 (0.19)	5.79 (0.09)	5.88 (0.17)	7.10 (1.14)
HOMA2 IR	1.60 (0.90)	1.71 (0.83)	1.48 (0.67)	2.14 (0.85)	2.44 (1.24)
HOMA2 Beta (%)	125.4 (50.9)	98.1 (37.2)	112.6 (33.9)	105.8 (38.7)	87.3 (37.2)
AUC glucose (mmol L ⁻¹)	137.1 (109.5)	122.5 (85.9)	194.6 (112.9)	211.7 (105.0)	386.3 (193.7)
AUC insulin (mmol L ⁻¹)	42.3 (30.4)	46.0 (29.4)	48.7 (21.4)	50.8 (20.8)	37.6 (31.5)
eGFR (MDRD mL min ⁻¹ 1.73 m ⁻²)	94.5 (18.0)	92.7 (19.8)	95.6 (21.7)	94.7 (19.7)	95.7 (17.6)
ASAT (U L ⁻¹)	23.6 (4.9)	23.5 (6.5)	25.1 (5.5)	25.3 (4.9)	29.9 (14.0)
ALAT (U L ⁻¹)	28.6 (13.4)	28.3 (14.7)	33.7 (18.5)	30.4 (10.1)	42.1 (25.8)
Cholesterol (mmol L ⁻¹)	4.6 (1.0)	5.1 (1.2)	5.2 (1.0)	4.8 (1.1)	4.1 (0.9)
HDLc (mmol L ⁻¹)	1.12 (0.29)	1.13 (0.23)	1.16 (0.16)	1.08 (0.29)	1.05 (0.23)
Triglycerides (mmol L ⁻¹)	1.08 (0.44)	1.58 (0.91)	1.10 (0.42)	1.79 (1.17)	1.40 (0.62)

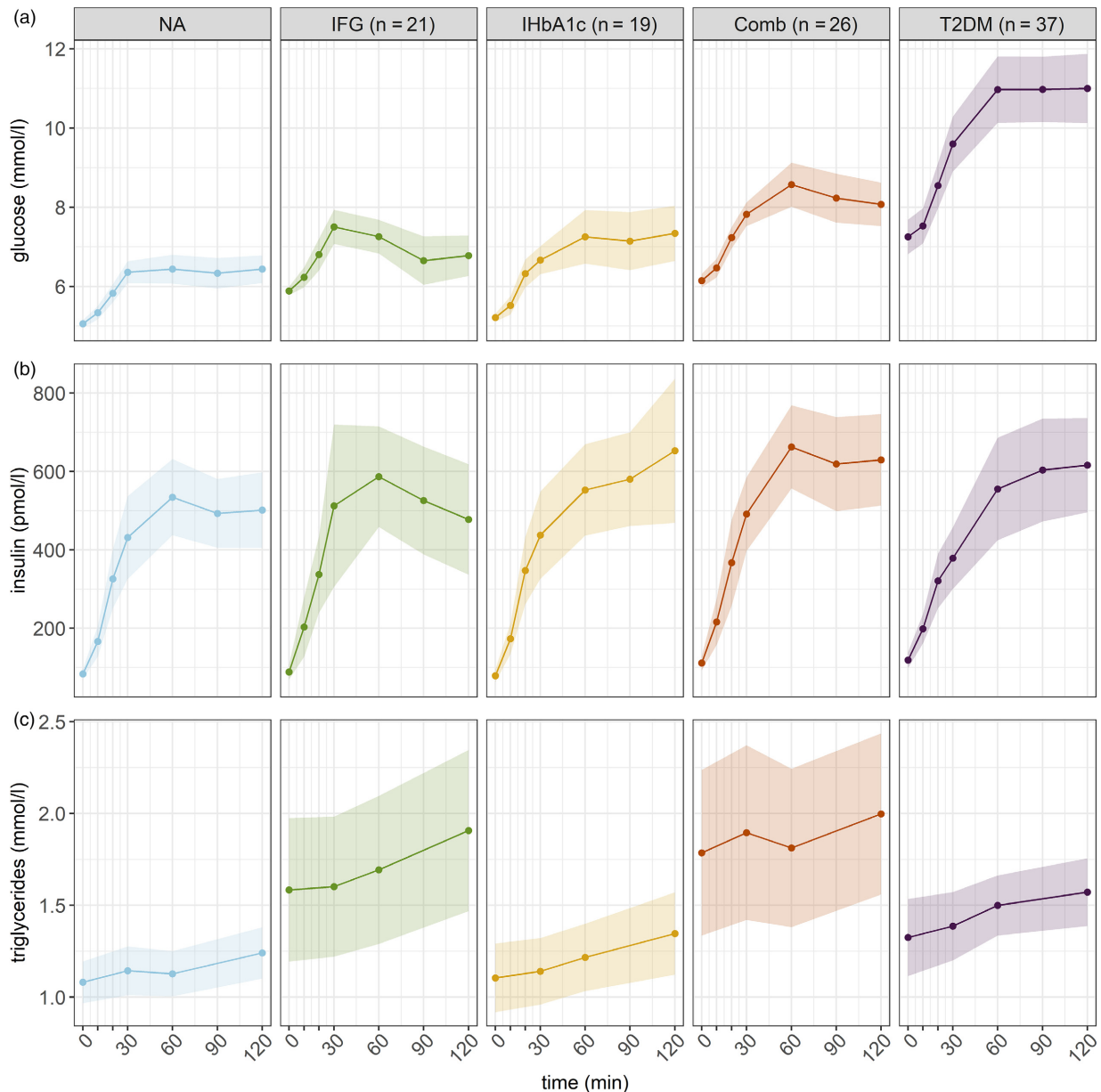


Fig. 2 Glucose, insulin and triglycerides measurements during 2-hour 7-sample mixed meal test, stratified by glycaemic classification, as formulated in the American Diabetes Association criteria: normoglycemic (Healthy), impaired fasting glucose (IFG), increased haemoglobin A1c (IHbA1c), combination of IFG and IHbA1c (Comb) and type 2 diabetes mellitus (T2DM). Values are presented as means with 95% confidence intervals. (a) glucose curves; (b) insulin curves; (c) triglycerides curves.

and in follow-up. The laparoscopic procedures give proper access to different adipose compartments, as well as liver and intestine for biopsy and, if the hepatoduodenal ligament is not too much

embedded in fatty tissue, to the portal vein for fine needle blood sample as well. Any haemorrhages can readily be detected and addressed surgically, minimizing the expected adverse events. In the

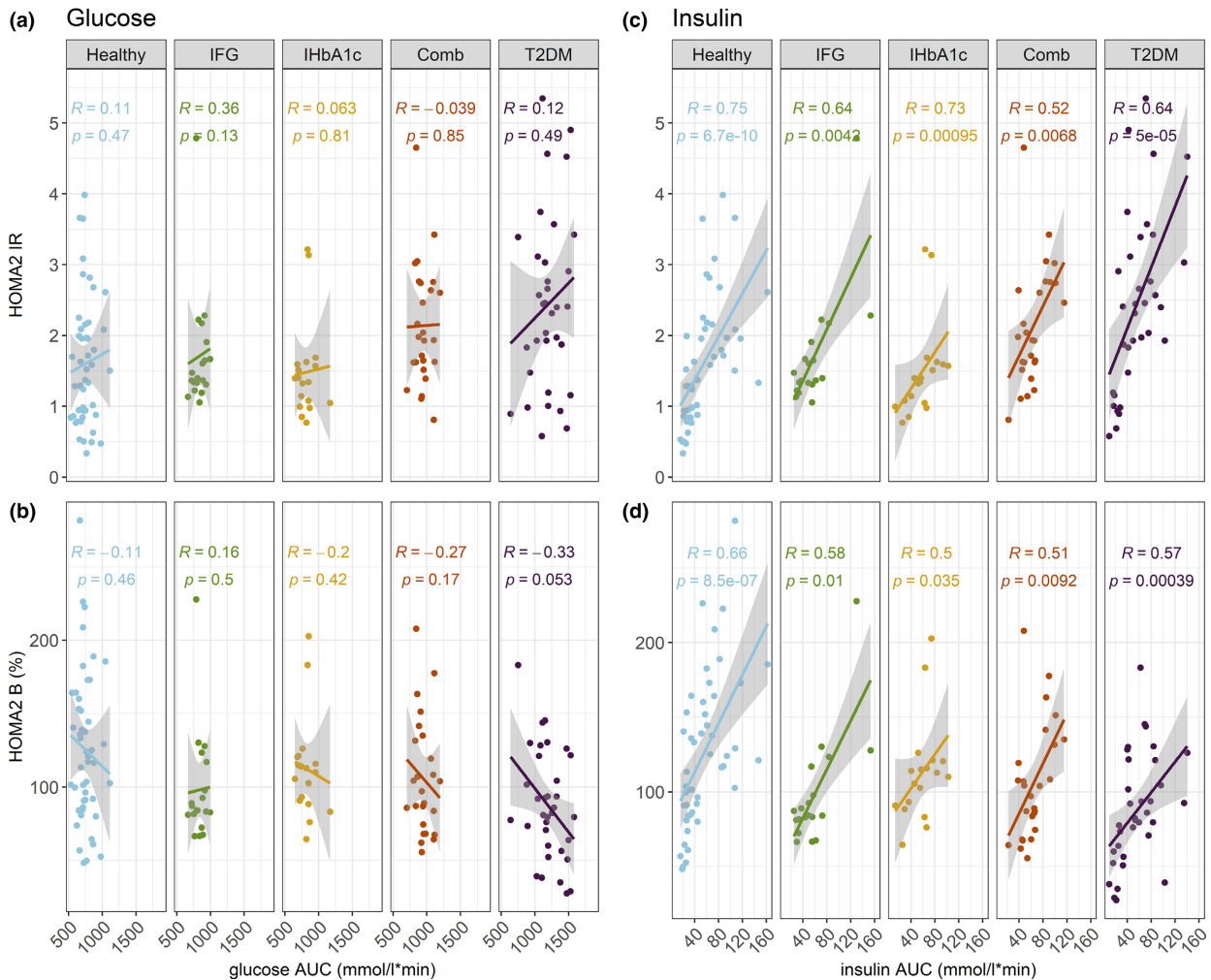


Fig. 3 Area under the curve (AUC) of insulin and glucose during mixed meal test and HOMA2 insulin resistance (IR) and beta cell function (B), stratified by glycaemic classification, as formulated in the American Diabetes Association criteria: normoglycemic (Healthy), impaired fasting glucose (IFG), increased haemoglobin A1c (IHbA1c), combination of IFG and IHbA1c (Comb) and type 2 diabetes mellitus (T2DM). Points are individual values, solid line represents linear regression, banded area is 95% confidence interval. (a) Glucose AUC and HOMA2 IR. (b) Glucose AUC and HOMA2 B (%). (c) Insulin AUC and HOMA2 IR. (d) Insulin AUC and HOMA2 B. Correlation coefficient (R) and P-values calculated with Spearman's rank correlation test.

hands of our surgical team, mortality of routine LRYGB is low (0.03%) and two-year follow-up is high (71%) [24]. During the first two years of inclusion, portal vein sampling could be performed safely in about one out of three cases. Other studies with similar bariatric surgery cohorts with invasive assessments showed that a majority of patients remains interested in participating during two years of follow-up [29]. Furthermore, up to 10% of bariatric surgery patients need additional

surgery within two years after primary procedure (for example revision surgery or cholecystectomy), which opens up the possibility for renewed biopsies and blood sampling [30].

However, studying bariatric patients has some limitations intrinsic to the surgical procedure. Biopsies and portal vein blood are taken under general anaesthesia and therefore potentially influenced by anaesthesia medication. For example,

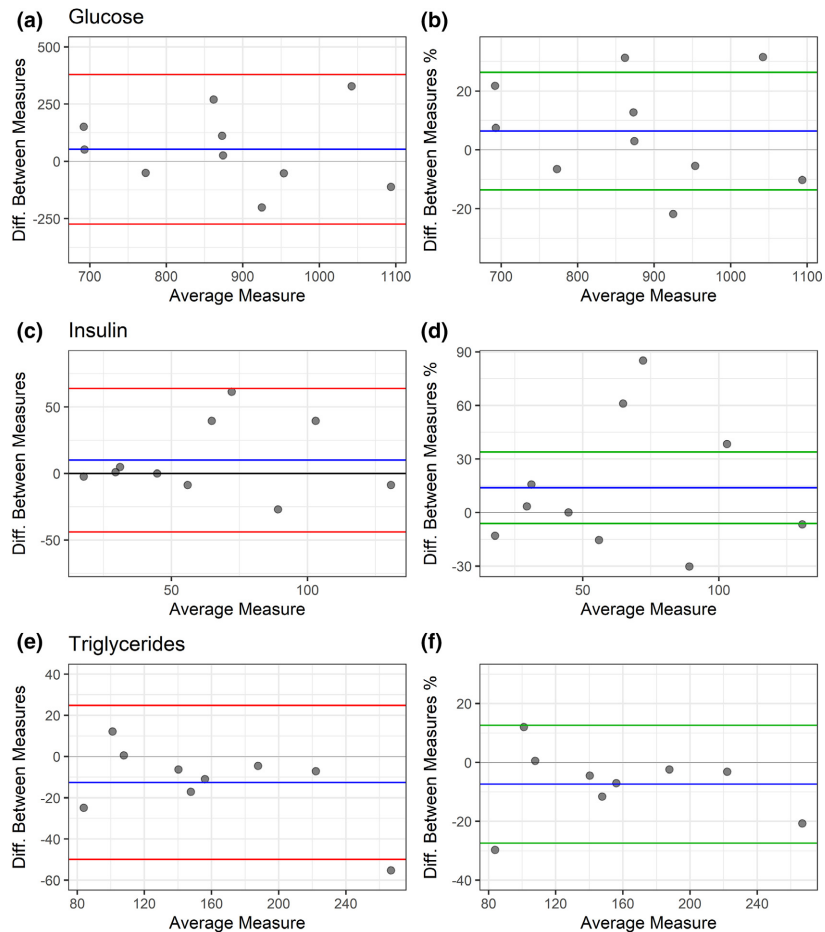


Fig. 4 Reproducibility of mixed meal test (MMT). Bland Altman plots of MMT (repeated within 1 week) for glucose, insulin and triglycerides. Blue line is mean of difference between measurements, red line is $\pm 1.96 \times \text{SD}$ of mean difference, and green line is $\pm 20\%$ of mean difference. (a) Glucose area under the curve (AUC) in $\text{mmol L}^{-1} \times \text{time}$. (b) Glucose AUC per cent change. (c) Insulin AUC in $\text{mmol L}^{-1} \times \text{time}$. (d) Insulin AUC per cent change. (e) Triglycerides AUC in $\text{mmol L}^{-1} \times \text{time}$. (f) Triglycerides AUC per cent change.

these drugs will be found in portal vein plasma and might accumulate in fatty tissues during surgery and, most importantly, will be metabolized by the liver. Furthermore, patients are routinely urged by their bariatric surgeon to lose as much weight as possible before the operation to reduce the surgical risk. It can be expected that such forced weight loss will influence metabolism, gene expression and gut microbiota. Although no standardized diet is prescribed, we nevertheless choose to exclude those patients that lose more than 5% in six months (or more than 3% in one month) prior to surgery. Another limitation is the fact that many patients using medication for obesity-related diseases will need less or even no medication after bariatric

surgery, which might be a confounder for outcome measurements.

In a separate analysis of the MMT results in a subset of included patients, we showed that the preoperative MMT has a good intraindividual reproducibility, which makes it a better estimate for glycaemic regulation than the oral glucose tolerance test [31]. We also showed that the MMT is able to represent the underlying metabolic dysregulation well, evident in the different curves and the steady state model assessment. The differences observed in the curves correspond well with the pathophysiology. First, impaired fasting glucose (IFG) is consistent with hepatic insulin

resistance as is evident, apart from the increased glucose, by increased baseline insulin and a decreased suppression of apo B production, resulting in increased triglycerides. An initial quick rise in glucose is followed by a steady decline of both glucose and insulin, as peripheral insulin resistance remains largely normal [32]. Second, the increased haemoglobin A1c (IHbA1c) group corresponds with peripheral insulin resistance, represented by a steady increase until the 2-hour time point of both glucose and insulin with relatively normal triglyceride levels. Finally, the group with a combination of IHbA1c and IFG (Comb) and the T2DM group show both characteristics, with the T2DM group reaching higher glucose levels. The HOMA2-IR and HOMA2-B values showed a good correlation with the AUC postprandial insulin, but not with the AUC postprandial glucose, which reiterates the suggestion that they are used best in combination with other clinical parameters [33].

With regard to the use of the MMT in postoperative follow-up, it must be noted that the anatomical changes affecting gastric emptying and resorption might impede the comparison of the MMT before and after surgery. However, the MMT is biologically a more relevant test than the glucose tolerance test, as one is rarely solely exposed to glucose without fat and proteins. Studies with a similar follow-up using intravenous glucose tolerance test and euglycaemic-hyperinsulinaemic clamp showed an improvement in insulin sensitivity in all patients, with least improvement for TDM2 patients [34, 35]. One other study assessing meal response after a follow-up of more than one year was cross-sectional, but with smaller numbers [36]. Outcome of the MMT in our BARIA study can provide further insight in the metabolic response following a meal after bariatric surgery. Another limitation of the MMT in bariatric patients is that the test can provoke early dumping, a well-known side effect of LRYGB and LOGB due to loss of pyloric regulation, which makes a heavy caloric MMT hard to endure for some patients in the first years of their follow-up.

We believe that the different subclasses of T2D are different paths of progression to the disease, with, in some individuals, a simultaneous existence of several pathways [2]. The underlying molecular mechanisms that lead to these different trajectories are probably different. Similarly, the reversibility and the therapeutic intervention that has the

greatest effect on their progression may vary. To the best of our knowledge, there are no successful therapeutic modalities specifically aimed at targeting short-chain fatty acids (SCFAs), bile acids, amino acid-derived metabolites, neural pathways and lymphoid cells with the aim of improving glucose metabolism. There have been several trials using specific SCFA as supplements to improve glucose metabolism and weight loss [37, 38]. The effects of the intervention in these studies as well as in faecal microbiota transplantation studies are usually limited with only few showing great improvement [7] where other groups found less efficacy of donor FMT (but were also using different FMT applications), but did observe the similar relation between FMT efficacy and decreased faecal microbiota diversity at baseline [39]. A better understanding of which molecular mechanisms need to be targeted in which patients will lead to a better personalized treatment.

With the comprehensive systems approach of the BARIA longitudinal cohort study, we aim to provide more understanding in to how the (small) intestinal microbiota affects our metabolism, especially with regard to NAFLD and T2DM. Moreover, we aim to identify leads that drive weight loss and psychological improvement upon surgery, thus identifying the causal factors connecting beneficial changes in metabolism, microbiota and immunological tone that will be of value to find new diagnostic and therapeutic leads to control the obesity-associated disease epidemic.

Lessons learned so far

During our study, we encountered a few learning points, which, we hope, future researches planning similar research can benefit from and not run into the same problems. We based the feasibility of our protocol on previous studies detailing MMT after RYGB surgery [40, 41]. In our study so far, a relatively large number (38 out of 134 participants) of participants exhibited adverse effects during the MMT at the one year after bariatric surgery (nausea, diarrhoea, dizziness and weakness). We suspect these adverse effects to be related to dumping syndrome. The symptoms were not of a severity that we found a need for extra diagnostic tests. None of the subjects experienced loss of consciousness, and there was no need for extended stay in the hospital beyond the normal testing time. Another valuable learning point was related to subject follow-up. In order to achieve a dropout rate of <20%, extensive

contact with participants had to be maintained. Many participants needed to be contacted via telephone several times, for reminders to schedule every visit. The amount of manpower and time necessary for that was greater than we anticipated.

At the 6-week and 6-month collection time-points, we collect blood for fasting glucose measurement, as well as anthropometric measurements and changes in medication. Our initial aim was to also collect blood at the 2-week time-point. During our try-out phase, we discovered that having these measurements was too demanding for our patients during this initial recovery period at 2 weeks. Nutrition questionnaires were also reported as stressful by our patients, and we have chosen to only include these in the large (1 and 2 year) collection time-points.

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Conflicts of interest

MN is in the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands and Kaleido Bioscience, USA; FB is in the Scientific Advisory Board of MetaboGen AB, Sweden. None of these are directly relevant to the current paper. There are no patents, products in development or marketed products to declare. The other authors declare no conflicts of interests.

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