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ANGPTL8 as a new determinant of type 2 diabetes remission after bariatric surgery

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Short running title: ANGPTL8 predicts T2D remission

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Abbreviations

ANGPTL8: Angiopoietin-like protein 8

SAT: Subcutaneous adipose Tissue

VAT: Visceral adipose Tissue

WAT: White adipose tissue

T2DM: Type 2 diabetes mellitus

ANGPTL3: Angiopoietin-like protein 3

TG: Triglycerides

HDL: high density lipoprotein

LDL: low density lipoprotein

RYGB: Roux-en-Y gastric bypass surgery

SG: Sleeve gastrectomy

LGCP: Laparoscopic greater curvature plication

BMI: Body Mass Index

HOMA-IR: Insulin Resistance Homeostatic Model Assessment

EWL: Excess weight loss

WL: Total weight loss

AWL: Alterable weight loss

WHR: Waist-hip ratio

ABSTRACT

This work aimed to explore the link between ANGPTL8 and weight loss after metabolic surgery. In the cross-sectional study (n=100), circulating ANGPTL8 concentrations were significantly lower in morbidly obese than in lean subjects, and strikingly lower in morbidly obese patients with type 2 diabetes mellitus (T2DM). Conversely, ANGPTL8 expression in subcutaneous adipose tissue (SAT) was higher in morbidly obese patients, particularly in those with T2DM, whereas its expression in visceral adipose tissue (VAT) was unchanged. The main predictors for circulating levels of ANGPTL8 were BMI and T2DM, whereas ANGPTL8 expression in SAT was determined by the presence of T2DM. The prospective cohort studies before and one year after bariatric surgery in morbidly obese patients with (n=45) and without (n=30) T2DM, revealed a significant increase of circulating ANGPTL8 levels one year after bariatric surgery. Intriguingly, this increment, which was predicted by basal ANGPTL8 concentrations, appeared as a determinant of T2DM remission. In conclusion, circulating ANGPTL8 levels have an inverse relationship with SAT expression. Low basal levels of ANGPTL8 rebound after bariatric surgery. The increment in ANGPTL8 concentrations at one month of follow-up after weight loss emerged as a significant predictor of the T2DM remission at one year of follow-up.

INTRODUCTION

Angiopoietin-like protein 8 (ANGPTL8; also known under a number of different aliases [1-5]) is a secreted protein initially reported to be highly expressed in liver, but is also widely distributed in a variety of tissues. The finding that ANGPTL8 promoted β-cell proliferation and expansion of the β-cell mass suggested that it may be a promising agent in the treatment of metabolic syndrome and type 2 diabetes mellitus (T2DM) [4]; however, follow-up studies have questioned its role in β-cell function [3, 6, 7]. Consequently, its physiological function is unclear and its molecular targets remain largely unknown [8, 9]. Circulating levels of ANGPTL8 is associated with intrinsic energy intake and expenditure, and in addition to its potential importance in glucose homeostasis, a role in lipid metabolism has been demonstrated [1, 2, 10]. The interaction of ANGPTL8 with angiopoietin-like protein 3 (ANGPTL3) promotes inhibition of lipoprotein lipase activity and ultimately modulates serum levels of triglycerides (TG), high-density lipoprotein cholesterol (HDL-cholesterol) and low-density lipoprotein cholesterol (LDL-cholesterol) [1, 11].

There are conflicting reports regarding the concentration of circulating ANGPTL8 in obesity or T2DM [12-16]. Moreover, results obtained on the relationship between ANGPTL8 and lipid profile or insulin sensitivity are ambiguous and inconclusive. One animal model study reported that ANGPTL8 does not have a role in glucose metabolism [3], although a recently published study points to a potential role for this protein in the amelioration of insulin resistance by increasing the insulin-mediated phosphorylation of several components of the insulin-signaling cascade [17]. Differences in the degree of insulin-resistance as well as different methodological approaches used to detect ANGTPL8 [18] have been claimed as possible explanations for the discrepancies

between previous studies that have measured circulating concentrations of ANGTPL8 in patients with obesity or T2DM.

In addition to the liver, white adipose tissue (WAT) is a substantial source of ANGPTL8. Studies in murine models of obesity suggest that ANGPLT8 is exclusively expressed in mature adipocytes [2]; however, data on human WAT are scarce. A previous study suggested that *ANGPTL8* expression in human adipose tissue (AT) is induced by insulin, but also reported a paradoxical decrease in circulating plasma concentrations of ANGPTL8 during insulin infusion in a euglycemic-hyperinsulinemic clamp study [19]. Few studies have explored the regulation of ANGPTL8 in the context of metabolic disease and, to date, ANGPTL8 expression in AT in obese or T2D individuals and its regulation after bariatric surgery in T2D patients is not well understood.

In the current study, we compared circulating ANGPTL8 levels in lean and obese patients, with and without T2DM. We also investigated changes in ANGPTL8 levels in a prospective manner following bariatric surgery, the intervention currently considered as the most effective treatment for morbid obesity and obesity-associated T2DM [20, 21]. We analyzed the impact of surgery on ANGPTL8 concentrations in patients with morbid obesity, both with and without T2DM at the time of admission. In addition, we explored WAT expression of ANGPTL8 and its relationship with circulating levels in lean and morbidly obese individuals.

MATERIAL AND METHODS

STUDY DESIGN AND PATIENTS

The study comprised three different clinical sub-studies: 1) a cross-sectional study with lean and morbidly obese patients, with and without T2DM, undergoing bariatric surgery; 2) a prospective randomized controlled trial of patients with T2DM undergoing metabolic surgery and 3) a confirmatory prospective study with morbidly obese patients undergoing bariatric surgery without T2DM. All studies were conducted in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Cross-sectional study - cohort 1:

Design: Observational single point study.

Participants: One hundred subjects (60 women and 40 men) were included in the cross-sectional study (20 lean, 35 morbidly obese patients without T2DM and 45 morbidly obese patients with T2DM). The obese subjects were recruited from patients scheduled for bariatric surgery for weight loss at the Endocrinology Departments at the University Hospital Joan XXIII (Tarragona, Spain) and University Hospital Bellvitge (Barcelona, Spain). Lean subjects were selected from an adipose tissue biobank registry at the University Hospital Joan XXIII. Subjects with signs of infection were excluded.

Intervention: All patients had fasted overnight before collection of blood and AT samples. Visceral AT (VAT) and subcutaneous (SAT) samples from the same individuals were obtained during the bariatric surgical procedure. Control (lean) samples were obtained from the biobank from patients undergoing laparoscopy surgery for hiatus hernia repair or cholecystectomy. The study was approved by the respective

local Ethics Committees review boards of the participating Hospitals. All participants gave their written informed consent to participate in the study.

Prospective study - cohort 2:

Design: Prospective single center, non-blinded, randomized controlled trial, including morbidly obese patients with T2DM (This trial was registered at www.controlledtrials.com as ISRCTN 14104758 (completed 25/01/2016)).

Participants: Forty-five patients (30 women and 15 men) were consecutively recruited among patients undergoing bariatric surgery at the Department of Endocrinology, Bellvitge University Hospital, Spain. The inclusion criteria were age 18-60 years, T2DM and BMI 35-42 kg/m^2. Clinical and anthropometric data and blood samples were collected before surgery and at one month and one year after bariatric surgery. The study was approved by the local Ethics Committee of Bellvitge University Hospital. All subjects gave written informed consent before study entry (the basal data of this cohort was used for the analysis of the cross-sectional study included in cohort 1 for T2DM patients).

Randomization: Patients were randomly assigned (1:1:1) to undergo either Roux-en-Y gastric bypass surgery (RYGB), sleeve gastrectomy (SG) or laparoscopic greater curvature plication (LGCP). Allocation of patients was assigned by simple randomization using envelopes, with stratification according to baseline levels of HbA_{1c} (greater or lower than 7%) to avoid differences in metabolic control across the groups during randomization.

Sample size: It was calculated to detect a 20% difference in ANGPTL8 concentration before and 1 month after surgery, and accepted a (α error of 0.05, a power of 0.80). We calculated a minimum of 10 subjects per arm, but taking into account the possibility of loss at follow-up, 15 patients were finally assigned.

Interventions: From May 2012 to February 2014, patients were consecutively randomized to RYGB (n=15), SG (n=15) and LGCP (n=15). The procedures were performed by the same surgeon at the same center.

Outcomes: To evaluate the rate of T2DM remission after surgery, we used the American Diabetes Association (ADA) criteria, considering complete T2DM remission if HbA1c <5.7% without treatment, partial remission if HbA1c 5.7-6.5% with withdrawal of treatment and non-remission if HbA1c >6.5% and/or still under treatment one year after surgery [22].

Prospective study - cohort 3:

Design: Prospective single center study including morbidly obese patients without T2DM.

Participants: Thirty subjects (22 women and 8 men) with age ranging from 23 to 59 years that met the criteria for surgical treatment of obesity at the time of inclusion (BMI >40 kg/m^2 or BMI >35 kg/m^2 and obesity-related illness) were consecutively recruited among patients undergoing bariatric surgery who attended at the Department of Surgery, Haugesund Hospital, Norway.

Intervention: Standardized laparoscopic bypass surgery (RYGB) was performed on all of the patients by the same team of surgeons at Haugesund Hospital. Anthropometric measurements and collection of fasting venous blood samples were performed the day before and one year after bariatric surgery. The study was approved by the Western Norway Regional Ethics Committee. All subjects gave written informed consent before study entry.

STUDY PROCEDURES

Analytical determinations

ANGPTL8

Circulating plasma levels of ANGPTL8 in cohorts 1 and 2 (Spain) were measured using a commercially available ELISA kit (Wuhan Eiaab Science, Wuhan, China; catalog no. E11644h) with an intra-assay coefficient of variation (CV) <6.5% and an inter-assay (CV) <9.2% (provided by the manufacturer) [23-25]. ELISA validation was previously carried out in our laboratory by western blotting and a good correlation was observed between both methods [25]. Measurement of plasma ANGPTL8 in cohort 3 (Norway) was performed using the Human ANGPTL8 ELISA kit (CSB-EL028107HU; Cusabio, Wuhan, China) with an intra-assay CV <8% and an inter-assay CV <10% (provided by the manufacturer) [26].

Gene expression analysis

Total RNA was extracted from SAT and VAT using the RNeasy lipid tissue mini kit (QIAGEN Science). One microgram of RNA was reverse transcribed with random primers using the reverse transcription system high capacity cDNA kit (Applied Biosystems). Quantitative ANGPTL8 (Hs00218820_m1) gene expression was evaluated with Taqman low-density arrays (Applied Biosystems; microfluidic cards) on a 7900HT fast real-time PCR system. The relative expression of each transcript was measured using the 2^{-ddCt} method. Peptidylprolyl isomerase A (PPIA, cyclophilin A, Hs04194521_s1) was used for normalization of gene expression levels and calculation of dCt values [27].

Statistical analyses

All data were tested for normality (Shapiro-Wilk test). Data are shown as mean (SD) (normally distributed) and median (25^{th}-75^{th} quartiles) (non-normally distributed). One-way analysis of variance (ANOVA) was used to compare groups in the cross-sectional study. The least square difference test was used for *post hoc* analyses. Paired t-test, Wilcoxon signed-rank test or paired-ANOVA were used for paired analysis in the two prospective cohorts as appropriate. Pearson's and Spearman's correlation coefficients were used to analyze the relationship between parameters as described. To determine which variables were associated with circulating ANGPTL8 and *ANGPTL8* WAT gene expression (cohort 1) and with changes of circulating ANGPTL8 after bariatric surgery **(**prospective studies: cohorts 2 and 3), multiple linear regression analyses were employed (stepwise backward selection procedures). All variables associated in the univariate analysis with ANGPTL8 were included in their respective models. To determine if ANGPTL8 was associated with T2DM remission after bariatric surgery (prospective cohort 2), logistic regression analysis was employed (stepwise backward selection procedures). Two-tailed p-values <0.05 were considered statistically significant. The calculations were made using STATA v.13.1 for Mac (StataCorp LP, College Station, TX).

RESULTS

Circulating levels and WAT expression of ANGPTL8 are differentially regulated in obesity: cross-sectional study.

A total of 100 subjects were included in cohort 1 (clinical characteristics are shown in **Table 1)**. Circulating levels of ANGPTL8 decreased across the three groups, with morbidly obese patients with T2DM having the lowest concentrations (101.6±18.8 pg/mL), significantly different to lean controls (892.7±183.1 pg/mL; p<0.001) and to morbidly obese patients without T2DM (548.0±63.3 pg/mL; p<0.001). The difference between lean patients and obese patients without T2DM did not reach statistical significance (p=0.244) (**Figure 1A**). Univariate analysis is depicted in **Supplementary Table 1.**

ANGPTL8 gene expression was analysed in SAT and VAT from the same patients. In contrast to our observations for serum levels, *ANGPTL8* expression was higher in SAT from morbidly obese patients with T2DM than in equivalent samples from morbidly obese patients without T2DM (2.00±0.47 vs. 0.54±0.10; p=0.008) and from lean subjects (2.00±0.47 vs. 0.07±0.02; p<0.001). Morbidly obese patients without T2DM also exhibited higher *ANGPTL8* expression than lean subjects (0.54±0.10 vs. 0.07±0.02; p<0.001). No differences in *ANGPTL8* were observed in VAT between the three groups (**Figure 1B**).

In univariate analyses, *ANGPTL8* expression in SAT was positively associated with female gender (r=0.406; p<0.001), BMI (r=0.430; p<0.001), T2DM (r=0.493; p<0.001), fasting plasma glucose concentrations (r=0.418, p<0.001), insulin (r=0.416, p<0.001) and HOMA-IR (r=0.539; p<0.001) (**Figure 1C**). *ANGPTL8* expression in

SAT was negatively correlated with circulating ANGPTL8 concentrations (r=-0.283; p=0.013) **(Figure 1C)**. *ANGPTL8* expression in VAT was positively associated with age (r=0.256; p=0.015) and total cholesterol (r=0.269, p=0.013).

To find the best model to predict circulating changes in ANGPTL8 concentrations, we design a multiple linear regression analysis (including all of the univariate associations described above and adjusted by gender, age and the presence of T2DM). Only BMI (β=-0.262; p=0.006) and the presence of T2DM (β=-0.64; p<0.001) appear as significant variables influencing circulating levels of ANGPTL8. Moreover, the model that best predicted *ANGPTL8* expression in SAT was the presence of T2DM (β=0.327; p=0.005).

Circulating ANGPTL8 increases after bariatric surgery and predicts T2DM remission.

A total of 45 morbidly obese patients with T2DM were included in a one-year follow-up prospective study (cohort 2). Patients were randomly assigned to RYGB, SG or LGCP (**Supplementary Figure 1**) and their clinical characteristics were evaluated at baseline and at one month and one year after bariatric surgery **Table 2.** Baseline characteristics were similar between the three surgery groups. As expected, weight, BMI, waist and WHR were all decreased after bariatric surgery. The BMI related changes %EWL, %WL and the %AWL at one month and one year after bariatric surgery are shown in **Supplementary Figure 2.** Fasting plasma glucose, insulin concentrations, HOMA-IR, C-peptide and HbA1c were also improved.

Circulating concentrations of ANGPTL8 were increased at one month and at one year after bariatric surgery (101.6±18.8 pg/mL vs. 177.0±30.5 pg/mL vs. 160.6±30.4 pg/mL, respectively; *p for trend*=0.046) (**Supplementary Figure 3**). ANGPTL8 concentrations at one year after bariatric surgery were inversely associated with BMI (r=-0.366; p=0.016). Positive associations with weight loss metrics %EWL (r=0.364; p=0.017), %WL (r=0.345; p=0.023) and %AWL (r=0.349; p=0.022) were also observed at one year after bariatric surgery.

To understand how circulating ANGPTL8 concentrations were regulated after bariatric surgery, and the potential role of the different types of surgery, we tested by multiple lineal regression the influence of the change of different clinical and analytical parameters evaluated, and found that the %AWL at one year after bariatric surgery was the main predictor of its increase (β=-0.467; p=0.012).

Finally, to assess the potential role of circulating ANGPTL8 concentrations for T2DM remission, multiple ordered logistic regression models were performed. The total remission rate of T2DM at one year after bariatric surgery was 42.2%. All the models were adjusted for the different types of surgery and for those variables that were previously described to be associated with T2DM remission (**Supplementary Table 2)**. The best models showed that the type of surgery (RYGB), baseline C-peptide, the T2DM duration and the increment of ANGPTL8 concentrations one month after surgery were the main predictors of T2DM remission at one year of follow-up.

Circulating concentrations of ANGPTL8 are increased after bariatric surgery: one-year follow-up of morbidly obese patients without T2DM.

A total of 30 morbidly obese patients without T2DM were included in the prospective study (cohort 32). Patients were evaluated at baseline and at one year after bariatric surgery (clinical characteristics and laboratory data are shown in **Table 3)**. As expected weight, BMI, fasting plasma glucose, insulin concentrations, HOMA-IR, C-peptide, HbA1c and lipid concentrations were all decreased after bariatric surgery. Similar to the patients with T2DM in cohort 2, circulating concentrations of ANGPTL8 were also increased at one year after bariatric surgery.

The increase of circulating ANGPTL8 concentrations after one year was positively associated with %EWL (r=0.422; p=0.020), %WL (r=0.369, p=0.045) and %AWL (r=0.388; p=0.034), and similar to the finding in cohort 2 a multiple linear regression analysis revealed that higher %AWL, and also male gender were the main predictors of its increase (**Supplementary Tables 3 and 4)**.

DISCUSSION

In this study, we determined the circulating levels and WAT expression of ANGPTL8 in lean and morbidly obese patients and examined how massive weight loss after bariatric surgery might alter its levels. In particular, we assessed whether ANGPTL8 was likely to be relevant for T2DM.

In a cross-sectional analysis, we found that circulating ANGPTL8 concentrations were lower in morbidly obese patients with T2DM than in obese counterparts without T2DM and in lean subjects. Accordingly, we found an inverse relationship between plasma ANGPTL8 levels and markers associated with impaired glucose metabolism. Because these findings do not provide information about the dynamics of the alterations of ANGPTL8 during the processes that lead to obesity or T2DM, we examined the changes in ANGPTL8 levels in two patient cohorts of morbid obesity (with and without T2DM) that underwent bariatric surgery. Interestingly, circulating ANGPTL8 concentrations were significantly increased in both cohorts after a one-year follow-up. These results are in accordance with a previous case-control study showing that ANGPTL8 levels are significantly lower in extremely obese patients than in lean subjects [15]. Moreover, this study reported a decrease in circulating levels of ANGPTL8 with increasing BMI, which, in agreement with our data, was more evident in subjects with glucose derangement. Our results also fit with a very recent report showing that circulating ANGPTL8 levels increase after bariatric surgery [28]. However, in our study, we included an additional lean control group, enabling direct comparison of ANGPTL8 levels in the plasma of morbidly obese and lean subjects, which was not possible in the other study [28]. Taken together, the data support that circulating ANGPTL8 are high in lean and low in obese patients, especially in those with T2DM.

Accordingly, we observed that the lower the basal concentrations of ANGPTL8 the greater the increase after weight loss at one-year follow-up.

A previous study examined ANGPTL8 in obese subjects with metabolic syndrome after diet-induced weight loss. In this eight-week diet intervention study, subjects with lower ANGPTL8 pre-intervention levels had the greatest reduction in fat mass after dietary modification despite no differences in ANGPTL8 levels were observed after follow up [29]. In contrast to the findings of our study, basal levels of ANGPTL8 were higher in patients with metabolic syndrome than in lean controls; however, these patients were not in the range of massive obesity. Nonetheless, Pascual-Corrales and colleagues only found changes in plasma ANGPTL8 after surgery but not when weight was lost after hypocaloric diet [28]. Likewise, the majority of patients in the diet-arm of this study were not in the range of massive obesity. Still, the basal levels of ANGPTL8 were not different to those in the surgery group, making it difficult to draw clear conclusions when comparing both groups. Some authors have attributed these discrepancies in circulating levels of ANGPTL8 in the context of T2D and obesity to differences in the epitopes recognized by commercial assays [18]. ANGPTL8 undergoes proteolytic cleavage before secretion, leading to different circulating levels of the C- or the N-terminal end of the protein. We considered this possibility in our study, but irrespective of the different detection methods used in the studied cohorts, we observed the same trend and relationship with metabolic parameters, particularly evident for the elevated levels of ANGPTL8 after bariatric surgery. The EIAAB kit applied in cohort 2 is widely used [30] and the results were validated on a western blot, and the Cusabio kit used in cohort 3 it has been also previously used [28].

Gathering the trends observed in these studies, it seems plausible that low pre-intervention ANGPTL8 levels may be associated with a better response in metabolic profile after weight loss mainly in T2DM patients or in those with metabolic syndrome. In line with this hypothesis, the increment of circulating ANGPTL8, as early as at one month of follow-up in the cohort 3, appeared as one of the main determinants of diabetes remission (independent of weight loss) in association with other well-known predictors, such as the type of bariatric surgery, the prior β-cell function (higher C-peptide) and the duration of T2DM (**Supplementary Table 2**) [31, 32]. This observation strengthens the potential role of ANGPTL8 in the glucose homeostasis. Supporting this observation, a recent study characterized the molecular mechanisms underlying the regulation of glucose metabolism by ANGPTL8 in a human hepatic cell line [17]. The authors suggested that differences in circulating levels observed in clinical studies might be due to the different capacity of the insulin-responsive target tissues depending on the stage of the insulin-resistance process. Accordingly, at early stages the liver might respond to the increment of insulin flux by increasing ANGPTL8 production, but at more advanced stages associated with an inadequate β-cell function the concentration of ANGPTL8 progressively decreases. This hypothesis may explain the behaviour of ANGPTL8 observed in our T2DM cohort. Thus, the improvement in insulin-resistance observed after weight loss would facilitate a better response to insulin in the liver, increasing circulating ANGPTL8 concentrations early in the first month after surgery.

Regarding the relationship between WAT expression and circulating levels of ANGPTL8, we observed a significant inverse association in SAT. In line with these observations insulin infusion induces a paradoxical response in ANGPTL8 levels in healthy subjects [19]. Although the underlying mechanisms for the inverse association of

circulating levels and *ANGPTL8* expression remain to be elucidated, these results suggest a local effect of ANGPTL8 on AT, independent of obesity. Of note, in contrast to murine WAT, where ANGPTL8 has been detected exclusively in adipocytes, ANGPTL8 is expressed both in mature adipocytes and in the stroma-vascular fraction in human WAT (**Supplementary Figure 4**). Preliminary experiments from our laboratory indicate that macrophages express and secrete ANGPTL8, pointing to a possible paracrine role for ANGPTL8 in AT that warrants further investigation. Along these lines, ANGPTL8 has been recently described as a stress-response protein that downregulates adipose triglyceride lipase in mammalian cells [33]. This novel role for ANGPTL8, linking lipid homeostasis and metabolic stress, might underscore its elevated expression in AT from obese and T2DM patients, opening a new field of investigation.

In summary, the present study shows that plasma ANGPTL8 concentrations are highest in lean subjects and lowest in morbidly obese patients with T2DM. *ANGPTL8* is expressed in all the components of the WAT and its expression correlates negatively with its circulating levels, and positively with T2DM as its main determinant factor. Finally, our results suggest that massive weight loss but also metabolic status are important regulators of ANGPTL8 levels being their increment after bariatric surgery one of the main predictors of T2DM remission independently of weight loss.

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Figure Legend

Figure 1. Circulating and mRNA ANGPTL8 levels change with BMI. (A) ANGPTL8 plasma circulating levels are significantly lower in morbidly obese patients (Ob-M) with and without T2DM than in lean subjects. **(B)** *ANGPTL8* expression in subcutaneous (SAT) and visceral (VAT) adipose tissue exhibits distinctive profiles. Data are represented as mean±SEM. $^{\#}p<0.001$ for post hoc test (lean vs. morbid-obese with T2DM). $^{*}p<0.01$ for post hoc test (morbid-obese without T2DM vs. morbid-obese with T2DM). $^{**}p<0.001$ for post hoc test (lean vs. morbid-obese without T2DM). **(C)** Associations between *ANGPTL8* SAT expression and BMI, fasting plasma glucose, insulin, HOMA-IR and ANGPTL8 circulating levels.

Table 1. Clinical and laboratory data for the patients included in the cross-sectional study (cohort 1)

	Lean	Morbidly obese without T2DM	Morbidly obese with T2DM	*p for trend*
n	20	35	45	
Age (yrs)	49.5 (12.1)	43.1 (9.9)	49.9 (8.0)‡	0.008
Sex (M/F)	13/7	12/23	15/30	0.038
BMI (kg/m^2)	22.2 (2.5)	41.1 (3.2)*	39.5 (1.9)†,‡	<0.001
Glucose (mmol/L)	5.0 (4.2-5.4)	5.0 (4.8-5.4)	8.5 (6.4-11.6)†,‡	<0.001
Insulin (mIU/L)	6.1 (3.8-7.7)	14.2 (11.8-19.2)*	16.0 (11.1-19.0)†	<0.001
HOMA-IR	1.2 (0.8-1.6)	3.2 (2.5-4.7)*	5.6 (3.6-9.1)†,‡	<0.001
HbA1c (%)	-	-	7.2 (6.3-9.3)	-
Total cholesterol (mmol/L)	5.0 (4.3-5.2)	3.1 (2.9-3.5)*	4.8 (4.3-5.4)‡	<0.001
HDL-cholesterol (mmol/L)	1.5 (1.4-1.7)	0.8 (0.6-1.0)*	1.1 (1.0-1.3)†,‡	<0.001
LDL-cholesterol (mmol/L)	2.9 (2.3-3.2)	2.0 (1.7-2.3)*	2.8 (2.3-3.3)‡	<0.001
Triglycerides (mmol/L)	1.1 (0.8-1.2)	0.9 (0.6-1.1)	1.7 (1.2-2.0)†,‡	<0.001

Data are presented as mean (SD) or median (25th-75th) quartiles as appropriate. T2DM, type 2 diabetes; BMI, body mass index; HOMA-IR, insulin resistance homeostatic model assessment. *$p<0.05$ for LSD post hoc test comparing lean vs. morbidly-obese without T2DM. $^{†}p<0.05$ for LSD post hoc test comparing lean vs. morbidly-obese with T2DM. $^{‡}p<0.05$ for LSD post hoc test comparing morbidly-obese without T2DM vs. morbidly-obese with T2DM.

Table 2. Clinical characteristics of the whole population before, 1 month and 1 year after bariatric surgery (cohort 2)

	Baseline	1 month	1 year	p for trend
n	45	45	44	-
Age (yrs)	49.4 (8.0)	-	-	-
Gender (M/F)	15/30	-	-	-
Hypertension (n, %)	30 (66.7)	21 (46.7)*	21 (46.7)†	0.005
Antihypertensive drugs (n, %)	30 (66.7)	21 (46.7)*	21 (46.7)†	0.005
Dyslipidemia (n, %)	33 (73.3)	14 (31.1)*	13 (28.9)†	<0.001
Lipid lowering drugs (n, %)	32 (71.1)	10 (22.2)*	14 (31.1)†	<0.001
Type 2 diabetes				
T2DM (n, %)	45 (100.0)	30 (66.7)*	15 (33.3)†,‡	<0.001
T2DM treatment (n, %)	45 (100.0)	19 (42.2)*	13 (28.9)†	<0.001
Insulin (n, %)	17 (37.8)	10 (22.2)*	5 (11.1)†	<0.001
Anthropometric measurements				
Weight (kg)	103.6 (11.0)	89.8 (11.3)*	75.1 (13.4)†,‡	<0.001
BMI (kg/m^2)	39.5 (1.9)	34.2 (2.3)*	28.6 (4.1)†,‡	<0.001
Waist (cm)	117.9 (7.9)	108.8 (7.6)*	96.0 (9.9)†,‡	<0.001
WHR	0.96 (0.91-1.02)	0.96 (0.91-1.0)	0.91 (0.86-0.97)†,‡	<0.001
Blood pressure				
SBP (mmHg)	134.0 (18.5)	128.4 (17.9)	129.0 (19.4)	0.261
DBP (mmHg)	81.1 (14.3)	81.4 (13.4)	77.2 (13.0)	0.216
Lipid profile				
Total cholesterol (mmol/L)	4.76 (4.27-5.38)	4.39 (3.66-5.16)*	4.61 (3.95-5.26)	0.032
HDL-cholesterol (mmol/L)	1.13 (1.01-1.33)	1.02 (0.90-1.15)*	1.46 (1.23-1.60)†,‡	<0.001
LDL-cholesterol (mmol/L)	2.75 (2.28-3.32)	2.72 (1.90-3.38)	2.60 (2.05-3.26)	0.230
Triglycerides (mmol/L)	1.70 (1.17-1.99)	1.42 (1.13-1.79)*	0.96 (0.73-1.38)†,‡	<0.001

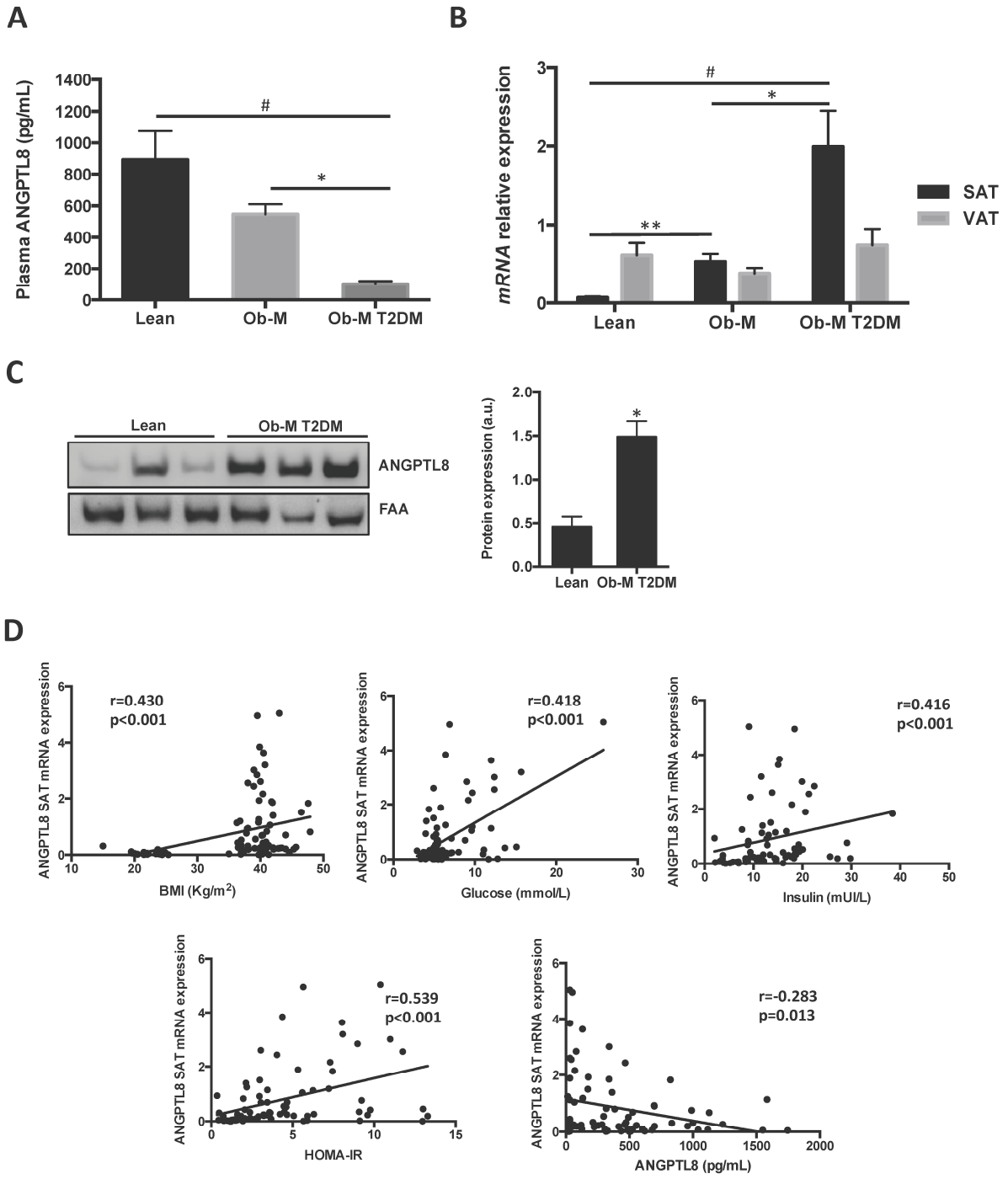
Data are presented as mean (SD) or median (25th-75th) quartiles as appropriate. T2DM, type 2 diabetes; BMI, body mass index; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, insulin resistance homeostatic model assessment. *$p<0.05$ for LSD post hoc test comparing basal measurements vs. 1 month after surgery. $^{\dagger}p<0.05$ for LSD post hoc test comparing baseline measurements vs. 1 year after surgery. $^{\ddagger}p<0.05$ for LSD post hoc test comparing variables 1 month after surgery vs. 1 year after surgery.

Table 3. Clinical and laboratory data for morbidly obese patients without T2DM before and 1-year after bariatric surgery (cohort 3)

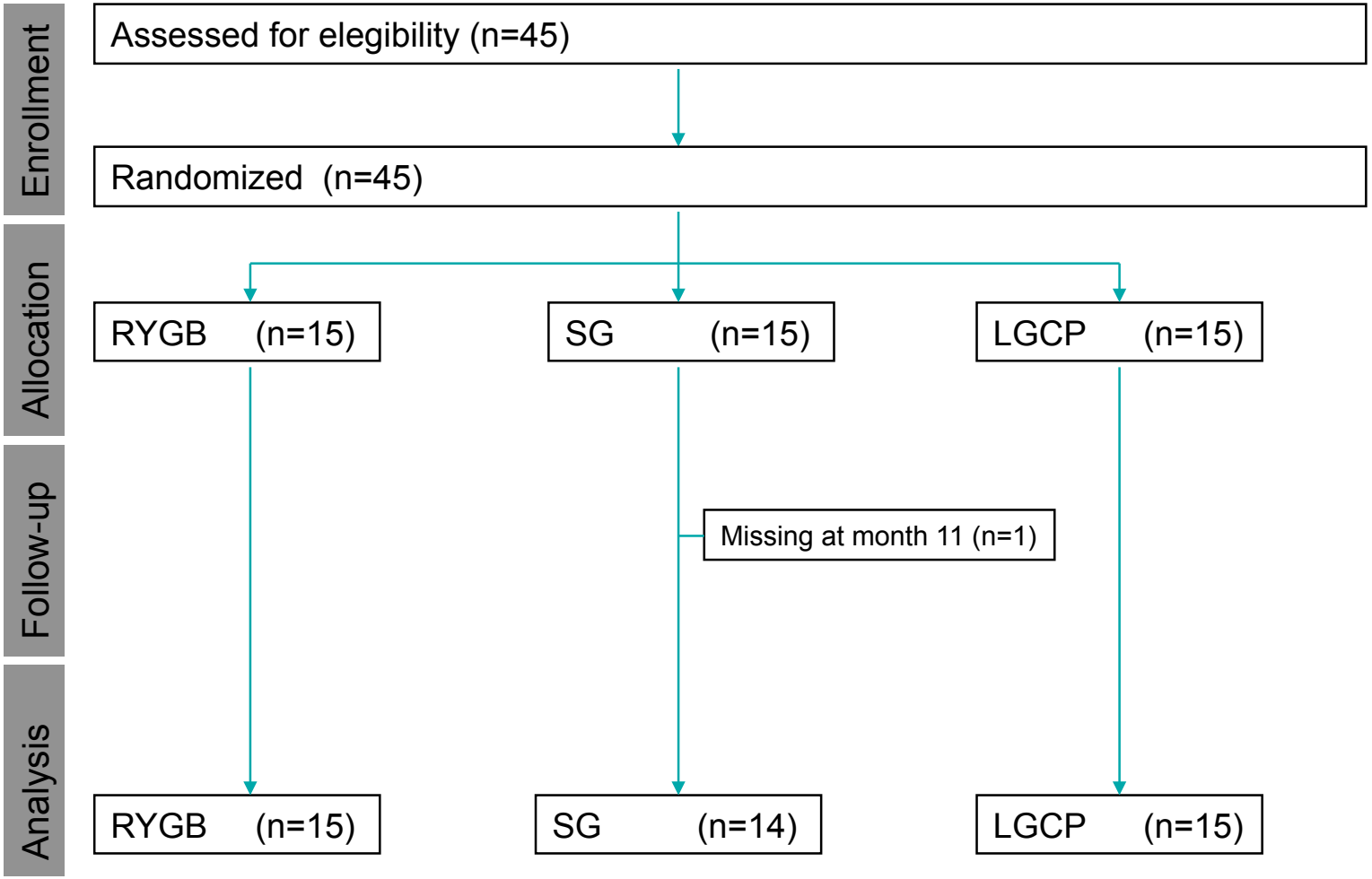
	Before	**1 year**	***p***
n	30	30	-
Age (yrs)	41.9 (9.5)	-	-
Sex (M/F)	8/22	-	-
Weight (kg)	117.6 (18.0)	82.1 (13.4)	<0.001
BMI (kg/m^2)	40.1 (4.1)	28.1 (3.8)	<0.001
%EWL (%)	-	84.3 (66.4-97.9)	-
%WL (%)	-	31.5 (26.9-33.0)	-
%AWL (%)	-	46.3 (41.1-50.2)	-
Glucose (mmol/L)	5.3 (5.0-5.5)	4.7 (4.6-4.9)	<0.001
Insulin (mIU/mL)	13.1 (9.9-18.8)	4.2 (3.1-6.0)	<0.001
HOMA-IR	3.0 (2.5-4.1)	0.8 (0.6-1.3)	<0.001
C-peptide	1.2 (1.0-1.3)	0.6 (0.5-0.8)	<0.001
HbA1c (%)	5.2 (5.1-5.3)	5.1 (5.0-5.3)	0.002
Total cholesterol (mmol/L)	4.74 (0.97)	4.27 (0.82)	0.002
HDL-Cholesterol (mmol/L)	0.89 (0.76-1.01)	1.24 (1.11-1.45)	<0.001
LDL-Cholesterol (mmol/L)	3.10 (2.80-3.80)	2.65 (2.09-3.30)	<0.001
Triglycerides (mmol/L)	1.40 (1.19-1.66)	0.86 (0.63-1.25)	<0.001
Circulating ANGPTL8 (ng/mL)	6.76 (3.83-10.80)	10.55 (7.47-14.38)	<0.001

Data are presented as mean (SD) or median (25^{th}-75^{th}) quartiles, as appropriate. BMI, body mass index; %EWL, percentage excess weight loss; %WL percentage total weight loss, %AWL percentage alterable weight loss; HOMA-IR, insulin resistance homeostatic model assessment.

Figure 1

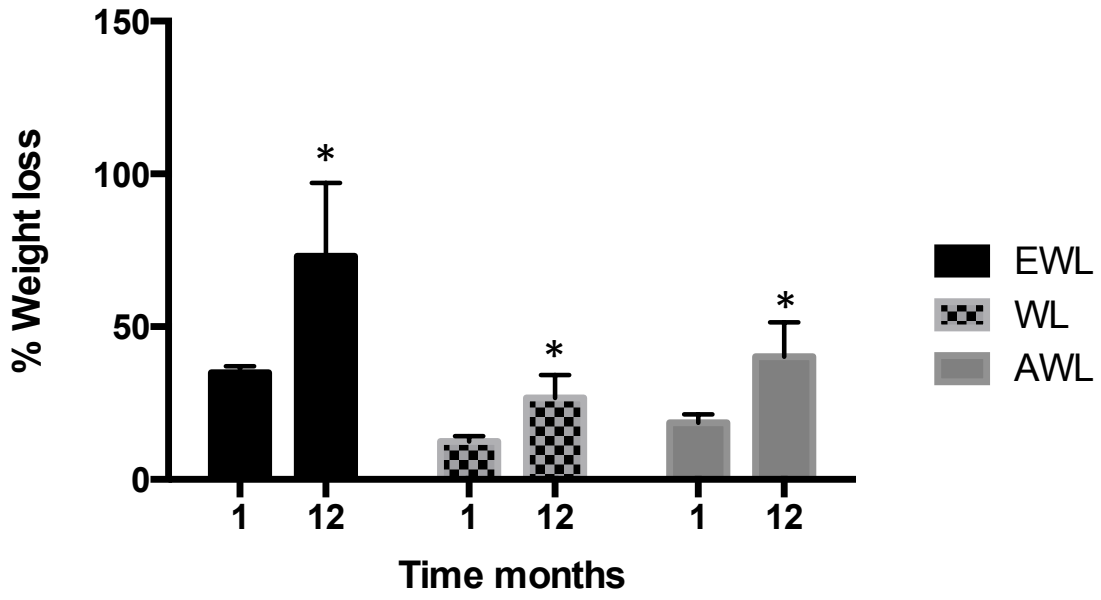

A
Plasma ANGPTL8 (pg/mL)
1400
1200
1000
800
600
400
200
0
#
*
Lean
Ob-M
Ob-M T2DM
B
mRNA relative expression
3
2
1
0
#
*
**
SAT
VAT
Lean
Ob-M
Ob-M T2DM
C
Lean
Ob-M T2DM
ANGPTL8
FAA
Protein expression (a.u.)
2.0
1.5
1.0
0.5
0.0
*
Lean
Ob-M T2DM
D
ANGPTL8 SAT mRNA expression
r=0.430
p<0.001
BMI (Kg/m2)
r=0.418
p<0.001
Glucose (mmol/L)
r=0.416
p<0.001
Insulin (mUI/L)
r=0.539
p<0.001
HOMA-IR
r=-0.283
p=0.013
ANGPTL8 (pg/mL)

Supplementary Figure 1



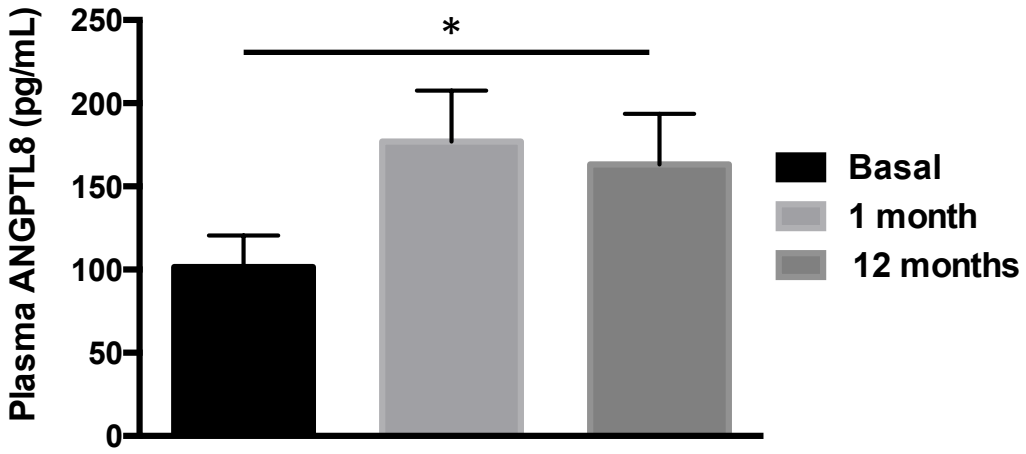
Supplementary Figure 1. 1-year follow-up study. The patients were randomly assigned and evaluated at baseline, 1 month and 1 year after bariatric surgery. One SG patient voluntarily withdrew from the study at 11 months. Follow-up compliance was 97.78% and 44 patients completed the 1-year follow-up.

Supplementary Figure 2



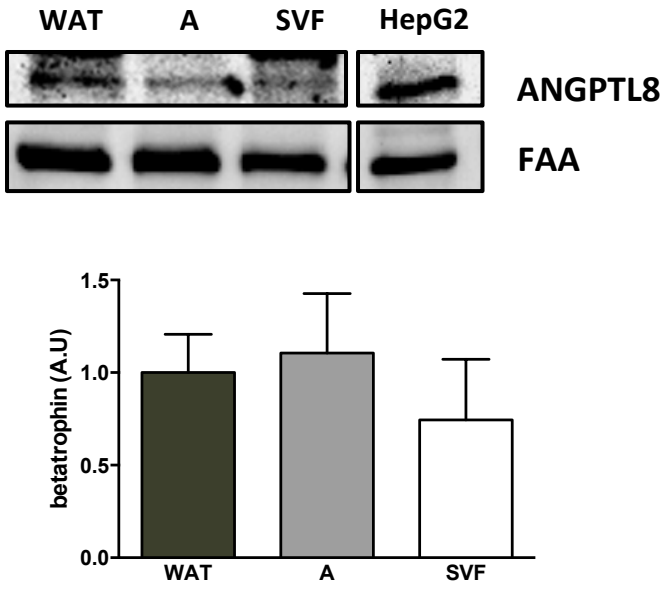
Supplementary Figure 2. %EWL, the %WL and the %AWL were 34.9 (28.8-37.9)%, 12.3 (10.7-14.3) and 18.5 (16.3-21.3)% at 1 month after bariatric surgery and 73.1 (56.7-97.1)%, 26.6 (22.9-34.1)% and 40.2 (33.7-51.4)% at 1 year after bariatric surgery, respectively. Data are represented as median (25^{th}-75^{th} quartiles). *$p<0.001$ (1 year vs. 1 month).

Supplementary Figure 3



Supplementary Figure 3. ANGPTL8 circulating levels were increased one month after surgery and were maintained after 1 year follow-up (101.6±18.8 pg/mL vs. 177.0±30.5 pg/mL vs. 160.6±30.4 pg/mL respectively; *p for trend*=0.046). Data are represented as mean±SEM.

Supplementary Figure 4



Supplementary Figure 4. ANGPTL8 protein expression was examined in total white adipose tissue (WAT), adipocytes (A) and stroma-vascular fractions (SVF) of a total of 8 individuals. A representative western blot is shown. Fumarylacetonacetase (FAA) was used as a loading control. HepG2, a liver derived cell line, was used as a positive control. Quantification is shown vs WAT. Data are represented as mean±SEM.

Supplementary Table 1. Univariate analysis between ANGPTL8 circulating levels and biochemical parameters and presence of T2DM in cohort 1.

	Circulating ANGPTL8	
	r	**p**
T2DM	-0.724	<0.001
Fasting glucose	-0.637	<0.001
HOMA-IR	-0.448	<0.001
Total cholesterol	-0.427	<0.001
LDL-cholesterol	-0.363	0.002
TG	-0.297	0.007

Supplementary Table 2. Ordered logistic regression models for T2DM remission prediction (Prospective study - Cohort 2)

	OR (95% CI)	**p**
Model 1: LR χ^2= 62.38; p<0.001		
Type of surgery		
Sleeve gastrectomy (vs. gastric by-pass)	0.001 (0.001-0.125)	0.005
Gastric plication (vs. gastric by-pass)	0.001 (0.001-0.029)	0.003
Duration of T2DM	0.966 (0.943-0.990)	0.006
Baseline C-peptide	18.292 (1.947-171.9)	0.011
ΔANGPTL8 at 1 month	1.870 (1.152-2.035)	0.011
Previous insulin treatment	0.017 (0.001-0.436)	0.014

Variables included in the models were: age, sex, type of surgery, %AWL at 1 month, duration of T2DM, previous insulin treatment and baseline C-peptide.

Supplementary Table 3. Univariate analysis between basal and one year after bariatric surgery ANGPTL8 circulating levels and biochemical parameters in cohort 3.

	Basal circulating ANGPTL8	
	r	**p**
Insulin	-0.503	0.005
Fasting glucose	-0.516	0.004
HOMA-IR	-0.435	0.013

	1-year circulating ANGPTL8	
	r	**p**
BMI	-0.356	0.050
Insulin	-0.392	0.032

Supplementary Table 4. Clinical and analytical changes after bariatric surgery associated with the increment of circulating ANGPTL8 concentrations (Prospective study - Cohort 3)

ΔANGPTL8 (R^2=0.384; p=0.014)					
	B (unstandardized)	**SE**	**95% CI**	**Beta (standardized)**	**p**
%AWL	0.265	0.127	0.005-0.525	0.489	0.046
Gender	-5.647	2.103	-9.957- -1.338	-0.416	0.012
Constant	5,192	5.741	-6.567-16.951	-	0.373

Variables included in the model: age, gender, %AWL and the differences 1 year-baseline (total cholesterol, HDL-cholesterol, triglycerides, fasting plasma glucose, HbA1c, C-peptide and HOMA-IR).

Supplementary information

STUDY DESIGN AND PATIENTS

Prospective study - cohort 2:

Participants: **Exclusion criteria were as follows:** type 1 diabetes mellitus, positive auto antibodies, secondary T2DM after injury or hormonal diseases (Cushing's syndrome or acromegaly); acute metabolic complications, ketosis, ketoacidosis or hyperosmolar state over the last six months; serious infection that could affect blood glucose control during the 4 weeks prior to inclusion; cardiovascular events (episode of heart failure, angina pectoris, myocardial infarction or stroke) within 6 months prior to the study; history of liver disease (chronic active hepatitis or cirrhosis) and/or abnormal liver function (ALT and/or AST 3 times above the upper normal value); altered renal function (creatinine greater than 1.4 mg/dl in women and 1.5 mg/dl in men); anticoagulant therapy; congenital or acquired abnormalities of the digestive tract (atresia, stenosis, etc); pregnancy, nursing or desired pregnancy in the 12 months following the inclusion; recent history of neoplasm (<5 years) except basal cell skin cancer; glucocorticoid use by oral or intravenous route for more than 14 consecutive days in the last three months; alcoholism, drug addiction or major psychiatric disorders; refusal to participate in the study.

STUDY PROCEDURES

Anthropometric measurements and weight loss after bariatric surgery

Body weight and height were measured. BMI was calculated with the Quetelet index (weight in kg/height in meters squared). Body circumference was measured with a non-stretchable band for the waist midway between the lower rib margin and the iliac crest and, for the hip circumference, over the greater trochanters and recorded to the nearest 0.5 cm. Blood pressure (the average of two measurements) was determined by sphygmomanometer aneroid (Omron®) with the patient seated after 5 minutes of rest.

To evaluate weight loss after bariatric surgery in the two prospective cohorts, we used three different formulas: the percentage excess of weight loss (%EWL= 100* [baseline BMI - final BMI]/[baseline BMI - 25]), the percentage (total) weight loss (%WL=100* [baseline BMI - final BMI]/baseline BMI) and the percentage alterable weight loss (%AWL= 100* [Baseline BMI - final BMI]/ [baseline BMI -13]).

Analytical determinations

Cross-sectional study – cohort 1 (Spain): Fasting plasma glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol and TG were determined by standard enzymatic methods (ADVIA 2400 Chemistry autoanalyzer. Siemens Healthcare, Earlangen, Germany). Insulin was determined by immune assay using an AVDIA Centaur XP autoanalyzer (Siemens Healthcare, Earlangen, Germany).

Prospective study - cohort 2 (Spain): Blood samples were collected after overnight fasting, before bariatric surgery, 1 month and 1 year later. Fasting plasma glucose, total cholesterol and TG were determined on a Hitachi 737 autoanalyzer (Boehringer Mannheim, Marburg, Germany) using standard enzymatic methods. HDL-cholesterol concentrations were determined by the standard enzymatic methods after precipitation with PEG-6000. Plasma insulin was analyzed by immunoassay (Coat-A-Count Insulin; Diagnostic Products Corp., Los Angeles, CA).

The homeostatic model assessment (HOMA)-IR index was used to evaluate insulin resistance in all the sub-studies and was calculated as the product of fasting plasma insulin (mIU/mL) and fasting plasma glucose (mmol/L) divided by 22.5 [1].

Prospective study - cohort 3 (Norway): Blood samples were collected after overnight fasting the day before bariatric surgery, and one year later. Fasting plasma glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol and TG were analyzed at the Department of Clinical Chemistry, Haukeland University Hospital, Bergen, Norway. Serum lipid levels and fasting glucose were assayed using an Architect 1600 chemistry analyzer (Abbott, Chicago, IL, USA). HbA1c levels were measured using high-performance liquid chromatography (HPLC) with a Tosoh G8 HPLC autoanalyzer (Tosoh Bioscience, Tessenderlo, Belgium). Fasting serum insulin and C-peptide levels were analyzed using a chemiluminescence assay on the Siemens immulite 2000 Immunoassay system (Siemens AG, Erlangen, Germany) at the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway.

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