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Titolo:

Biliopancreatic diversion in patients with type 2 diabetes and moderate obesity: impact and mechanisms.

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Dedico essa tese à Fabiana, minha eterna namorada, que me reconheceu ao nosso primeiro encontro, que me apoia, me segue e me ama. Dedico à minha filha Laura que com apenas sete aninhos me disse: "vou porque è importante para você", abriu mão da sua vidinha para me acompanhar. Dedico também a Sofia que com sua alegria e amor ajuda a manter unida a nossa família e me diz sempre: "você é o melhor papai do mundo!".

Dedico aos meus pais, a tudo que fizeram por mim, ao amor que me deram e que me ensinaram a dar!

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"Enquanto você se esforça pra ser, um sujeito normal e fazer tudo igual
Eu do meu lado aprendendo a ser louco, um Maluco total, na loucura real
Controlando a minha maluquez, misturada com minha lucidez
Vou ficar, ficar com certeza, Maluco beleza!"
Raul Seixas

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Abstract

Context Diabetes remission is frequent after biliopancreatic diversion (BPD) in morbidly obese patients with type 2 diabetes (T2DM). Data, mechanisms, and clinical indications in nonobese T2DM patients are scanty.

Objective To assess remission and investigate insulin sensitivity and ß-cell function after BPD in non-morbidly obese patients with long-standing T2DM.

Design, setting and patients Clinical research study comparing 15 T2DM patients (age 55±1 years, duration 16±2 years, BMI=28.3±0.6 kg/m^2, HbA_{1c}=8.6±1.3%) with 15 gender-, age-, and BMI-matched nondiabetic controls. Before surgery, and 2 months and one year later, a 3-hour OGTT, a 5-hour mixed meal test, and a 3-hour euglycemic clamp were performed.

Intervention BPD (gastric resection, distal jejunum anastomosed to remaining stomach, biliopancreatic tract anastomosed to ileum 75cm from the ileocecal valve).

Results Glycemia improved in all patients, but remission (HbA_{1c}<6.5% and normal OGTT) occurred in 6/15. Insulin resistance (19.8±0.8 $\mu mol \cdot min^{-1} \cdot kg_{ffm}^{-1}$, p<0.001 *vs* 40.9±5.3 of controls) resolved already at 2 months (34.2±2.8) and was sustained at one year (34.7±1.6), although insulin-mediated suppression of endogenous glucose production remained impaired. In contrast, ß-cell glucose sensitivity (19[12] $pmol \cdot min^{-1} \cdot m^{-2} \cdot mM^{-1}$ *vs* 96[73] of controls, p<0.0001) rose (p=0.02) only to 31[26] at one year, and was lower in non-remitters (16[18]) than remitters (46[33]).

Conclusions In nonobese patients with long-standing T2DM, BPD improves metabolic control but induces remission in only ~30% of patients. Peripheral insulin sensitivity is restored early after surgery, and similarly in remitters and non-remitters, indicating a weight-independent effect of the operation. The initial extent of ß-cell incompetence is the main predictor of the metabolic outcome.

1. INTRODUCTION

1.1. Epidemiology of Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is known a long time but still today is not completely understood. Affecting million of individuals T2DM is responsible, each year, for about 5% of all deaths worldwide. Diabetes results from the interaction of genetic predisposition and environmental factors (Stumvoll, 2005). Several genetic defects are involved in alteration of glucose metabolism. Environmental factors could be responsible by enhance or not these genetic predispositions leading to T2DM (Modan, 1986).

According to the International Diabetes Federation (IDF) the number of people with type 2 diabetes is rising rapidly worldwide. It is associated with the economic development, ageing populations, increasing urbanization, dietary changes, reduced physical activity and changes in other lifestyle patterns. Diabetes prevalence in the population is increasing around the world: 366 million of persons were diagnosed as diabetic in 2011 and by 2030 this number will rise to 552 million (IDF Atlas, 2011).

There are three main types of diabetes: insulin dependent diabetes mellitus (IDDM) or type 1 diabetes (T1DM), non insulin dependent diabetes mellitus (NIDDM) or T2DM and gestational diabetes (GDM). Others types are also recognized and are resulting of malnutrition and therefore named as related diabetes mellitus (MRDM) or as consequence of certain conditions or syndromes and known as secondary diabetes (Bennett, 1992).

Type 2 diabetes is the most common type of diabetes. Results from an impairment of normal glucose homeostasis that progresses and becomes a chronic disease exhibiting the hyperglycaemia and glucose intolerance as principal symptoms (DeFronzo, 1992). Impairment in glucose metabolism is lead to the incapacity of the body in produce enough insulin to maintain euglycaemia or to responding to its effects, generating an acute increasing and chronically higher concentration of glucose and insulin in the blood.

T2DM usually occurs in adults, but actually is also seen in children and adolescents, as result of children obesity and unhealthy dietary behaviour. People who develops T2DM (90%), are usually middle-aged (45-64 years), not elderly (>65 years), 80% live in low and middle-income countries. Type 2 diabetes mellitus usually occurs after that the subject have progressed from the normal glucose tolerance (NGT) state to impaired glucose tolerance (IGT) and finally, to T2DM. Because symptoms may take years to appear or be recognized, meanwhile body is progressively damaged by the excess of blood glucose (Stumvoll, 2005).

Diagnosis of T2DM is based on measurements of fasting plasma glycaemia (FPG) and glycosilated haemoglobin (Hb_{A1c}%) but oral glucose tolerance test (OGTT) is the standard method to diagnose incipient diabetes. When diabetes becomes chronic, derived hyperglycaemia can leads to serious damage to many of the body's systems. Bring to debilitating micro and macrovascular diseases as nephropathy, retinopathy and neuropathy.

Cardiovascular complications constitute the major cause of morbidity and mortality in patients with diabetes. Intensive glycaemic control can prevents the development and progression of microvascular complications in patients with type 1 or type 2 diabetes (Brown, 2010).

1.2. Natural History of Type 2 Diabetes Mellitus

In subjects with normal tolerance to glucose, plasma glucose concentrations are maintained within a narrow range as result of simultaneous release of glucose into circulation and uptake of glucose from plasma by the cells stimulated by insulin hormone (DeFronzo, 1992). Metabolism of glucose is an essential step to sustain life by generation of energy to the body and to avoid long glucose contact with systems that could be damaged. Glucose flux through from intestine and liver to bloodstream and finely into the cells is the initial condition to generate energy and store energy (Tripathy, 2010). Defects in one or more of these systems could cause impairment in sensitization of tissues by glucose, insulin secretion and insulin

stimulated glucose uptake originating hyperglycaemia and disequilibrium in the glucose homeostasis (Leahy, 2005). Because in T2DM the body exhibit impaired ability to respond to insulin (DeFronzo, 1992), the pancreas must to increase its secretion of insulin to offset the insulin resistance maintaining the normal glucose tolerance. The progression from NGT to impaired glucose tolerance (IGT) to T2DM is characterized by progressive hyperinsulinaemia. ß-cell dysfunction starts when pancreatic ß-cells fails to maintain hyperinsulinaemia necessary to cope insulin resistance leading to the development of IGT and eventually overt T2DM (Kan, 1993). Insulin resistance is observed in skeletal muscle, liver and adipocytes (Cusi, 2010). The relative contribution of defects in insulin action and ß-cell function for the pathogenesis of T2DM remains still unclear (Tripathy, 2010).

To avoid the progression from NGT to IGT and finally to T2DM, β-cell must be able to regulate its secretion of insulin hormone in precise doses to cope insulin resistance presented by peripheral tissues. In fact, only insulin resistance is not sufficient to cause loss of homeostasis in glucose metabolism if β-cell function is preserved enough to offset insulin resistance. This is commonly observed in obese subjects with NGT. In whom insulin output can be 10-fold higher than in lean individuals and returns to normal levels after weight-loss. However, β-cell dysfunction is necessary and sufficient to produce hyperglycaemia.

Whether obese insulin resistant subjects, shown an increase in β-cell mass. Decrease by 80-90% in β-cell mass is required before sufficient insulinopenia develops to cause overt T2DM (Ferrannini, 2010). It seems that other factors in addition to β-cell lost must be responsible for the impairment of insulin secretion. Many acquired factors also play a role in the pathogenesis of the disease and information from DNA transcript: protein and metabolite profiles may better capture the genetic influences on metabolism than studying single genes.

1.3. Obese Type 2 Diabetes Mellitus

Most subjects with T2DM are obese. The common occurrence of obesity in T2DM, and the observation that obesity precedes the deterioration of glucose homeostasis, lead to the hypothesis that, at least for obese subjects, obesity was the risk condition resulting in T2DM (DeFronzo, 1992).

Prospective studies among Pima Indians demonstrate that obesity precedes the development of T2DM (Tulloch-Reid, 2003). Obesity therefore must be viewed as a risk factor for the development of the disease rather than a consequence, or complication, of the diabetic condition. The increase in the prevalence of obesity appears to be associated with an increased prevalence of risk factors for cardiovascular disease and T2DM, including hypertension and reduced glucose tolerance (Meisinger, 2006). Duration of obesity is an additional risk factor for the development of T2DM. Although obesity is a major risk factor for T2DM in Pima Indians, the presence of diabetes in one or both parents has been shown to be a major dependent risk factor (Mallikarjun, 2009). Obese, adult Pima offspring of a diabetic parent are at much greater risk of developing T2DM than the obese, adult offspring of two non-diabetic parents. Obesity was formerly believed to be a risk factor for T2DM as a result of being associated with insulin resistance. Prando et al. have shown that, on average, obese subjects are more insulin resistant than lean subjects (Prando, 1998).

Diabetes risk is determined not only by the degree of obesity (Eckel, 2011). The adiposity in T2DM patients is predominantly located around the centre of the body, in the abdomen, subscapular and triceps regions. Increased upper body fat, including visceral adiposity, as reflected in increased abdominal girth or waist-to-hip ratio, is associated with insulin resistance (Kahn, 2001) metabolic syndrome and cardiovascular disease. Although obesity is commonly associated with T2DM and has been clearly established to precede its development, it is neither a sufficient nor a necessary metabolic abnormality to cause T2DM. One or more of the other metabolic abnormalities that occur in subjects with T2DM, together

with genetic susceptibility, must also be present in the obese subject before T2DM develops. Obesity is associated with higher fat free acids (NEFA), low-grade inflammation: tumour necrosis factor alpha, interleukin-6 and protein C-reactive (TNFα, IL6, CRP), and lower adiponectin levels. Other potential mechanisms that have been put forward to explain the insulin resistance, include increased lipid oxidation and glucose toxicity (DeFronzo, 1992) impaired incretin effect observed in obesity and T2DM can contribute with β-cell dysfunction leading to the inability of insulin secretion to offset body's insulin resistance resulting in impairment of glucose homeostasis (Nauck, 1997).

At least three mechanisms have been proposed to link obesity to insulin resistance and predispose to T2DM: 1) increased production of adipokines/cytokines, including TNFα, resistin and retinol binding protein-4 (RBP-4), that contribute to insulin resistance as well as reduced levels of adiponectin (Deng, 2010); 2) ectopic fat deposition, particularly in the liver and also in skeletal muscle, and the dysmetabolic sequelae (Larson-Meyer, 2011); and 3) mitochondrial dysfunction, evident by decreased mitochondrial mass or function. Mitochondrial dysfunction could be one of the most important underlying defects linking obesity to diabetes, both by decreasing insulin sensitivity and by compromising β-cell function (Bournat, 2010).

1.4. Nonobese Type 2 Diabetes Mellitus

It is not clear if nonobese and obese forms of T2DM have a similar natural history (Prando, 1998; Eckel, 2011). The obese form of T2DM is the most common, accounting for the majority of cases in caucasoides and other populations such as the Americans Indians, Mexican-Americans, Micronesians and Polynesians. On the other hand, in populations of Far East including Japan, China, Taiwan and Korea, the nonobese form is more common (Bennett, 1992 and IDF Atlas, 2011). Insulin resistance and impaired insulin secretion are usually present in patients with classic type 2 diabetes as well as in most patients with

impaired glucose tolerance. Both play important roles in determining if diabetes development and also in determining the magnitude of the accompanying hyperglycaemia and others metabolic abnormalities. Lean subjects, first-degree T2DM relatives showed impairment in insulin secretion whereas insulin sensitivity was normal (Pimenta, 1995). Lean subjects with normal glucose tolerance and first-degree T2DM relatives showed defective insulin secretion relative to insulin sensitivity, lower insulin sensitivity compared with controls and correlation between NEFA and insulin resistance (Perseghin, 1997). But not only obesity can be implicated in the development of insulin resistant state. Non obese individuals can present insulin resistance by numerous others mechanisms: high fat diets (Gupta, 2012), decreased physical fitness, increased visceral fat accumulation (Eckert, 2012), smoking, pregnancy and certain used medications (Gupta, 2012). It seems that in nonobese T2DM genetic susceptibility may contribute more to the onset of the disease than environmental component does (Rhodes, 2002). When matched for obesity, normal glucose tolerant, first-degree relatives of patients with T2DM had impaired insulin secretion but were not insulin resistant (Van Haeften, 1998; Pimenta, 1995). Normal glucose tolerant monozygotic twins of someone with T2DM had impaired β-cell function, but normal insulin sensitivity; moreover, monozygotic twins who had developed impaired glucose tolerance had the same degree of impaired insulin secretion as those who had maintained normal glucose tolerance, but they had greater body mass index and waist-to-hip ratio and were insulin resistant (Vaag, 1995). Prando and associates studied 147 nonobese and 215 obese subjects to assess the possible differences in insulin secretion and its link with treatment failure with oral hypoglycaemic agents. Obese group showed higher percentage of dietetic control, high insulin secretion with comparable levels of insulin sensitivity. As conclusion of this study obese have high insulin secretion than nonobese subjects, especially in the early years of disease and secondary failure in hypoglycaemic treatment is linked with progressive impairment in insulin secretion for both obese and nonobese T2DM individuals (Prando, 1998). Arner et al., studied elderly

patients divided in four groups nonobese and obese with and without diabetes. In nonobese hyperglycaemic subjects, however, there was no evidence of peripheral insulin resistance with marked secretory defect involving both the first and second phase response (Arner, 1991). Moreover obese diabetic patients were more insulin resistant than nonobese diabetic patients and showed only the first phase insulin response decreased. The second phase was slightly enhanced, in contrast with nonobese diabetic subjects that presented lower first-and second-phase insulin secretion (Felber, 2002).

1.5. Pathogenesis of Type 2 Diabetes Mellitus

The pathogenesis of T2DM is multi-factorial and includes both genetic factors and environmental elements that affect insulin secretion, insulin function and higher hepatic glucose production (DeFronzo, 2004). Genetically, the pancreas responsible to produce insulin at exactly doses, may cannot be able to produce enough insulin to offset the insulin resistance state, given rise to hyperglycaemia. The environment may impair the insulin ability to stimulate glucose uptake by the cells. It provokes hyperglycaemia that could or not be resolved by increase in β-cell function as reviewed by Ferrannini (Ferrannini, 2010). This dynamic equilibrium between β-cell function, represented by β-cell glucose sensitivity, i.e., the ability of β-cell in secret exactly amounts of insulin to offset increments in plasma glucose concentration and the ability of insulin-dependent tissues to recognize promptly insulin hormone and internalizes glucose molecules represent the two major defects observed during the progression of normal glucose tolerance to IGT and finally T2DM. They must be viewed simultaneously because any impairment in one will reflect in a defective response in the other.

In normal glucose tolerant subjects, the increase in insulin secretion that occurs simultaneously with insulin resistance is described by a hyperbolic relationship (Kahn, 1993). Not only insulin concentration in plasma is impaired but also qualitative changes in insulin

secretion are observed in insulin resistant state (O'Rahilly, 1988). Indeed, defects in insulin secretion pulses have been reported as inversely correlated with peripheral insulin sensitivity (Hunter, 1996). Proinsulin, molecule that can be used to measure the correct rate of insulin synthesis, is increased in states of impaired glucose tolerance (IGT and T2DM). Higher glucagon and proinsulin were associated with insulin resistant status (Ferrannini, 2007) reflecting the incapacity of β-cell to synthesises insulin correctly and the insulin to inhibit glucagon secretion by pancreatic α-cells.

Thus, we can conclude that type 2 diabetic subjects manifest multiple disturbances in glucose homeostasis, including: (1) impaired insulin secretion; (2) insulin resistance in muscle, liver, and adipocytes; and (3) abnormalities in splanchnic glucose uptake.

1.5.1. Insulin resistance

Insulin resistance is present when the biological effects of insulin are less than expected for glucose disposal in skeletal muscle, suppression of endogenous glucose production in liver and to mediate lipid metabolism on adipose tissue (DeFronzo, 1992). Insulin resistance is present in lean or obese patients with T2DM, in whom precedes and predicts the hyperglycaemia (Bogardus, 1991), contributing to metabolic syndrome (Reaven, 1988) or being responsible together with the hyperinsulinaemia by the insulin resistance syndrome (Ferrannini, 1992). Augmented ß-cell response represents a compensatory adaptation by the ß-cells to offset the defect in insulin action (Mari, 2005a). During the progression from NGT to IGT there is an increase in insulin resistance and secretion. In the long term, the increased demand for insulin led to ß-cell exhaustion, the development of fasting hyperglycaemia and overt diabetes (Abdul-Ghani, 2006).

Skeletal muscle is the primary site of insulin action in the postprandial state, accounting for about two thirds of glucose uptake. The liver is responsible by uptake the other one third of glucose, both via insulin dependent mechanisms (Abdul-Ghani, 2010). During

euglycaemic hyperinsulinaemic clamp, hyperinsulinaemia inhibits glucose production by the liver and insulin secretion (Ferrannini, 1999). So the glucose uptake stimulated by insulin during the clamp is represented almost only by the muscle metabolism. The skeletal muscle of lean T2DM and obese subjects with normal glucose tolerance (NGT) is resistant to insulin (Felber, 2002).

In nonobese healthy subjects with strong familiar history of T2DM (FH+ subjects) insulin resistance is present long before the development of impairment in glucose metabolism and as severe as in established T2DM (Mari, 2005b; Jensen, 2002).

Liver insulin resistance plays an important role in the T2DM pathogenesis. Mild-age subjects divided by glucose tolerance status were studied during OGTT and low-dose insulin infusion rate to assessing glucose rate of appearance (Ra) using [6-^{3}H] glucose as glucose tracer. Hepatic insulin resistance appears as an early and important defect that characterizes subjects with IGT and mild diabetes (Båvenholm, 2001). In the postabsorptive state liver is responsible to produce glucose to exactly match the obligatory needs of the brain and other tissues that utilize glucose independently of insulin, this uptake of glucose is not impaired in T2DM and is basically equal at basal and postabsorptive state. After glucose ingestion, insulin is released into the portal vein and carried to the liver where binds to specific receptors on the hepatocytes and suppresses hepatic glucose output. Liver insulin resistance results in impaired suppression of HPG contributing to hyperglycaemia. In T2DM subjects with moderate hyperglycaemia and higher fasting insulin plasma concentrations, an increase in basal HPG has been demonstrated been closely correlated with the degree of fasting hyperglycaemia (Ferrannini, 1999). Hepatic insulin resistance may drive an increase in insulin secretion to prevent an increase in hepatic glucose production (HGP) and hyperglycaemia. This chronic ß-cell demand may accelerate the progression to T2DM. Excessive HPG is an important factor in the development of fasting hyperglycaemia. Examining HGP rates in 200 men of similar age, with a range of glucose tolerance degrees, Tripathy et al. have showed that although in

absolute terms the endogenous glucose production (EGP) was not high until late in the stages of glucose intolerance, hepatic insulin resistance was already evident in subjects with IGT (Tripathy, 2004). Because augmented HPG occurs even in the presence of hyperinsulinaemia, a powerful inhibitor of HGP, it is believed that hepatic resistance to insulin is present in postabsorptive state and contributes to the excessive output of glucose by the liver. Because hyperglycaemia also suppresses HGP there may also be glucose resistance with respect to the inhibitory effect of hyperglycaemia on hepatic glucose output (Faerch, 2009).

Insulin also control fat metabolism stimulating fat storage and/or fat free acids (NEFA) release (Groop, 1989). Circulating NEFA levels are markedly increased in obesity and T2DM and evidences suggests a fundamental role of NEFA to induce insulin resistance in peripheral tissues. Prolonged exposition of skeletal muscle and myocytes to high levels of NEFA leads to severe insulin resistance by affecting intracellular signalling pathways in cells (Roden, 2004). When white tissue ability for storage circulating lipids is exceed, NEFA storage starts in others tissues such as liver and skeletal muscle. However, an increased mass stored triglycerides especially in adipocytes and in visceral or deep subcutaneous adipose tissue depots, leads to large adipose cells, which become resistant to the ability of insulin to suppress lipolysis. It leads to increased release and circulating levels of NEFAs which aggravates insulin resistance (decreases glucose uptake and oxidation) in skeletal muscle and liver. When these NEFAs accumulates in other organs than the adipose tissue (ectopic fat storage), they further promote insulin resistance in liver and muscle cells and damage the β-cells in the pancreas. This is known as “lipotoxicity”. The link between intra-hepatic lipids in non-alcoholic fatty liver disease (NAFLD) and hepatic insulin resistance seems tight as well. The excess of NEFAs also promotes hepatic triglyceride production and therefore the dyslipidemia usually seen in T2DM. Ectopic fat is strongly associated with insulin resistance (Gastaldelli, 2011).

Adipocytes synthesize and secrete a host of proteins that collectively are designated as adipocytokines that have local, autocrine and paracrine effects, as well as systemic effects. Low-grade inflammation is associated not only with obesity but also with cardiovascular disease and T2DM (Pickup, 1998). Circulating concentrations of inflammatory markers, such as CRP and IL-6 are independent predictors of the future development of type 2 diabetes in a prospective case-cohort study within the population-based MONICA/KORA Augsburg cohort (Herder, 2011), but failed in another cohort study: the Framingham Heart Study (Dallmeier, 2012). Tumour necrosis factor alpha (TNF-α) have direct effect on insulin sensitivity in skeletal muscle and it has been demonstrated *in vitro*, *in vivo* in animals and *in vivo* in humans (Hotamisligil, 2003). A number of other adipocytokines have been identified, including IL-6, plasminogen activator inhibitor-1 (PAI-1), resistin, IL-1, leptin, adiponectin, and visfatin, all of them exhibiting positive, such adiponectin (Pajvani, 2003) or negative (TNF-α, IL6, resistin) effects on insulin sensitivity. Activation of inflammatory pathways by adipocytokines has been shown to inhibit insulin signal transduction by causing serine/threonine phosphorilation of IRS-1 in multiple insulin-sensitive tissues, including muscle, liver and adipocytes (Hotamisligil, 1996). Thus, expression and secretion into plasma of these and other cytokines could provide a link between insulin resistance and low-grade inflammation in T2DM (Shoelson, 2006). There are several possible explanation for how inflammation in adipose tissue lead to systemic (muscle and liver) insulin resistance in humans: 1) decrease insulin responsiveness in adipose tissue leads to increased lipolysis and elevated NEFA concentrations, which are known to cause insulin resistance in muscle and liver; 2) adipokines released by adipocytes and resident macrophages are released into the circulation and act at distal sites (skeletal muscle and liver), and activate inflammatory (c-Jun N-terminal kinase (JNK), nuclear factor kappa-light-chain-enhancer of activated β-cells (NFK-β) pathways, to impair insulin signalling (Shoelson, 2006; Pajvani, 2003; Pickup, 1998).

1.5.2. Insulin Secretion

Abnormalities in insulin secretion are present in subjects with IGT and in patients with type 2 diabetes (Beck-Nielsen, 1994). Indeed, subjects with IGT can remain in these conditions for several years, or revert to NGT or progress to T2DM (Ferrannini, 2011). Diabetic subjects showed the same abnormalities observed in IGT people but without normal glycaemic control .

Usually during the progression from IGT to T2DM, basal insulin concentrations may be raised several fold than the normal value (Tripathy, 2004), especially in obese hyperglycaemic patients. However, basal β-cell secretory function must is not normal, because the prevailing plasma glucose level also must be taken into account. Hyperglycaemia is the major stimulus for insulin secretion, and when normal individuals are made hyperglycaemic by glucose infusion, circulating levels are much higher than those found in T2DM. Ehrmann et al. has shown that subjects with IGT secrete less insulin at any given glucose level than NGT subjects matched for a similar degree of insulin resistance and obesity (Ehrmann, 2002).

Normal insulin secretion is biphasic and is composed by an early burst of insulin release for about 10 minutes, followed by a graded and progressive release of insulin that persists parallel with hyperglycaemic stimulus. Henquin, studying this phenomenon showed that the first phase insulin secretion is represented by the release of insulin secretory granules. There are a reserve of insulin localized near to the plasmatic membrane of the β-cell and immediately releasable (Henquin, 2000). Glucose is a powerful generator of insulin secretion by stimulation of both pathways involved in insulin secretion: the triggering pathway that occurs early and starts when glucose enters inside β-cells by facilitated diffusion. Glucose is then metabolized, generating by glycolysis a rise in ATP-to-ADP ratio, closure of ATP-sensitive K+ (KATP) channels, membrane depolarization, opening of voltage-operated Ca2+ channels, Ca2+ influx, rise in cytoplasmic free Ca2+ concentration ([Ca2+]i), and activation

of the insulin exocytosis. The mechanism of the amplifying pathway is less known. Increase in β-cell metabolism also generates amplifying signals that augment the efficacy for the $Ca2^{+}$ on the exocytose machinery. Stimulatory hormones and neurotransmitters modestly increase the triggering signal and strongly activate amplifying pathways biochemically distinct from that set into operation by nutrients. Many drugs can increase insulin secretion in vitro, but only few have a therapeutic potential (Henquin, 2004).

This biphasic response is identifiable after intravenous glucose load (IVGTT) (Seltzer, 1967). T2DM subjects have reduced first phase response to oral or intravenous glucose load already at an early stage of their disease (Swinburn, 1995). However, loss of first phase insulin secretion does not appear to be the primary defect responsible for T2DM even if the loss of first phase of insulin secretion have been linked with the increased in EGP observed in T2DM (Luzi, 1989).

Although insulin resistance is considered the initiating event in the pathogenesis of T2DM, β-cell dysfunction must be present for the hyperglycaemia initiation (Ferrannini, 2010). Initially in the T2DM progression increments in FPG can be observed and correlated with insulin resistance. At this point, a compensatory mechanism is already present. In fact, when β-cell function is expressed in relation to insulin resistance observed that β-cell dysfunction is present longer before T2DM (Gastaldelli, 2004 a). As long as the β-cell is able to secrete sufficient amounts of insulin to offset the severity of insulin resistance, glucose tolerance remains normal (Gastaldelli, 2011).

2. Bariatric Surgery

Diet, exercise and prescription medication remain the cornerstones of type 2 diabetes mellitus therapies, but the long-term success rates of lifestyle and drug modifications are disappointing. Despite a vast variety of pharmacological therapies, long-term glycaemic control is difficult, and use of large number of drugs introduces a proportionate risk of

hypoglycaemia. Moreover, most diabetes medications promote weight gain and weight gain in turn, exacerbate the severity of T2DM.

In cases where classic strategies prove insufficient, several types of gastrointestinal (GI) surgery offer alternatives to treat obesity and T2DM. Among severely obese patients, bariatric operations cause significant sustained weight loss, improve obesity-related comorbidities, and reduce mortality in the long term. Currently, bariatric surgery is considered appropriate for individuals with a body mass index (BMI) >35 kg/m^2 and serious obesity-related comorbidities, including T2DM. Operations involving intestinal bypasses exert particularly dramatic effects on diabetes. Mounting evidence indicates that these remarkable effects result not only from weight loss but also from weight-independent antidiabetic mechanisms. Consequently, conventional bariatric procedures and new experimental GI operations are being used to treat T2DM associated with obesity and, increasingly, among less obese or merely overweight patients.

Bariatric procedures were initially classified as restrictive, malabsortive, or combined, reflecting the purported mechanism of weight loss. Restrictive procedures reduce the volume of the stomach to decrease food intake and induce early satiety. Malabsortive procedures, such as biliopancreatic diversion (BPD), shorten the small intestine to decrease nutrient absorption. Combined procedures, such as the Roux-en-Y gastric bypass (RYGB), incorporate both restrictive and malabsortive elements.

2.1. Diabetes Resolution and Improvement after bariatric surgery

Observational evidences suggest that bariatric surgery is associated with 60% to 80% rate of diabetes resolution, defined as discontinuation of all diabetes-related medications and blood glucose levels within the normal range (Vetter, 2009). Recovery from type 2 diabetes was established in 76,8% of the patients who underwent bariatric surgery (Buchwald, 2009). In the surgically treated group of SOS study, T2DM had disappeared in 72% after 2 years. In

the conventionally treated group it was 21%. These percentages seem related to the operative procedures that are used.

The remarkable control of diabetes in severely obese patients together with results from experimental studies showing that GI operations can improve diabetes in both obese and nonobese animals have lead to the concept that surgery may be beneficial for moderately obese patients with T2DM.

2.2 Mechanisms of diabetes remission after gastrointestinal surgery

2.2.1 Weight loss effects

Weight reduction leads to a decline in insulin resistance, better metabolic regulation of patients with T2DM, lower blood pressure and less atherogenic lipid profile (Sjöström, 2004). The Diabetes Prevention Program reported a 58% reduction of the diabetes incidence after modest weight loss of 5.6 kg on average (Knowler, 2002). The 'Swedish obese subjects' (SOS) study has shown that long-term weight reduction, achieved by bariatric surgery improves the cardiovascular risk profile and decreases the overall mortality (Knowler, 2002). In the Pima Indians with normal tolerance to glucose long-term weight changes have been associated with reciprocal change in insulin action (Weyer, 2000). However, weight loss did not explain all amelioration observed in morbidly obese subjects after bariatric surgery.

Observations that glycaemic control improves early after surgery, before important weight loss, has led to the recognition that bariatric surgery induces modifications that are independent of weight loss. Comparisons between BPD and RYGB effects on glucose metabolism realized by Muscelli et al. demonstrated differentiated changes in insulin sensitivity in face of paired weight loss (Muscelli, 2005). Camastra et al. reported that RYGB surgery promotes early (one month) improvement in glucose metabolism by increasing hepatic insulin sensitivity more than in peripheral tissues. Moreover, studying the same subjects after two years, improvement in peripheral IS was correlated with weight loss

(Camastra, 2011). Others investigated the effect of BPD surgery and found that diversely of RYGB, IS and first phase insulin secretion improved in a few days after surgery (Salinari, 2009).

Weight loss has been also linked to amelioration of β-cell function and insulin secretion. Homeostasis model (HOMA-β), Acute insulin response (AIR) and mathematical modelling were used to assess this parameter (Briatore, 2008; Ferrannini, 2009; Camastra, 2011). Morbidly obese T2DM patients restored first phase insulin secretion earlier after BPD (Briatore, 2008). Using mathematical modelling to assess the β-cell function after BPD and RYGB surgery, Mari et al. showed that all of 3 mathematical model parameters improved after weight loss induced by surgery (Mari, 2006). Among these parameters, β-cell glucose sensitivity is the main determinant of IGT and T2DM, rate sensitivity and potentiation being relatively minor factors.

Finally, several peptides expressed and releases by adipose tissue are metabolically active and are also changed by bariatric surgery.

2.2.2. Effects of BPD on Insulin Sensitivity

Insulin resistance is present when the biological effects of insulin are less than expected for one or more of the insulin action: glucose disposal in skeletal muscle, suppression of endogenous glucose production primarily in liver (Rizza, 2010) and to mediate lipid metabolism on adipose tissue (Ferrannini, 1999). Most of available evidence suggests that insulin resistance is the primary metabolic disturbance in T2DM and that the augmented ß-cell response represents a compensatory adaptation by the ß-cells to offset the defect in insulin action (Ferrannini, 1992; Arner, 1991). Most T2DM are obese and obese normoglycaemic subjects are also insulin resistant. Overweight and the adiposity are determinants of insulin sensitivity (Ferrannini, 1997) and loss of weight by diet or surgery is able to enhance IS (Sjöström, 2013). It is known that weight loss promoted by bariatric

surgery improve IS in subjects with NGT, IGT and T2DM. However some investigators have found greater defect on insulin secretion (AIR) than in IS in non obese T2DM patients (Arner, 1991). Subjects with non-morbidly obesity (BMI < 35 kg/m2) showed an improved IS after loss of 5kg in average and only 1.5 kg of fat mass (Chiellini, 2009). Suggesting that more than the quantity, site of fat accumulation instead of amount of fat gained or lost is very important. Moreover when fat is removed selectively by surgeries as liposuction, does not promote improvements in IS as could be expected (Ferrannini, 2009). Ectopic fat has been linked with IR independently of total body fat mass (Gastaldelli, 2011). On the other hand, omental adipose tissue is lipolytically active and releases a large quantity of fatty acids. These fatty acids are converted into triglycerides. The liver in turn produces increased free fatty acids and releases the same into the systemic circulation which contributes to peripheral insulin resistance directly as well as by increasing muscle triglyceride. Removal of visceral fat from diabetic mice have restored IS (Gabriely, 2002) but RYGB plus omentectomy showed no additional improvement in IS that observed in control group (Lima, 2010).

Two groups of obese patients (with or without T2DM) underwent BPD demonstrated that the obese T2DM group had before surgery high FPG, IR (measured by HOMA-IR) and lesser AIR (IVGTT). One month after surgery FPG and HOMA-IR were reduced at levels similar to those observed in the obese non-diabetic control group while AIR increases only in T2DM subjects, but without achieves normal values (Briatore, 2008). Two years after BPD surgery obese non diabetic have showed levels of IS greater than lean control subjects and greater than obese non diabetic subjects underwent RYGB (Muscelli, 2005). NGT, IGT and T2DM subjects were submitted to BPD and compared to two control groups, lean and obese subjects that underwent abdominal surgery for other reasons (mainly cholecystectomy and abdominal hernia). IS did not change in the control groups while increased only 10 days after BPD surgery in all patients before any significant weight loss. The improvement in IS could not be explain by changes in NEFA, adiponectin or leptin concentrations (Mari, 2006).

Obesity is a state of heightened inflammation as noted by increased serum levels of inflammatory markers like TNF-α and IL-1. These molecules induce insulin resistance by inhibition of insulin signal transduction. Interleukin-6 (IL-6) and TNF-α have been shown to contribute to hepatic insulin resistance in animal models (Barbuio, 2007). Bariatric surgery has been shown to decrease the levels of IL-1, whereas there is no consensus about the change in TNF-α and IL-6 after bariatric surgery (Bruun, 2007; Vazquez, 2005).

Results as presented above leads to the hypothesis that some unknown regulator factors of insulin sensitivity are directly affected by BPD as well as by RYGB surgery. In fact, Rubino et al. showed that exclusion of duodenum from the alimentary transit in non-obese rats Goto-Kakyzaky (T2DM model) improved glucose metabolism as observed in BPD surgery (Rubino, 2004 b).

2.2.3. Effects of BPD on β-cell function

Maintenance of normal glucose tolerance is dependent on the finely tuned balance between insulin secretion and insulin action (Reaven, 1989 e Wogensen 1991 e Ferrannini, 1997). NGT subjects show insulin sensibility that varies greatly but nevertheless glucose tolerance remains normal in function of β-cell compensation that offset tissue resistance to insulin. Biliopancreatic diversion is able to enhance both IS and β-cell function earlier after surgery and these effects of BPD have been observed after long term (Pontiroli, 2010; Muscelli, 2005). The progression from normal glucose tolerance to IGT and finally to T2DM is characterized by progressive hyperinsulinaemia, loss of β-cell mass that leads to the incapacity of β-cell to offset tissue insensitivity. A basic genetic defect in the insulin gene has been proposed to explain the disturbance in insulin secretion but anyone was able to prove or find any significant abnormalities (Das, 2006). The most likely explanation for the acquired defect in insulin secretion relates to the concept of glucose toxicity (Del Prato, 2009). The pathological sequence that leads to T2DM would be the superposition of obese-related insulin

resistance upon a β-cell with a geneticatilly limited capacity to compensate (Gerich, 2000). Studying obese and non obese T2DM subjects Prando el al. found that mainly in the earlier years of disease obese T2DM showed higher levels of insulin secretion than non obese T2DM subjects; that BMI was more related with insulin resistance and that after several years (10 years) the differences in insulin secretion between obese and non obese were negligible (Prando, 1998; Arner, 1991). The impact of bariatric surgery in obese subjects with or without T2DM has been done using the acute insulin response (AIR), homeostasis model (HOMA-B), insulinogenic index and mathematical modelling. All authors described improvements in β-cell function after BPD and after RYGB when the comparison has been made. Obese NGT and T2DM subjects have been studied for AIR using glucose and arginina stimulus (Briatore, 2010). One month after BPD fasting insulin, Homa-IR, AIR for glucose and arginine improved but AIR in T2DM did not were similar for NGT and T2DM.

Effects of BPD on β-cell function has been explored using mathematical modelling to assess insulin secretion by deconvolution of C-peptide proposed by Van Cauter (Van Cauter, 1992) and revised by Mari A. (Mari, 2008) and to assess the relationship between insulin secretion and glucose concentration (β-cell glucose sensitivity - BCGS). After BPD obese subjects showed improvements in all parameters of mathematical model. BPD induces a reduction of insulin secretion rate at fasting and during meal or OGTT stimulation. Guidone et al, found a correlation between decrease in insulin sensitivity and β-cell glucose sensitivity (Guidone, 2006). Camastra et al, studying morbidly obese T2DM subjects before and after Roux-en-Y showed that patients were more insulin resistant (IR) with decreased β-cell function when compared with control group. Patients revert completely the IR and β-cell function proportionally with weight loss (Camastra, 2007).

2.3. The entero-insular axis

Food intake, transit and absorption are regulated by a complex network including the gastrointestinal system, the liver and the brain (Cummings, 2007 b). Surgical rearrangements of gastrointestinal tract may can change hormones concentrations and per consequence, alteration in glucose metabolism. Changes in gastro-intestinal hormones (GI) have been analysed for those bariatric surgeries that alter food transit. Glucagon-like peptide 1 (GLP-1) is a potent insulin agonist that is secreted by L-cells of the distal ileum in response to ingested nutrients and is inactivated by the enzyme dipeptidyl peptidase IV (DPP-IV). GLP-1 potentiates insulin release in a glucose dependent manner. Insulin increases affect α-cells inhibiting glucagon secretion (Holst, 2007). GLP-1 response to a mixed meal or OGTT is impaired in obesity and T2DM and increases after RYGB and BPD (Rubino, 2004a; Laferrère, 2008; Mingrone, 2009). Muscelli et al. have quantified the incretin effect on insulin secretion during a mixed meal test followed by an intravenous glucose test that duplicate previous MTT glycaemic values but without meal stimulation. GLP-1 and GIP account for 50% to 60% of nutrients stimulated insulin release (Muscelli, 2006) increases β-cell mass trough regulation of proliferation, neogenesis, and apoptosis (Hansotia, 2005). It is not clear what the precise mechanisms is by which GLP-1 release is raced up by anatomical rearrangements that either by-pass the duodenum and upper jejunum (RYGB) or exclude the larger part of the entire gastrointestinal tract from food transit (BPD). Moreover GLP-1 also delays digestion and blunts postprandial glycaemia and acts on the central nervous systems to induce satiety and decrease food intake (Gutzwiller, 1999).

Glucose dependent insulinotropic peptide is secreted by K-cells of the proximal gut in response to carbohydrate and lipid rich meals. It acts on pancreatic β-cells to increase insulin secretion through the same mechanism as GLP-1, although it is less potent, and also stimulates lipoprotein lipase activity (Meier, 2002). But does not affect gastric emptying or satiety.

Others non-incretins gut peptides that also modulate glucose metabolism have changed your plasma profile after bariatric surgery. Neuropeptide YY (PYY), like GLP-1 is secreted by the L-cells of the distal intestine. Peptide YY is present in 2 molecular forms: PYY 1-36 and PYY 3-36 a cleavage product. PYY increases satiety and delays gastric emptying through neuropeptide Y-receptors subtypes in the central and peripheral nervous systems. Intravenous PYY 3-36 increases satiety and decreases food intake in humans (Vetter, 2009). Ghrelin is another hormone secreted in response to meal stimulation by cells from gastric fundus and proximal intestine and acts on the hypothalamus to regulate appetite. Systemic ghrelin levels increases before a meal and decreases afterward, stimuling appetite, and food intake and suppressing energy expenditure and fat catabolism. There is a negative correlation between body weight and serum ghrelin levels. Increases in ghrelin level after surgery or diet suggest its role in body weight regulation (Karra, 2010).

Rubino et al. found that excluding a short segment of proximal intestine from food passage improved glucose tolerance, whereas restoring duodenal transit re-established glucose intolerance. This observation has lead to the foregut hypothesis, which holds that contact of nutrients with duodenal mucosa generates signals (hormonal and/or neural) that interfere with glucose metabolism and insulin action; by-passing duodenal passage (as RYGB and BDP) would remove this inhibition (Rubino, 2004 b).

3. Protocol End-points

We hypothesizing that changes promoting by BPD on intestinal limb and as consequence on entero-insular axis will be able to decrease plasma glucose concentration, promoting improvements in HbA_{1c}%, in insulin secretion (ISecr) and action (M value). Similar as observed in morbidly obese subjects (BMI≥40kg/m^2) after bariatric surgery, we expect that changes are to be stable in the long time. Based in observations in morbidly obese and T2DM very obese subjects (BMI≥35kg/m^2) we expect great reduction or disappearance

of insulin resistance (IR) and improvement of β-cell function, represented here by parameters obtained from mathematical model applied on MMT data (Fasting insulin secretion, Total insulin secretion, beta-cell glucose sensitivity, rate sensitivity and potentiation factor) with consecutive improvement of clinical T2DM symptoms and others components of metabolic syndrome. Bariatric surgery is actually used as weight loss treatment in patients with BMI $\geq$40 kg/m^2 or BMI $\geq$35 kg/m^2 with comorbidities. To our patients excessive weight loss is not desirable. Surgery was re-designed to avoid it. We have targeted a BMI not lesser than 22 kg/m^2 as acceptable final body mass index.

To assess metabolic effects of BPD in non morbidly obese subjects we have used 3 different stimulus.

1. Oral glucose tolerance test (OGTT),
2. Mixed meal tolerance test (MTT),
3. Euglycaemic hyperinsulinaemic clamp (CLAMP).

During the tests we measured:

- plasma glucose response to standard OGTT in order to determine glucose tolerance status before and post surgery,
- plasma glucose, Fat free acids, insulin, C-peptide, glucagon and GLP-1 response to a mixed meal stimulation,
- insulin secretion during MTT using C-peptide deconvolution and β-cell function by mathematical modelling,
- whole body glucose uptake by euglycaemic hyperinsulinaemic clamp and inhibition of hepatic glucose production (EGP) by tracer methods,
- Lipid profile : total cholesterol, HDL and LDL-cholesterol and triglycerides,
- Liver profile: ALT, AST an GGT
- Body weight, waist and hip circumference

Results obtained by this protocol can be used to better understanding the pathophysiological mechanisms of T2DM; BPD effects on T2DM and mechanisms of T2DM remission after bariatric surgery.

4. MATERIAL AND METHODS

4.1. Study Population

Fifteen patients (6 men, 9 women, 55±1 years, BMI=28.3±0.6 kg/m^2, range 24.5-33.1 kg/m^2) with T2DM (duration 16±2 years, range 6-27 years, HbA_{1c}=8.5±0.4%, range 6.8-11.5%) on treatment with metformin+sulfonylureas (Oral Hypoglicemiants Agents - OHA, n=6) or OHA plus insulin (n=9, mean dose=30 IU/day, range 10-90 IU/day) participated in the study. All of them had shown history of poor metabolic control under OHA treatment and comorbidities linked to T2DM. Patients were studied at baseline (*Pre*), ~2 months after surgery (*Post1*), and one year after surgery (*Post2*). Each patient underwent a 3-hour oral glucose tolerance test (OGTT), a 5-hour mixed meal test (MTT), and a euglycaemic hyperinsulinaemic clamp (CLAMP) on separate days over two weeks (**Fig. 1**). The tests were performed after an overnight fast; antidiabetic medications were withdrawn 3 days before testing. When required to avoid hyperglycaemia, regular insulin was used until 8 hours before the metabolic tests.

Fifteen nondiabetic volunteers, matched to the patient group by gender, age (51±3 years), and BMI (30.2±0.9 kg/m^2), served as a control group; they all received the MTT, and eight of them also received the euglycaemic clamp.

Patients were submitted to the metabolic tests and surgery at the San Martino Hospital - Genova, Italy from 2008 to 2012 instead control group subjects were studied at Santa Chiara Hospital - Pisa in the same period.

4.2. Subjects Enrolment

Inclusion criteria:

- Both genders, age between 35 and 65 years old;

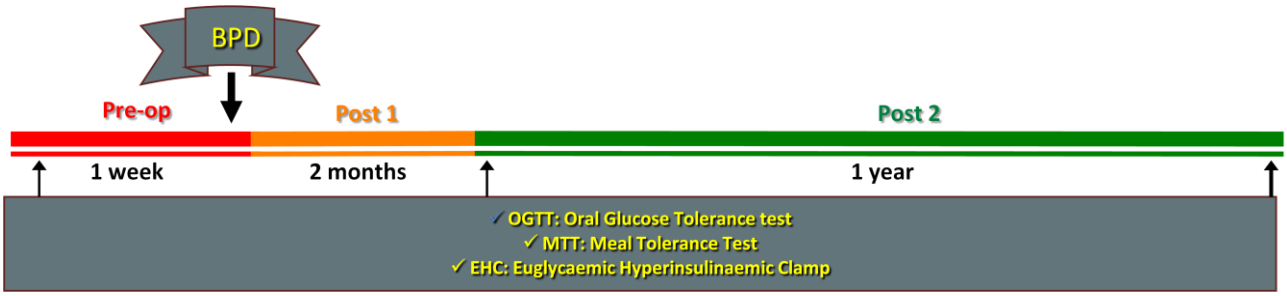
  Type 2 diabetes diagnoses longer than 5 years, poor glycaemic control ($HbA_{1c} \geq 8\%$) on standard of care diabetes therapy;
- Presence of some comorbidities (dyslipidemia, hypertension, neuropathy, retinopathy, cardiovascular disease or stroke),
- Possibility to participate to the follow-up protocol.

Exclusion criteria:

- Anti-glutamic acid decarboxylase (GAD) antibodies or plasma C-peptide lesser than 0.5 ng/ml,
- Liver cirrhosis, renal failure, history of malignancy or malignant neoplasm in place, severe inflammatory, neurological or cardiovascular complications in act,
- Pregnancy or any condition that at the discretion of the head of the study could represent risk to the patient or could affect the protocol results.

Diabetes remission was defined as an $HbA_{1c} \leq 6.5\%$, fasting plasma glucose (FPG) ≤ 7 mmol/l, and a 2-hour plasma glucose ≤ 11.1 mmol/l during the OGTT without any anti-diabetic medication.

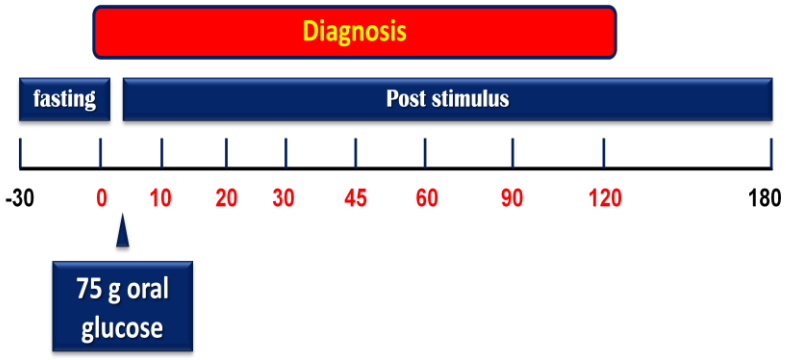
Fig. 1: Schematic protocol representation.



4.3. Oral Glucose Tolerance Test (OGTT)

A standard 75-g oral glucose tolerance test (OGTT) was performed in each patient (**Fig. 2**). Venous blood samples were collected at -30, 0, 30, 60, 90, 120, and 180 min after glucose load from catheter localized in antecubital vein for determination of plasma glucose concentrations. Patients were studied after a rest period and keep lying for all study. Results obtained from OGTT were used to evaluate a) glucose tolerance status before and after surgery and b) T2DM remission after surgery.

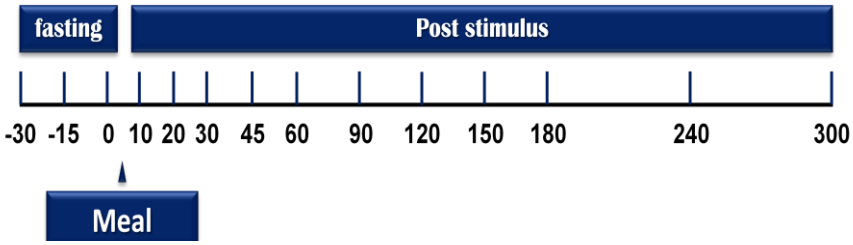
Fig. 2: Schematic representation of oral glucose tolerance test - OGTT



4.4. Mixed Meal Tolerance Test (MMT)

Meal stimulation was performed, in a separate day, at fasting state, following a 30 minutes stabilization period. Patients were invited to eat one boiled egg, 50-g of parmesan cheese, and 75-g of an aqueous glucose solution. The MMT composition was: 54% carbohydrate, 30% lipid, 16% protein, totalizing 561 kcal (**Fig. 3**). Venous blood samples were collected at -30, 0, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, and 300 min from a catheter placed at antecubital vein. Plasma samples were drawn and stored for future measurements. Plasma glucose insulin and C-peptide concentrations were assayed to assess ß-cell function using C-peptide deconvolution associated to mathematical modelling to reconstruct insulin secretion rates. Time course of plasma glucagon, GLP-1 (glucagon-like peptide 1) and Fat free acids (NEFA) were also assessed.

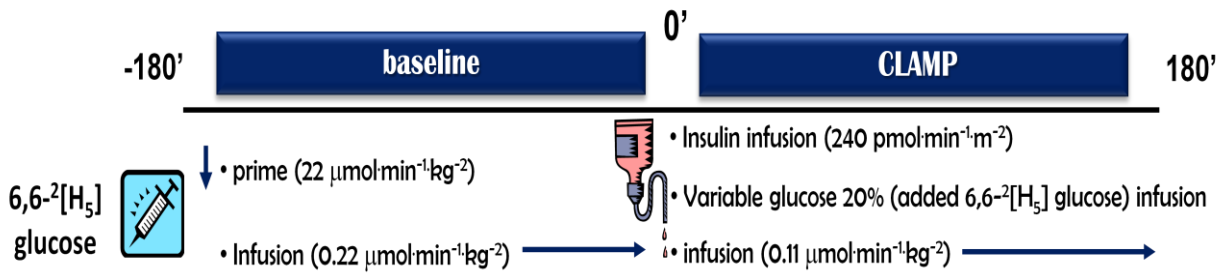
Fig. 3: Schematic representation of mixed meal tolerance test - MMT



4.5. Euglycaemic Hyperinsulinaemic clamp

Euglycaemic Hyperinsulinaemic clamp was performed after 3 hours basal period (**Fig. 4**). Primed-continuous infusion of 6,6-$^{2}[H_5]$ glucose (0.22 μmol·min^{-1}·kg^{-1}; prime: 22.0 μmol/kg*[FPG/5]) were started at -180 minutes in an antecubital vein and continued throughout the study. At time -120, -40, -20, -10 and 0 min blood samples were obtained from an arterialised vein for measurement of glucose, NEFA, insulin, C-peptide and for tracer determinations. At time zero, a primed-continuous insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) insulin infusion (240 pmol·min^{-1}·m^{-2}) was started and continued for 180 minutes. At time zero, 6,6-$^{2}[H_5]$ glucose was decreased (from 0.22 to 0.11 μmol·min^{-1}·kg^{-1}) and continued until the end of the clamp. During the clamp, a variable rate of 20% of dextrose solution, enriched with 6,6-$^{2}[H_5]$ glucose, was infused to maintain euglycaemia. Plasma glucose levels were measured every 5 minutes throughout the clamp. Patients that had FPG ≥5.5 pmol/l received the same glucose clamp technique described above with a minimum (0.5 mg·min^{-1}·m^{-2}) of 20% glucose solution infusion until reach the euglycaemia or until the time 80 min, when plasma glucose concentration were clamped at the plasma glucose level observed at this time. Blood samples were drawn at each 20 min for measurement of glucose, NEFA, insulin, C-peptide and for tracer determinations. Additional plasma samples were stored to future determinations.

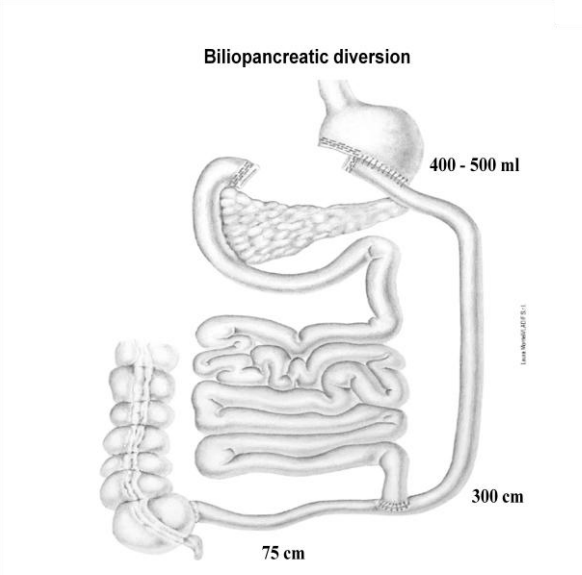
Fig. 4: Schematic representation of euglycaemic hyperinsulinaemic clamp - CLAMP



4.6. Surgery

Patients were submitted to a modified biliopancreatic diversion (BPD) procedure. During BPD a 60% gastric resection with stapled closure of the duodenal stump was made resulting in a residual stomach volume of ~400-500 ml; the small bowel was then transected ~2.5 meters from the ileocecal valve, and the distal end was anastomosed to remaining stomach forming the alimentary limb. The proximal end of the ileum, comprising the remaining small bowel carrying the biliopancreatic juice and excluded from food transit, was anastomosed to the alimentary limb at ~75 cm from the ileocecal valve forming the common limb where nutrients and biliopancreatic juice mix (**Fig. 5**).

Fig. 5: Schematic representation of modified BPD for non-morbidly obese T2DM subjects.



4.7. Analytical procedures

Plasma glucose was measured by the glucose oxidase technique (Analox GM-9, Analox Instruments USA Inc, Lunenburg, MA, USA). Serum insulin and C-peptide were measured by a specific time-resolved immunofluorometric assay (TR-IFMA) (AutoDELFIA, Insulin and C-peptide kit, Wallac Oy, Turku, Finland). Plasma glucagon was assayed by radioimmunoassay (Millipore Corporation, Billerica, MA, USA), total plasma glucagon-like peptide-1 (GLP-1) was assayed by ELISA technique (Millipore Corporation, Billerica, MA, USA). Blood samples for C-peptide, insulin and glucagon were drawn using a protease inhibitor (5 μl/ml of 10 mg/ml gabexate mesilate); for GLP-1 measurements blood was drawn into tubes containing a DPP-IV inhibitor (Diprotin A, 10 mM, Sigma Aldrich, Saint Louis, MO, USA). No significant cross-reactivity was declared by kits manufacturers. The sensitivity ranges are: 0.003 nmol/l and 1.39 pmol/l for the C-peptide and insulin assay, respectively, 18.5 pg/ml for glucagon, and 1.5 pM for GLP-1.

Gas chromatography/mass spectrometry - GCMS (GC, Hewlett Packard 5890 series II plus and MS, Hewlett Packard 5972) was used to determine 6,6-[$^{2}H_{2}$]glucose enrichment in the plasma. The method employs deproteinization of plasma samples with methanol (for liquid chromatography - Merck KGaA, Germania) followed by a derivatization of the labelled and non-labelled glucose with a solution of acetic anhydride (for liquid chromatography - Sigma Aldrich, Saint Louis, MO, USA) and pyridine (for liquid chromatography - Merck KGaA, Germania) in order to produce the corresponding glucose penta-acetate derivative. Samples were injected in GCMS in a solution of ethyl-acetate (for liquid chromatography - Sigma Aldrich, Saint Louis, MO, USA). The ratio between labelled and non-labelled glucose was used to calculate the endogenous glucose production.

Fat free acids (NEFA) were measured by standard spectrophotometric methods (NEFA kit, Wako Chemicals GmbH, Neuss, Germany) on a Synchron Clinical System CX4

(Beckman Instruments, Fullerton, USA). Triglycerides, serum total cholesterol, HDL and LDL-cholesterol were assayed in central laboratory by standard techniques.

4.8. Insulin secretion

Van Cauter described at 1991 a two-compartment model that estimates insulin secretion rates from C-peptides concentrations in plasma (Van Cauter, 1991) after determination of C-peptide clearance using *iv* injection of biosynthetic C-peptide in a broad range of individuals with different status of glucose tolerance and BMI. Based on these findings, a simple procedure to derive standard parameters for C-peptide clearance taking into account degree of obesity, sex, and age was defined. The model β-cell function was resolved from the MTT using a mathematical model that describes the relationship between insulin secretion and glucose concentration (Mari, 2006). The model expresses insulin secretion (in pmol per min per square metre of body surface area) as the sum of two components. The first component represents the dependence of insulin secretion on absolute glucose concentration at any time point during the MTT, and is characterised by a dose-response function relating the two variables. The characteristic parameter of the dose-response, *i.e.*, the mean slope within the observed glucose range, is denoted as *Beta-cell glucose sensitivity* (BCGS) by analogy with insulin sensitivity (slope of the dose-response of insulin-mediated glucose uptake *vs.* insulin concentrations). Thus, glucose sensitivity as used here is not meant to measure the multiple cellular phenomena responsible for glucose sensing (or stimulus/secretion coupling) but only as a metrics for the *in vivo* output of all glucose sensing pathways.

In the mathematical model, the dose-response is modulated by a potentiation factor, which accounts for the fact that during an acute stimulation insulin secretion is higher on the descending phase of hyperglycaemia than at the same glucose concentration on the ascending phase. The second insulin secretion component, denoted as rate sensitivity, represents the dependence of insulin secretion on the rate of change of glucose concentration. The model

parameters were estimated from glucose and C-peptide concentration by regularised least-squares.

4.9. Data and Statistical Analysis

Insulin sensitivity was calculated as the M value during the final 40 min of the 3-hour clamp (normalized to the fat-free mass, $\mu mol \cdot min^{-1} \cdot kg_{ffm}^{-1}$) as well as the ratio of the M value to the mean plasma insulin concentration measured during the same interval (M/I, in units of $\mu mol \cdot min^{-1} \cdot kg_{ffm}^{-1} \cdot mM^{-1}$).

Glucose, C-peptide, glucagon and GLP-1 areas under the time-concentration curves (AUC) were calculated by the trapezium rule.

The pre-hepatic insulin-to-glucagon molar concentration ratio was estimated by the following formula: $(ISR(t)/hPF + [I(t)])/\{[Gg(t)] * (1 + MCR_{Gg}/hPF)\}$ where ISR(t) is the insulin secretion rate at time t, hPF is hepatic plasma flow; [I(t)] and [Gg(t)] are the measured (peripheral) plasma concentrations of insulin and glucagon at time (t); and MCR_{Gg} is the metabolic clearance rate of glucagon. The hPF was estimated by multiplying the cardiac index ($3.2\ L \cdot min^{-1} \cdot m^{-2}$) by a plasma-to-blood ratio of 0.6, and by assuming that hepatic blood flow is 30% of cardiac index (= $0.576\ L \cdot min^{-1} \cdot m^{-2}$). MCR_{Gg} was taken to be $0.537\ L \cdot min^{-1} \cdot m^{-2}$ (Fisher, 1976).

Data are given as means ± SEM (or median and interquartile range for variables with a non-normal distribution). Groups were compared by the Mann-Whitney test. BPD effect was analyzed using by Wilcoxon signed rank test. Time series were analyzed by ANOVA for repeated measures.

4.10. Ethical Considerations

The study was approved by the local ethics committee. The nature and purpose of the study were carefully explained to all participants before they provided written consent to participate.

5. RESULTS

5.1. Anthropometrical and fasting data

At baseline, patients had higher fasting plasma glucose (FGP), fat free acids (NEFA) and triglycerides than controls (CT group), but similar BMI (**Table 1**).

Surgery caused weight loss (an average of -12 kg at 2 months, -14 kg at one year), while HbA_{1c} decreased from 8.6±1.3 to 6.7±0.9% at 2 months to 6.0±1.0% at 1 year. Waist and hip decreased was proportioned to weight loss and was associated with the time after surgery. The ratio between waist and hip (W/H ratio) decreased from 1.01±0.09 to 0.96±0.08 at 2 months and 0.94±0.04 at 1 year (p=0.0001) (**Fig. 6**).

FPG decreased after surgery about 60% in both follow-ups, but, remained higher than in CT group (p=0.0007 at *Post 1* and *Post 2*) (Table 1). By the defined remission criteria, diabetes was in full remission in 6 patients, at 1 year. At 1year, only remitter patients showed FPG at normal levels (5.7±0.5 mmol/l; p = ns *vs.* CT), whereas in non-remitters FPG was 8.3±0.4 mmol/l, p=0.005 *vs*. CT group (**Table 5**).

Fasting plasma insulin, C-peptide (FPC-pep) and NEFA decreased early after surgery (*Post1*) and did not change in the long term (*Post2*). Fasting plasma glucagon and GLP-1, similar to CT group at baseline, were not affected by BPD (Table 1).

Total and LDL cholesterol at baseline were similar to the CT group, and decreased to levels significantly lower than in the later group at both post surgery evaluations. HDL cholesterol decreased only at *Post 1*, whereas plasma triglycerides, higher than in the CT

group at baseline, did not change after the BPD (Table 1). The lipid profile and all its changes were similar between remitters and non remitters (**Table 6**).

Two months after surgery, all patients stopped OHA and 8 remained on insulin therapy alone (mean dose=14 IU/day); at 1 year, 6 patients were still on insulin therapy (mean dose=12 IU/day).

5.2. Oral glucose tolerance test data

The plasma glucose response to the OGTT was largely improved already at 2 months, and changed little thereafter (**Fig. 7**). Two hours plasma glucose, similar to the FPG, also decreased about 60% at *Post 1* and *Post 2*, from 21±1.4 mmol/l at baseline to 13±0.8 and 12±0.9 mmol/l respectively (p=0.008 and p=0.004 *vs. Pre*). After surgery, the OGTT glucose response changed mainly regarding the plasma concentration and little the time course of the curve (Fig. 7). Maximal plasma concentrations were reached around 90' and 120' minutes after glucose loading. Mean values for glucose peak were 27±1.5 mmol/l at baseline, 18±0.8 and 18±0.8 mmol/l at two months and one year after surgery respectively (p=0.001 by ANOVA). Area and incremental area under plasma glucose curve decreased early after surgery and did not change after 1 year (p=0.001 by ANOVA) (Fig. 7).

5.3. Mixed meal tolerance test data

Results from MMT showed prompt improvement in glucose metabolism already at two months after surgery and stable time course at 1 year. Therefore, biliopancreatic diversion caused clear improvement in the plasma glucose response to meal stimulation (**Table 2 and Fig. 8**). Before surgery, the plasma glucose AUC was significantly higher than in control group (5.5±0.1 and 1.8±0.4 $mol.l^{-1}.h^{-1}$; $p \leq 0.001$). One year later AUC glucose decreased significantly compared to baseline values, to 3.1±0.2 ($mol.l^{-1}.h^{-1}$) p=0.001, but was yet higher

than in CT group $p \leq 0.001$. At baseline, insulin response to MMT was blunted in diabetic patients (Fig. 8 and Table 2). After surgery, decreased to levels lower than in the control group, both, at fasting and after meal stimulation, and assessed as area under the curve or incremental AUC. The decreased response was observed soon at 2 months and did not change at 1 year ($p<0.01$ for both follow-up periods).

Glucagon and GLP-1 responses to MMT were also investigated. While AUC glucagon (p= ns *vs.* CT) showed a little but significant increment 2 months after surgery that returned to baseline levels at 1 year after surgery; AUC GLP-1, similar to control group at baseline, increased significantly at both *Post1* and *Post2* evaluations ($p=0.002$ and $p=0.03$, respectively). The elevated plasma NEFA response decreased during the MMT till to reach levels similar to the control group at 1 year. Analysing the incremental area under the curves from MMT for glucagon, GLP-1 and NEFA we observed different responses by each one. The incremental area of glucagon increased significantly at 2 months after surgery returning to levels similar to the control group at 1 year. The same response was also observed for incremental area of NEFA, while GLP-1 incremental area, greater than the control group at baseline, increased even more in both follow-up periods (**Fig. 9** and Table 2).

The estimated mean prehepatic insulin-to-glucagon molar concentration ratio was significantly higher in patients than controls, and after surgery decreased significantly to similar values (Table 2). However, the profile of the curves remained blunted as compared to the CT group (**Fig 15**).

Results from MMT were also divided by remitters and non remitters according our strict stated criteria (**Table 7**). Analysing these results, we observed that both groups, remitters and non-remitters, were very similar at baseline. However, remitters showed higher incremental AUC insulin and C-peptide and lower AUC GLP-1 than non remitters. At 2 months follow-up remitters showed lower plasma glucose AUC and higher insulin and C-peptide responses. Finally, at one year follow-up, plasma glucose values were lower in

remitters and C-peptide response was still higher when compared with non remitters. All of other variables studied were similar between the groups (Table 7).

5.4. β-cell function during MMT

Insulin secretion assessed by mathematical modelling and deconvolution of C-peptide during MMT showed the blunted and protracted profile typical of T2DM (**Fig. 10**), such that fasting insulin secretion was higher than in controls but total insulin output during the 5 hours of the MMT was similar (**Table 3**). All three parameters of ß-cell function, *i.e.,* glucose sensitivity, rate sensitivity, and potentiation, were severely compromised in the patients at baseline; following surgery, each of them improved significantly (**Table 3**), but only potentiation was normalized. In particular, ß-cell glucose sensitivity was still markedly depressed in comparison with the control group (**Fig. 11-12**). At baseline, parameters of ß-cell function as fasting ISR as well total ISR were higher in those that remitter of T2DM than in non-remitters. This difference was not observed 2 months after surgery. But at this moment ß-cell glucose sensitivity (BCGS) was higher in remitters that in non-remitters even if without statistical significance p=0.07. However, at one year we observed significant difference in BCGS, 46[33] to 16[18] ($pmol \cdot min^{-1} \cdot m^{-2} \cdot mM^{-1}$) for remitters and non-remitters respectively, p=0.006 (**Table 8**).

5.5. Euglycaemic hyperinsulinaemic clamp data

Before surgery, insulin sensitivity was markedly depressed in patients as compared to controls (p=0.0002), but it doubled at 2 months post surgery, and remained essentially unchanged at 1 year (p<0.0001 by ANOVA repeated measures testing) (**Fig. 13 and Table 4**). Insulin concentration obtained during the clamp steady state was lower after surgery than at baseline. These results agreed to the increase in insulin clearance observed during the clamp (data not show).

Before surgery, EGP was higher in patients than controls both under fasting conditions and during the clamp. At both times after surgery, fasting EGP was significantly lower than preoperatively, and no longer different from controls. Post-operatively, during the clamp EGP was significantly better than before surgery, but still higher than in controls (**Fig. 14** and Table 4). Before surgery, insulin-mediated glucose disposal was markedly depressed in patients as compared to controls, whether in absolute terms (*i.e.*, µmol/min from the tracer-determined Rd) or as the M value from the clamp (p=0.0002). At 2 months postsurgery, insulin-mediated glucose disposal had increased to levels no longer significantly different from controls, and remained essentially unchanged at 1 year.

None of the insulin action parameters were different between remitters and non remitters and the observed improvements were similar in these groups at both post surgery evaluations (**Table 9**).

6. DISCUSSION

We studied non-morbidly obese subjects (BMI<33 kg/m^2) characterized by long-standing, poorly controlled T2DM in spite of active pharmacological treatment. As expected, they were strongly insulin resistant in peripheral tissues (as reduced insulin-mediated glucose uptake under euglycaemic conditions), in the liver (as elevated fasting EGP and reduced EGP suppression during the clamp), and in adipose tissue (manifest as raised NEFA in the face of hyperinsulinaemia). Furthermore, all indices of ß-cell function were severely compromised; in particular, ß-cell glucose sensitivity (*i.e.*, the intrinsic ability of the ß-cell to sense and respond to dynamic glucose changes in a timely and quantitatively appropriate fashion) was only 20% of that of age- and BMI-matched nondiabetic controls. Insulin release was therefore sustained at near-normal levels by the elevated glucose concentrations. The hormonal milieu of these abnormalities included hyperinsulinaemia – compensatory to the degree of insulin resistance – and a heightened glucagon response to the meal.

Biliopancreatic diversion improved diabetes control in all patients and induced diabetes remission, according our strict criteria, in 40% of them. Although not all patients experimented complete remission of T2DM, all of them showed ameliorations in glucose metabolism: minor fasting plasma glucose and glycaemic excursion during OGTT, diminution of Hb_{A1c}%, better daily glycaemic control with pharmacological treatment and minor insulin secretion (Fig. 6 and 7) both at fasting state and during MMT stimulus. Nocturnal long-action insulin treatment was chose as antihyperglycemic therapy for who still diagnosed diabetic by OGTT after surgery. Furthermore, there was an important improvement in the lipid profile, particularly LDL cholesterol (Table 1).

Surgery induced weight loss but not excessively. Patients did not exhibit BMI lower than 20 kg/m^2 or related difficulties to eat or vomit. Waist and hip ratio decreases (Fig. 6) but the body architecture observed before surgery: high deposit of abdominal fat, keep on even if weight was reduced one year after surgery. Blood pressure, hepatic enzymes or blood test were not altered in pre-operatory state (normal ranges) and did not change significantly after surgery (data not show).

Withal there was almost a complete normalization of peripheral insulin sensitivity, augmented suppression of EGP during clamp and diminution of NEFA stimulated by insulin and an improvement in ß-cell function parameters that was still lower than observed in CT group.

One year after the surgery, glucose levels at fasting and in response to OGTT were still higher than in CT group, while fasting insulinaemia and insulin response to the meal test were lower (Table 2). We can observe (Fig. 8 and 9) a decrease in the plasmatic concentrations but the curves time did not change, remaining flatter than those observed in the CT group. BPD possibly did not alter gastric emptying; observed as the peak of plasma glucose concentration during stimulation, in the OGTT and MTT. When insulin secretion was analyzed by C-peptide deconvolution (Fig. 10) there is a little change in the curve profile one

year after surgery. Total insulin secretion decreases in comparison with baseline followed by a drop during the last 3 hours.

Similar results were observed by ß-cell parameters. In fact, ß-cell glucose sensitivity, potentiation and rate sensitivity improved after surgery but were still much reduced. Final ßCGS values corresponds to only 30% of the control values, demonstrating the severe insulin secretion dysfunction observed before surgery is still present at the late follow-up (Fig. 12). Studying obese diabetic subjects before and after Roux-an-Y bypass, Nannipieri showed that BCGS was the stronger indicator of diabetes remission (Nannipieri, 2011).

Remitters and non remitters groups were very similar at baseline. Minor fasting and total ISR evidenced a worse ß-cell dysfunction in non remitters. In fact, BCGS did not differed at baseline 20 [4] and 11 [17] for remitters and non remitters respectively, but were higher in remitters in the *Post2* (p=0.006). Remitters had similar age, diabetes duration, baseline BMI and HbA_{1c} as non-remitters, but lost slightly more weight (16 *vs.*13 kg). The difference was very small even if, in both follow-up evaluations had final weight lower than the CT group. Importantly, at follow-up, insulin sensitivity, fasting insulin secretion and β-cell function were lower in non remitters after surgery (Table 9).

Several works have showed high rates for T2DM remission after bariatric surgery in morbidly obese (BMI≥40kg/m^2), mainly with procedures that greatly restrain food intake (RYGB) or absorption (BPD). These procedures share the common feature of an early delivery of nutrients to the distal small intestine, bypassing duodenum and in part the jejunum. Just because different procedures induced specific alterations in gastrointestinal tract and in the response to the meal T2DM remission have been explained as result of various transformations promoted by surgery.

The first effect of bariatric surgery that collaborates to the improvement in glucose homeostasis is the weight loss. Is well known that body fat in specially, visceral adiposity is linked with diminution of insulin action (Kahn, 2001), hyperglycaemia, increase in EGP and

levels of circulating NEFA that in their turn can impair β-cell function by lipotoxicity (Groop, 1989; Gastaldelli, 2004b; Roden, 2004). A prospective study of ten obese patients with T2DM undergoing biliopancreatic diversion, with re-study after 2 years, demonstrated weight change from 136 to 89 kg. This was accompanied by a fall in the FPG, HbA_{1c}, improvement in IS and β-cell function (Camastra, 2007). It is certain that massive weight loss plays important role in consolidating the long-term antidiabetic effects of bariatric surgery, but only weight loss cannot explain all amelioration seen after surgery. Our patients have loss only 4 kg at the first follow-up time (*p*= ns) but improved M value from 38 [8] to 82 [41] ($\mu mol.min^{-1}kgffm^{-1}.pmol^{-1}$) (p=0.0007). Previous studies have been show that in non-diabetic morbidly obese patients undergoing BPD levels of insulin sensitivity were higher than those of lean controls; this improvement in insulin sensitivity was not correlated with weight loss in BPD treated patients. In contrast, in the patients treated with Roux en Y, the improvement of M value was predicted by the general relation between glucose disposal and body mass. In this way, the underlying mechanisms to the metabolic improvement should be quite different between these surgical procedures (Muscelli, 2005). Our patient group was very insulin resistant at pre-operatory state and showed an improvement in IS early after surgery, when weight loss was only by 12%. Relationship with improvement in insulin sensitivity as M value correct by insulin during clamp steady state (M/I) was observed only at one year taking in account the difference (Δ) between M/I and BMI from baseline and one year (*p*=0.001). It is important to attempt that early after surgery the relationship did not observed.

As discuss above, fasting plasma glycaemia and glucose levels in response to OGTT falls after bariatric surgery. Diminution of hyperglycaemia contributes to amelioration of insulin signalling, action and β-cell function. Acute mild hyperglycaemia decreases insulin secretion in normal subjects (Toschi, 2002) whereas insulin induced mild hypoglycaemia lowered insulin secretion during both glucose and arginine stimulus. Hyperinsulinaemia is commonly observed in obese with IGT or T2DM and in naïve diabetics (Ferrannini, 1997) in

response to hyperglycaemia but not in lean diabetic subjects (Eckell, 2011). Progression of IGT to T2DM is associated with impairment in β-cell function and in insulin action (Reaven, 1989). Intensive glucose lowering treatment showed to be able to reduce harmful effects of hyperglycaemia on β-cell function (Alvarsson, 2003). Bariatric surgery causes rapid diminution in plasma glucose concentrations, promptly enhance IS and β-cell function in morbidly obese subjects (Camastra, 2007; Camastra, 2011 and Muscelli, 2005). After surgery our patients have had rapid diminution of glucose concentration, increase in IS and β-cell function, similar as observed in morbidly obese patients. However, β-cell function remain still lower than in CT group (Fig. 11), demonstrating that dysfunction in lean diabetic is different that observed in obesity. Maybe due to genetic predisposition to β-cell dysfunction being more present in lean diabetics than in obese with T2DM (Arner, 1991). Thus, alterations promoted by bariatric surgery in obese are more effective than in lean subjects.

Only few studies have reported the efficacy of gastrointestinal surgery in non-morbidly obese diabetic patients after biliopancreatic diversion (Chiellini, 2009), Roux-en-Y gastric bypass (Lee, 2008), gastric banding (Coehn, 2006) or others procedures such duodenal-jejunal exclusion (Geloneze, 2009), ileal interposition (DePaula, 2011); both in humans and animals models (Rubino, 2004 b and Zhang, 2011). They reported similar or higher rates of diabetes remission, using less restrict definition for diabetes remission or using non gold standard methods to evaluate insulin sensitivity and insulin secretion.

The rationale to offer metabolic surgery to nonobese subjects with T2DM is that alterations promoted by surgery in gastrointestinal limb can induce changes in gastrointestinal hormones that influence positively glucose homeostasis. There are two main hypothesis: the hindgut hypothesis and foregut hypothesis. The hindgut hypothesis also called the "lower intestinal hypothesis" suggests that rapid delivery of nutrients to the distal bowel improves glucose metabolism by enhancing GLP-1 and other anorexigenic gut peptides (Ferrannini, 2010). This intestinal rearrangement speeds the delivery of nutrients to the distal intestine,

which causes exaggerated GLP-1 and PYY levels and improves glucose tolerance and insulin secretion without affecting body weight and food intake. In rodents, ileal interposition caused GLP-1 increment also with intact stomach (Cummings, 2007 a). Our patients demonstrated after surgery an important increment in plasma GLP-1 unchanged plasma glucagon. The time course GLP-1 plasma curves changed and passed to exhibit a peak in plasma concentration after meal, similar as we can observe in CT group (Fig. 9). At the same time, insulin:glucagon ratio decreased (Fig. 15). It is important to attempt that insulin:glucagon ratio decreases even if total insulin secretion was lower than in pre-operatory state; contributing to reduce EGP. Augmented levels of GLP-1 also contribute to reduce EGP independently of glycaemia. Seghieri et al. have demonstrated that GLP-1 infusion at physiological post-prandial concentrations have direct on EGP inhibition in normal subjects during pancreatic clamp (Seghieri, 2013). Thus increase in GLP-1 observed after surgery could be partially responsible by augmented inhibition of EGP observed during euglycaemic hyperinsulinaemic clamp. On the other hand, there is the foregut hypothesis or the foregut exclusion theory, in which causing food bypass the duodenum and proximal jejunum prevents the secretion of as-yet unidentified putative signal that promotes insulin resistance and type 2 diabetes (Rubino, 2008).

From our study is not possible to clarify the mechanisms involved in diabetes control. However, we can speculate that the higher GLP-1 levels are the consequence of the rapid delivery of food to the distal parts of the gastrointestinal tract. The caloric restriction and the weight loss might have contributed to the insulin sensitivity increment. The withdrawn of some degree of glucose toxicity must also be considered among the factors. There are no other studies regarding the mechanisms of diabetes remission after BPD surgery in diabetic patients with BMI lower than 33kg/m^2.

LDL cholesterol decreased at the same proportion reported to morbidly obese patients submitted to BPD (Corradini, 2005) and can be attributed to the fat malabsorption described to this surgery.

One limitation of this study is the small number of participants. In this way, is not possible to rule out diabetes duration or initial insulin secretion dysfunction as predictive factors. It is possible that an earlier intervention could have higher diabetes remission percentages.

In summary, in nonobese patients with long-standing T2D biliopancreatic diversion improved metabolic control in all but induced remission in only one third of them. While modest weight loss contributed to the outcome, increases in both insulin sensitivity and ß-cell function were primarily responsible for bringing down glucose and HbA_{1c} levels. Peripheral insulin sensitivity was restored to control levels already 2 months after surgery, and similarly in remitters and non-remitters, indicating a weight-independent effect of the operation. Hepatic insulin sensitivity, however, was still compromised one year postsurgery, possibly because of an unfavourable prehepatic insulin-to-glucagon concentration ratio. In contrast, ß-cell dysfunction was still prevailing at one year, and its degree distinguished remitters from non-remitters. Thus, the initial extent of ß-cell incompetence emerges as the main predictor of the metabolic outcome of BPD, and may condition relapse of hyperglycaemia beyond one year postsurgery.

7. Publications

During my PhD I have worked in several protocols. Generally, protocols were designed to better understanding the pathophysiological events underlying T2DM and obesity, specially related with the effects of gastrointestinal surgeries on T2DM resolution. We also assessed the physiological basis of these diseases by clinical trials, protocols designed by pharmaceuticals industries to investigate pharmacological effects of their

products on T2DM and obesity - "Sitagliptina" - Merck Sharp & Dome, "Exenatide" - Amylin Corporation and "SGLT-2- inhibitor - Boehringer Inglheim.

This thesis has been submitted to the publication at The Journal of Clinical Endocrinology & Metabolism and it is available in electronic version at Pubmed Central at http://www.ncbi.nlm.nih.gov/pubmed/23666972 with the title: Biliopancreatic diversion in nonobese patients with type 2 diabetes: impact and mechanisms; as well an two another manuscripts, titled: Visceral fat resection in humans: effect on insulin sensitivity, beta-cell function, adipokines, and inflammatory markers. Submitted to the Obesity (Silver Spring) and also available at Pubmed Central at http://www.ncbi.nlm.nih.gov/pubmed/23404948 and Human Insulin Resistance is Associated with Increased Plasma Levels of 12α-hydroxylated Bile Acids. Submitted to the Diabetes journal and available at Pubmed Central at http://www.ncbi.nlm.nih.gov/pubmed/23884887.

Publications realized from the beginning of the PhD until today are presented below.

Biliopancreatic Diversion in Nonobese Patients With Type 2 Diabetes: Impact and Mechanisms

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Context: Diabetes remission is frequent after biliopancreatic diversion (BPD) in morbidly obese patients with type 2 diabetes (T2D). Data, mechanisms, and clinical indications in nonobese T2D patients are scanty.

Objective: The objective of the study was to assess remission and investigate insulin sensitivity and β-cell function after BPD in nonobese patients with long-standing T2D.

Design, Setting, and Patients: This was a clinical research study comparing 15 T2D patients (aged 55 $\pm$ 1 years, duration of 16 $\pm$ 2 years, body mass index of 28.3 $\pm$ 0.6 kg/m^2, glycosylated hemoglobin 8.6% $\pm$ 1.3%) with 15 gender-, age-, and body mass index-matched nondiabetic controls. Before surgery and 2 months and 1 year later, a 3-hour oral glucose tolerance test, a 5-hour mixed-meal test, and a 3-hour euglycemic clamp were performed.

Intervention: The intervention included a BPD (distal gastrectomy, proximal ileum anastomosed to remaining stomach, biliopancreatic limb anastomosed to ileum 50 cm from the ileocecal valve).

Results: Glycemia improved in all patients, but remission (glycosylated hemoglobin $<$ 6.5% and normal oral glucose tolerance test) occurred in 6 of 15 patients. Insulin resistance (19.8 $\pm$ 0.8 μmol $\cdot$ min^{-1} $\cdot$ kg$_{ffm}^{-1}$, $P < .001$ vs 40.9 $\pm$ 5.3 of controls) resolved already at 2 months (34.2 $\pm$ 2.8) and was sustained at 1 year (34.7 $\pm$ 1.6), although insulin-mediated suppression of endogenous glucose production remained impaired. In contrast, β-cell glucose sensitivity (19 [12] pmol$\cdot$min^{-1} $\cdot$ m$^{-2}\cdot$mM^{-1} vs 96 [73] of controls, $P < .0001$) rose ($P = .02$) only to 31 [26] at 1 year and was lower in nonremitters (16 [18]) than remitters (46 [33]).

Conclusions: In nonobese patients with long-standing T2D, BPD improves metabolic control but induces remission in only approximately 40% of patients. Peripheral insulin sensitivity is restored early after surgery and similarly in remitters and nonremitters, indicating a weight-independent effect of the operation. The initial extent of β-cell incompetence is the main predictor of the metabolic outcome. **(*J Clin Endocrinol Metab* 98: 2765–2773, 2013)**

Bariatric surgery has powerful effects to improve glucose metabolism (1, 2) and has been associated with reduced rates of type 2 diabetes (T2D) (3), cardiovascular events (4), and mortality (5, 6). Detailed metabolic studies have shown that adjustable gastric banding (7, 8), Roux-en-Y gastric bypass (9, 10), biliopancreatic diversion (11–14), and ileal transposition (15) all enhance both insulin action and β-cell function more effectively than conven-

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Abbreviations: AUC, area under the time-concentration curve; BMI, body mass index; BPD, biliopancreatic diversion; EGP, endogenous glucose production; FFA, free fatty acid; GLP-1, glucagon-like peptide-1; HbA_{1c}, glycosylated hemoglobin; HDL, high-density lipoprotein; MTT, mixed-meal test; OGTT, oral glucose tolerance test; OHA, treatment with metformin+sulfonylureas; T2D, type 2 diabetes.

tional medical therapy (7, 16–18). Although drastic weight loss and, in the early postoperative period, caloric restriction certainly make a contribution to better glucose tolerance, surgery itself may trigger weight-independent mechanisms that eventually translate into favorable metabolic effects. This postulate is based on early animal studies (19) and, in humans, on evidence that metabolic changes sometimes precede sizable weight loss or are disproportionate to the amount of weight lost (20). In this regard, the weight of evidence indicates that different bariatric procedures may engage putative weight-independent mechanisms to different extents or involve altogether different mechanisms (21, 22). In any event, this possibility has generated enthusiasm for bariatric surgery as a treatment for T2D (23) and has encouraged a broadening of the body mass index (BMI) range as an indication for surgery in diabetic patients (24).

Among bariatric surgeries, biliopancreatic diversion (BPD) induces the most dramatic changes in body weight and the highest diabetes remission rates (1). In very obese T2D patients (BMI $\geq$ 35 kg/m^2), BPD is followed by rapid normalization of insulin sensitivity and improved β-cell function (11–14). In T2D patients with a BMI less than 35 kg/m^2, an early trial of 5 subjects (25) showed achievement of good glycemic control 1 month after surgery, and a larger study of 30 patients followed up for up to 2 years (26) suggested differences in outcome, depending on initial BMI. In the present series of well-characterized patients with long-standing T2D and BMI less than 30 kg/m^2, we assessed remission at 1 year by stringent criteria and carried out detailed investigations of insulin sensitivity and β-cell function [combining oral glucose tolerance test (OGTT), mixed meal, and euglycemic insulin clamp with tracer glucose] before, 2 months, and 1 year after BPD in comparison with gender-, age-, and BMI-matched nondiabetic controls.

Materials and Methods

Study population

We studied Caucasian 15 patients (6 men, 9 women; aged 55 $\pm$ 1 years; BMI 28.3 $\pm$ 0.6 kg/m^2, range 24.5–29.9 kg/m^2) with T2D [duration 16 $\pm$ 2 years, range 6–25 years; glycosylated hemoglobin (HbA_{1c}) 8.5% $\pm$ 0.4% (66 $\pm$ 23 mmol/mol), range 8.0%–11.5% (47–98 mmol/mol)] on treatment with metformin+sulfonylureas (OHA, n = 6 of 15) or OHA plus insulin (n = 9 of 15, mean dose 30 IU/d, range 10–90 IU/d). Patients were engaged only in light physical activity, mostly occupational. Twelve patients were nonsmokers (5 were ex-smokers ~14 years) and 3 were smokers (1 pack/d). Each patient underwent a 3-hour OGTT, a 5-hour mixed-meal test (MTT), and a 3-hour euglycemic hyperinsulinemic clamp on separate days over 2 weeks. All tests were performed after an overnight fast; antidiabetic medications were withdrawn 3 days before testing. When required to avoid hyperglycemia, regular insulin was used until 8 hours before the metabolic studies. Each patient was studied at baseline (before), approximately 2 months after surgery (2 months), and 1 year after surgery (12 months). Diabetes remission was defined as an HbA_{1c} less than 6.5% (<44 mmol/mol), a fasting plasma glucose less than 7.0 mmol/L, and a 2-hour plasma glucose less than 11.1 mmol/L 2 hours into the OGTT without antidiabetic medication.

Fifteen nondiabetic volunteers from our database were matched to the patient group by gender, age (51 $\pm$ 3 years), and BMI (30.2 $\pm$ 0.9 kg/m^2, range 24.6–35.4 kg/m^2) and served as a weight-matched control group; they all received the MTT, and 8 of them also received the euglycemic clamp.

Study subjects were informed about the procedures and risks and provided their written consent. The protocol was approved by the institutional Ethics Committee of the University of Genova.

Surgery

With the BPD procedure, a two thirds distal gastric resection with stapled closure of the duodenal stump results in a residual stomach volume of 400–500 mL; the small bowel is then transected 300 cm from the ileocecal valve, and the distal end is anastomosed to remaining stomach (alimentary limb). The proximal end of the ileum, comprising the remaining small bowel carrying the biliopancreatic juice and excluded from food transit, is anastomosed to the common limb 50 cm from the ileocecal valve (26).

Oral glucose tolerance test

A standard 75-g OGTT was performed in each patient. Venous blood samples were collected at −30, 0, 30, 60, 90, 120, and 180 minutes after the glucose load for determination of plasma glucose concentrations.

Mixed-meal tolerance test

Meal stimulation was performed following a 30-minute stabilization period. Patients were invited to eat 1 egg, 50 g of parmesan cheese, and 75 g of an aqueous glucose solution (54% carbohydrate, 30% lipid, 16% protein, 561 kcal). Venous blood samples were collected at −30, 0, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, and 300 minutes for analytical measurements.

Euglycemic hyperinsulinemic clamp

Insulin (Humulin R; Eli Lilly, Indianapolis, Indiana) was infused at a rate of 240 pmol $\cdot$ min^{-1} $\cdot$ m^{-2} for 3 hours. At times −20, −10 and 0, 20, 40, 60, 80, 100, 120, 140, and 180 minutes, samples were drawn from a heated dorsal hand vein (to obtain arterialized blood) for the measurement of glucose tracers and insulin concentrations. Plasma glucose levels were measured every 5 minutes to adjust an exogenous glucose infusion. Patients with fasting hyperglycemia (>5.5 mmol/L) received only the insulin infusion until euglycemia or until time 80 minutes, when plasma glucose was clamped at the level attained at this time. Three hours before starting the clamp, a primed [22.0 μmol/kg*(fasting glycemia/5)]-continuous (0.22 μmol $\cdot$ min^{-1} $\cdot$ kg^{-1}) infusion of 6,6-$^2[H_2]$ glucose was administered via an antecubital vein catheter. At time zero, the tracer infusion rate was decreased (to 0.11 μmol $\cdot$ min^{-1} $\cdot$ kg^{-1}) and continued until the end of the clamp. During the clamp, a variable rate of 20% of

dextrose solution, enriched with 6,6-$^2[H_2]$ glucose, was infused to maintain euglycemia (9).

Analytical procedures

Plasma glucose was measured by the glucose-oxidase technique (Analox GM-9), plasma insulin and C-peptide by electrochemiluminescence (on a COBAS e411 instrument; Roche, Indianapolis, Indiana). Plasma triglycerides, free fatty acids (FFAs), and serum high-density lipoprotein (HDL) cholesterol were assayed in duplicate by standard spectrophotometric methods on a Synchron Clinical System CX4 (Beckman Instruments, Fullerton, California). 6,6-[$^2H_2$]glucose enrichment was measured by gas chromatography/mass spectrometry as described previously (27). Plasma glucagon was assayed by RIA, total plasma glucagon-like peptide-1 (GLP-1) by an ELISA method (Millipore Corporation, Billerica, Massachusetts). Blood samples for C-peptide, insulin, and glucagon were drawn using a protease inhibitor (5 μl/mL of 10 mg/mL gabexate mesilate); for GLP-1 measurements blood was drawn into tubes containing a dipeptidyl peptidase-IV inhibitor (Diprotin A, 10 mM; Sigma Aldrich, St Louis, Missouri).

No significant cross-reactivity was declared by the manufacturers of the kits. The sensitivity ranges are 0.003 nmol/L and 1.39 pmol/L for the C-peptide and insulin assay, respectively, 18.5 pg/mL for glucagon, and 1.5 pM for GLP-1.

Data analysis

Insulin sensitivity was calculated as the whole-body insulin-mediated glucose uptake (M value, micromoles per minute^{-1} per kilogram$_{ffm}{}^{-1}$) during the last 40 minutes of the clamp normalized to fat-free mass. The 6,6-$^2[H_2]$ glucose data were analyzed as previously described (27) to calculate glucose rates of appearance and disappearance in the fasting state and during the clamp. Endogenous glucose production (EGP) was obtained as the difference between the time course of the glucose rates of appearance and exogenous glucose infusion rate.

β-Cell function during the MTT was resolved using a mathematical model (28) describing the relationship between insulin secretion and glucose concentration. In brief, the model consists of 3 blocks: 1) a model fitting the glucose concentration profile, the purpose of which is to smooth and interpolate plasma glucose concentrations; 2) a model describing the dependence of insulin (or C-peptide) secretion on glucose concentration; and 3) a model of C-peptide kinetics, ie, the 2-exponential model proposed by Van Cauter et al (29), in which the model parameters are individually adjusted to the subject's anthropometric data. The dependence of insulin secretion rate on absolute glucose concentration at each time point during the MTT is described by a dose-response function relating the 2 variables. The main characteristic parameter of the dose response, ie, the mean slope within the observed glucose range, is denoted as β-cell glucose sensitivity by analogy with insulin sensitivity (slope of the dose response of insulin mediated glucose uptake vs insulin concentrations). The dose-response function is modulated by a time-dependent potentiation factor, indexed as the ratio of its value at 2 hours into the MTT to the corresponding basal value. The dependence of insulin secretion rate on the rate of change of plasma glucose levels is denoted as rate sensitivity.

The prehepatic insulin to glucagon molar concentration ratio was estimated by the following formula:

$$(ISR(t)/hPF + [I(t)])/\{[Gg(t)] * (1 + MCR_{Gg}/hPF)\}$$

where ISR(t) is the insulin secretion rate at time t, hPF is hepatic plasma flow; [I(t)] and [Gg(t)] are the measured (peripheral) plasma concentrations of insulin and glucagon at time t; and MCR_{Gg} is the metabolic clearance rate of glucagon. hPF was estimated by multiplying the cardiac index (3.2 L $\cdot$ min^{-1} $\cdot$ m^{-2}) by a plasma to blood ratio of 0.6 and by assuming that hepatic blood flow is 30% of the cardiac index (0.576 L $\cdot$ min^{-1} $\cdot$ m^{-2}). MCR_{Gg} was taken to be 0.537 L $\cdot$ min^{-1} $\cdot$ m^{-2} (30).

Statistical analysis

Data are given as means $\pm$ SEM (or median [interquartile range] for variables with a skewed distribution). Areas under the

Table 1. Metabolic Characteristics From MMT[a]

	Controls	Patients Before	Patients at 2 Months	Patients at 12 Months	P[b]
n (M/F)	15 (5/10)	15 (9/6)	15 (9/6)	15 (9/6)	–
Fasting glucose, mmol/L	5.3 $\pm$ 0.1	12.2 $\pm$ 0.5[c]	8.3 $\pm$ 0.6[c]	7.3 $\pm$ 0.4[c]	.0001
Fasting insulin, pmol/L	63 $\pm$ 7	105 $\pm$ 35[c]	46 $\pm$ 6	36 $\pm$ 5[c]	0.04
Triglycerides, mmol/L	1.0 $\pm$ 0.1	1.9 $\pm$ 0.2[c]	1.9 $\pm$ 0.2[c]	1.9 $\pm$ 0.3[c]	ns
Total cholesterol, mmol/L	4.8 $\pm$ 0.2	5.3 $\pm$ 0.4	3.5 $\pm$ 0.2[c]	3.5 $\pm$ 0.1[c]	.0003
HDL cholesterol, mmol/L	1.0 $\pm$ 0.1	1.2 $\pm$ 0.1	1.0 $\pm$ 0.1	1.1 $\pm$ 0.1	.007
LDL cholesterol, mmol/L	3.2 $\pm$ 0.2	3.3 $\pm$ 0.4	1.5 $\pm$ 0.1[c]	1.8 $\pm$ 0.1[c]	.0001
AUC glucose, mol $\cdot$ L^{-1} $\cdot$ h^{-1}	1.8 $\pm$ 0.4	5.5 $\pm$ 0.1[c]	3.4 $\pm$ 0.2[c]	3.1 $\pm$ 0.2[c]	.0001
AUC insulin, nmol $\cdot$ L^{-1} $\cdot$ h^{-1}	79 [73]	66 [42]	40 [12][c]	40 [23][c]	.003
AUC glucagon, μg $\cdot$ L^{-1} $\cdot$ h^{-1}	18 [9]	20 [10]	22 [18][c]	22 [8]	.14
Mean prehepatic insulin to glucagon ratio	8.8 [8.9]	17.6 [10.5][c]	13.4 [9.5]	9.6 [7.2]	.01
AUC FFA, mmol $\cdot$ L^{-1} $\cdot$ h^{-1}	70 [23]	155 [120][c]	124 [70][c]	79 [74]	.0001
AUC GLP-1, ng $\cdot$ L^{-1} $\cdot$ h^{-1}	15 [10]	15 [7]	26 [13][c]	24 [10]	.003

Abbreviations: F, female; LDL, low-density lipoprotein; M, male; ns, not significant.

[a] Data are mean $\pm$ SEM or median [interquartile range].

[b] $P \leq .05$ for the comparison of before and 2 and 12 months by repeated-measures ANOVA.

[c] $P \leq .05$ vs controls.

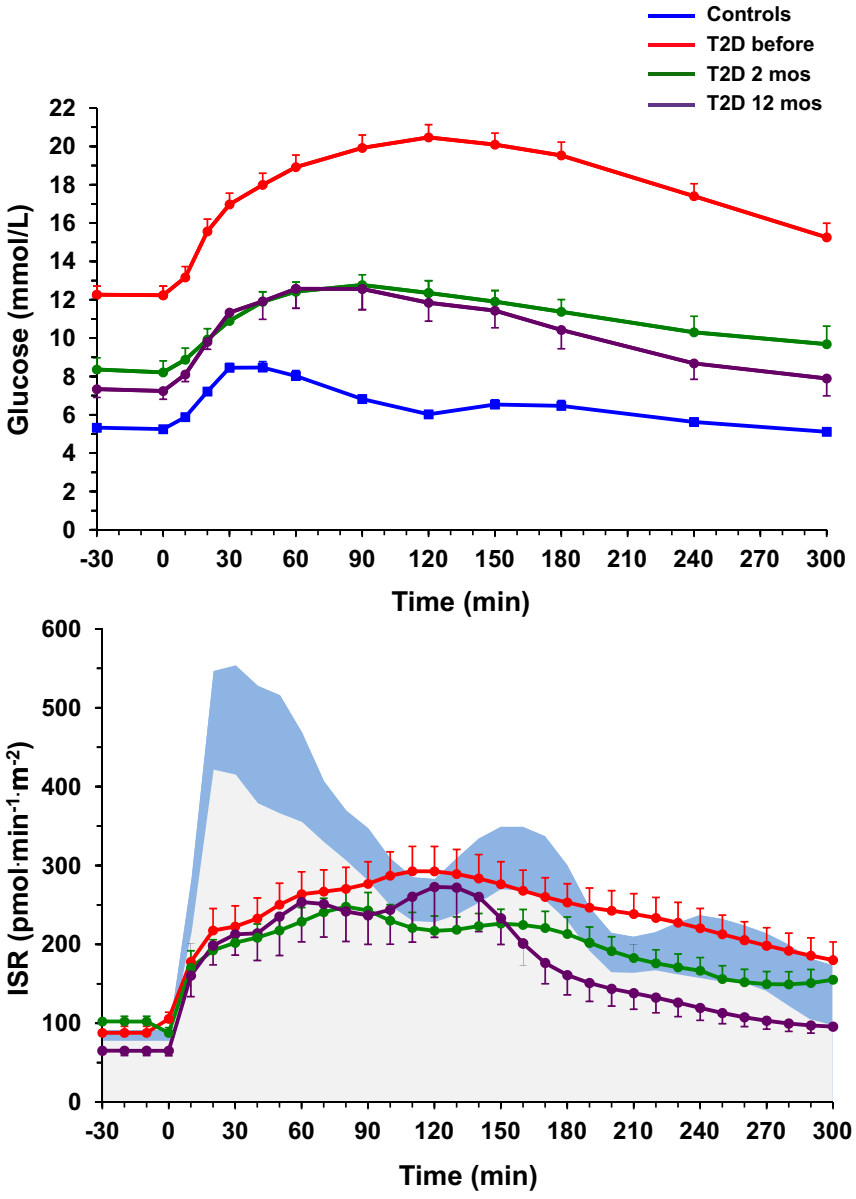

Figure 1. Time course of plasma glucose concentrations (top panel) and insulin secretion rates (bottom panel) in nondiabetic control subjects and diabetic patients before and 2 and 12 months after biliopancreatic diversion. Plots are mean ± SEM; the blue shaded area is mean ± SEM of controls. ISR, insulin secretion rate.

time-concentration curve (AUC) and incremental AUCs were calculated by the trapezium rule. Groups were compared by the Mann-Whitney test. Time series were analyzed by ANOVA for repeated measures; for these tests, variables with skewed distribution were log transformed. Group differences over time series were analyzed by 2-way ANOVA for repeated measures. Statistical analyses were performed using JMP 7.0 (SAS Institute, Cary, North Carolina); $P \leq .05$ was considered statistically significant.

Results

Surgery caused weight loss (an average of −12 kg at 2 months and −14 kg at 1 year), whereas HbA_{1c} decreased from 8.6% ± 1.3% to 6.7% ± 0.9% at 2 months to 6.0% ± 1.0% at 1 year ($P < .0001$). After surgery, patients remained in the hospital for 1 week on a low-carbohydrate diet. During this period, glycemia was carefully controlled with insulin. One week after surgery, no patient resumed OHA, and 8 were switched to long-acting insulin alone (mean dose 14 IU/d) titrated on fasting glycemia with the aim of maintaining HbA_{1c} at approximately 7%. At 1 year, 6 patients were still on insulin (mean dose 12 IU/d). By the defined criteria, diabetes remitted in 4 patients at 2 months and in a total of 6 patients at 1 year. At this time, low-density lipoprotein-cholesterol concentrations were markedly reduced (to levels significantly lower than in controls), whereas plasma triglycerides did not show significant changes and HDL cholesterol concentrations were marginally reduced.

At baseline, patients had higher fasting and postprandial plasma glucose concentrations than weight-matched controls (Table 1). In the patient group, the plasma glucose response to the MMT was much improved already at 2 months and changed little thereafter (Figure 1). Both fasting and postmeal plasma insulin declined significantly after surgery to levels that were lower than in the control group (Table 1). At baseline, insulin secretion showed the blunted and protracted profile typical of T2D (Figure 1), such that fasting insulin secretion was higher than in weight-matched controls, but total insulin output during the 5 hours of the MTT was similar (Table 2). All 3 parameters of β-cell function, ie, glucose sensitivity, rate sensitivity, and potentiation, were severely compromised in the patients at baseline; after surgery, each of them improved significantly (Table 2), but only potentiation was normalized. In particular, β-cell glucose sensitivity was still markedly depressed in comparison with the control group (Figure 2).

Before surgery, EGP was higher in patients than controls both under fasting conditions and during the clamp (Table 2). At both times after surgery, fasting EGP was

Table 2. β-Cell Function Parameters, EGP, and Insulin-Mediated Glucose Disposal[a]

	Controls	Patients Before	Patients at 2 Months	Patients at 12 Months	P[b]
Fasting ISR, pmol · min^{-1} · m^{-2}	83 [53]	115 [52][c]	83 [22]	61 [38]	.0005
Total insulin output, nmol · m^{-2}	77 [46]	80 [51]	62 [19]	52 [37][c]	ns
β-Cell glucose sensitivity, pmol · min^{-1} · m^{-2} · mM^{-1}	96 [73]	19 [12][c]	30 [14][c]	31 [26][c]	.02
Rate sensitivity, pmol · m^{-2} · mM^{-1}	934 [981]	187 [247][c]	605 [436]	453 [453][c]	.004
Potentiation factor, fold	1.52 [0.47]	1.29 [0.44][c]	1.11 [0.43]	1.49 [1.09]	.02
Clamp insulin, pmol/L	633 [363]	531 [132]	418 [63][c]	418 [107][c]	.009
Fasting EGP, μmol · min^{-1}	696 [91]	869 [239][c]	733 [213]	806 [233]	.0004
Clamp EGP, μmol · min^{-1}	−27 [116]	271 [133][c]	168 [170][c]	150 [75][c]	.0006
Insulin-mediated glucose disposal, μmol · min^{-1}	1608 [1480]	1235 [356][c]	1658 [558]	1701 [305]	.02

Abbreviations: ISR, insulin secretion rate; ns, not significant.

[a] Data are median [interquartile range].

[b] $P \leq .05$ for the comparison of before and 2 and 12 months by repeated-measures ANOVA.

[c] $P \leq .05$ vs controls.

significantly lower than preoperatively and no longer different from controls. Postoperatively, clamp EGP was significantly better than before surgery but still higher than in controls. Before surgery, insulin-mediated glucose disposal was markedly depressed in patients as compared with controls, whether in absolute terms (ie, micromoles per minute from the tracer determined glucose rates of disappearance; Table 2) or as the M value from the clamp ($P = .0002$, Figure 3). At 2 months after surgery, insulin-mediated glucose disposal had increased to levels no longer significantly different from controls and remained essentially unchanged at 1 year (Table 2 and Figure 3).

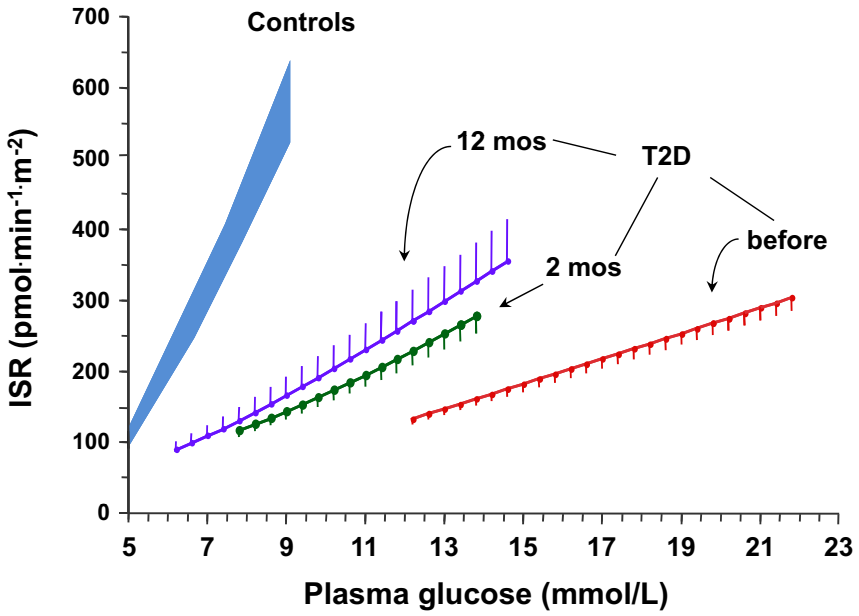

Figure 2. Insulin secretion rates as a function of concomitant plasma glucose concentrations during the mixed meal in nondiabetic control subjects and diabetic patients before and 2 and 12 months after biliopancreatic diversion. Plots are mean ± SEM; the blue shaded area is mean ± SEM of controls. ISR, insulin secretion rate.

Plasma FFA concentrations at fasting and during the meal were significantly higher in patients than controls before surgery and gradually reverted to normal levels after surgery (Table 1 and Figure 4). In contrast, plasma glucagon concentrations, which were higher in patients than controls at baseline, increased approximately 2-fold early after surgery ($P < .05$), and returned to presurgery levels at 1 year (Table 1 and Figure 4). The estimated prehepatic insulin to glucagon molar concentration ratio was significantly higher in patients than controls before surgery and decreased significantly after surgery (Table 1 and Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). At baseline, plasma total GLP-1 concentrations were similar in controls and patients; after surgery, GLP-1 responses to the MTT were markedly increased at 2 months and remained high at 1 year (Table 1 and Figure 4).

Remitters (n = 6) had similar age, diabetes duration, and baseline BMI, HbA_{1c}, and fasting plasma glucose levels as nonremitters (n = 9) but had lost slightly more weight at 1 year (16 vs 13 kg, P = ns). With regard to metabolic changes, insulin sensitivity improved to a similar extent in remitters and nonremitters, whereas β-cell glucose sensitivity increased significantly ($P = .01$) less in nonremitters (from 11 [17] to 24 [22] at 2 months to 16 [18] pmol · min^{-1} · m^{-2} · mM^{-1}

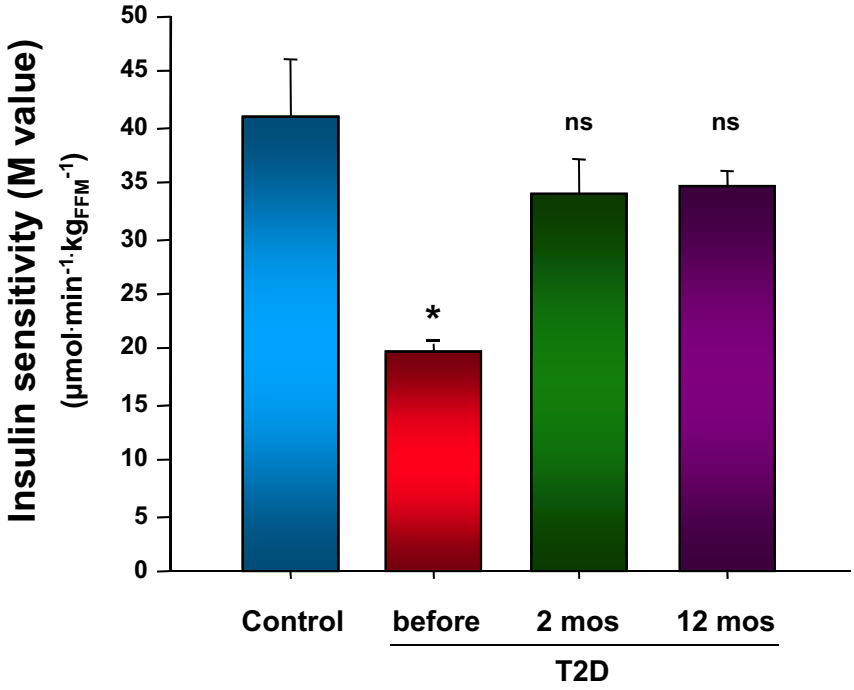

Figure 3. Insulin sensitivity (M value from the euglycemic clamp) in nondiabetic control subjects and diabetic patients before and 2 and 12 months after biliopancreatic diversion. Bars are mean ± SEM.

at 12 months) than in remitters (20 [4] to 32 [interquartile range 12] to 46 [33]). This was also the case for rate sensitivity (from 247 [127] to 508 [342] to 610 [697] pmol · m^{-2} · mM^{-1} in remitters, $P = .03$, vs 166 [293] to 692 [390] to 212 [455] in nonremitters).

Discussion

The patients in this study were nonobese but had long-standing, poorly controlled diabetes despite intensive antihyperglycemic therapy (which included insulin in two thirds of them). As expected, they presented profound insulin resistance, in peripheral tissues (as reduced insulin mediated glucose uptake under euglycemic conditions), in the liver (as elevated fasting EGP and reduced EGP suppression during the clamp), and in adipose tissue (manifest as raised FFAs in the face of hyperinsulinemia). Furthermore, all indices of β-cell function were severely compromised; in particular, β-cell glucose sensitivity (ie, the intrinsic ability of the β-cell to sense and respond to dynamic glucose changes in a timely and quantitatively appropriate fashion) was only 20% of that of age- and BMI-matched nondiabetic controls. Insulin release was therefore sustained at near-normal levels by the elevated glucose concentrations.

In the patient group as a whole, BPD induced a modest weight loss and a marked amelioration of glycemic control. By the stringent criteria adopted here, 6 of 15 patients were in remission at 1 year postoperatively, whereas considerable degrees of improvement were obtained in each of the other 9 patients. Correspondingly, both insulin resistance and β-cell dysfunction abated to a substantial and statistically significant extent in the group. Of note, β-cell glucose sensitivity increased in the face of a reduction in insulin output, thereby relieving the secretory burden on the β-cell.

Despite these improvements, at 1 year after surgery, when weight had stabilized, β-cell function was still, on average, only approximately 30% of that of nondiabetic controls (Table 2 and Figure 2), indicating that a substantial fraction of the β-cell dysfunction in long-standing diabetes cannot be reversed by this surgical approach. In line with this, full remission was observed in a minority of patients, and even in those patients, β-cell glucose sensitivity at 1 year was only approximately 50% of normal and rate sensitivity was 30% reduced. Presumably, in this kind of patients, diabetes may recur at a relatively high rate at later times after surgery as the disease progresses or whether weight is regained or insulin resistance worsens. In previous work in 15 nonobese (BMI < 30 kg/m^2) patients with long-term diabetes treated with BPD, follow-up at 2 years showed maintenance of satisfactory glycemic control, although the acute insulin response to iv glucose was still far from normal (26). Clearly, more data from longer periods of follow-up are needed to assess the durability of diabetes remission in nonobese T2D patients undergoing BPD. However, it is relevant to note that the outcome of this bariatric procedure may be more favorable in severely obese diabetic patients. We previously reported on a group of noninsulin-treated, morbidly obese T2D patients (with a mean BMI of 49.5 kg/m^2 and relatively preserved β-cell glucose sensitivity), in whom BPD induced near normalization of day-long glucose profiles and β-cell sensitivity 2 years after surgery (12). In another series of 15 obese (BMI 30–35 kg/m^2) patients, the outcome was intermediate between those of nonobese and severely obese T2D patients (26).

The question arises of whether the reduced efficacy of BPD in leaner as compared with very obese T2D patients is related to the smaller weight loss occurring in the former than in the latter. The insulin sensitivity data in the present patients do not fully support this explanation. In fact, the

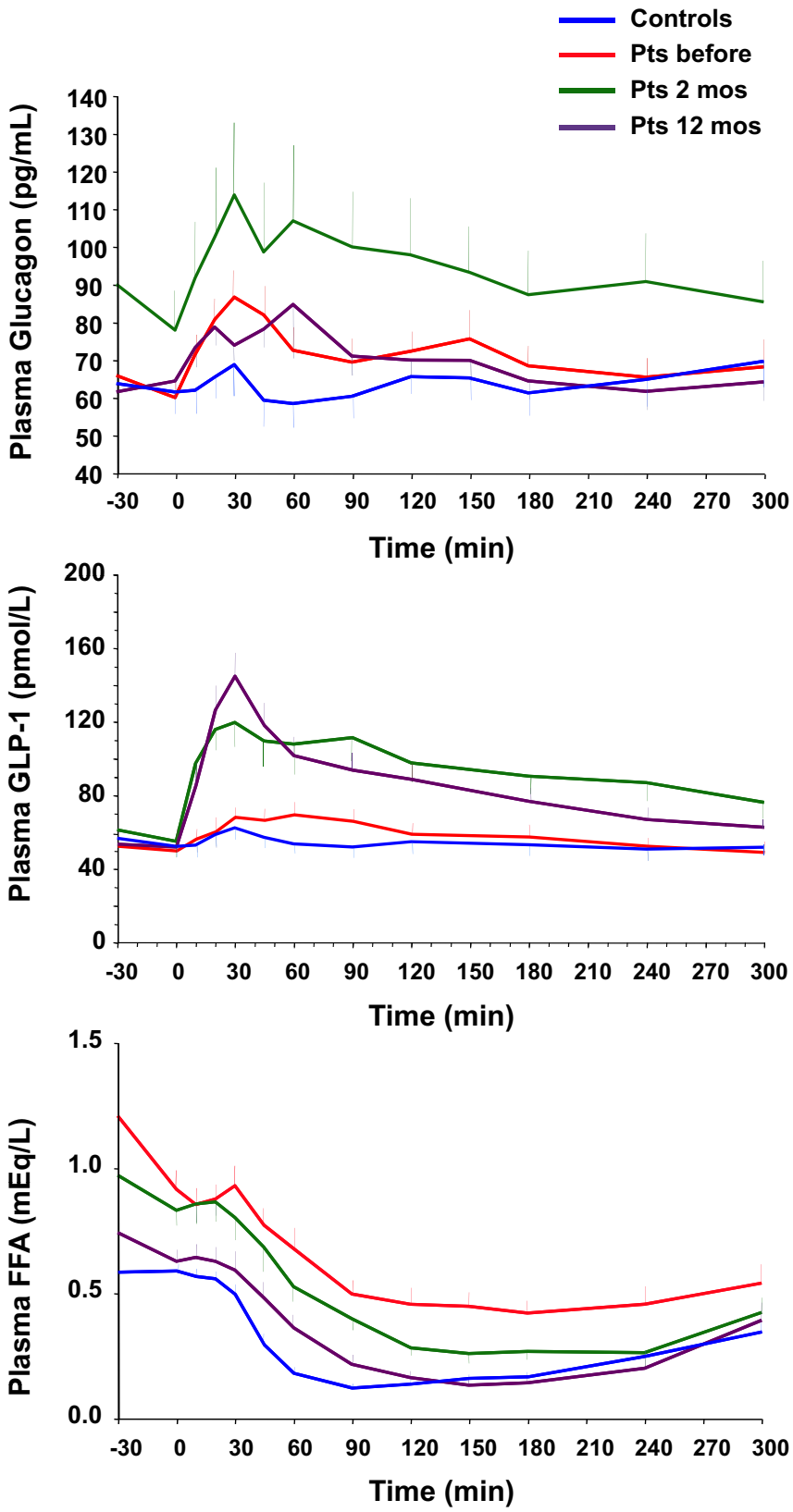

Figure 4. Time course of plasma glucagon, GLP-1, and FFA concentrations during the mixed meal in nondiabetic control subjects and diabetic patients before and 2 and 12 months after biliopancreatic diversion. Plots are mean ± SEM.

euglycemic clamp data show that insulin-mediated glucose disposal was restored to normal values already 2 months after surgery, when weight loss was incomplete, and, more importantly, equally in remitters and nonremitters. This was also the case in a previous series including nondiabetic, glucose-intolerant, and diabetic subjects with severe obesity (11), in whom clamp-derived insulin resistance resolved at even earlier times after BPD, regardless of weight loss and diabetes status. Therefore, the effect of BPD on peripheral insulin sensitivity is very likely to be, at least in part, independent of weight loss. Moreover, in the face of BPD-induced resolution of insulin resistance, the worse outcome of glycemic control of the nonobese T2D patient presumably is the consequence of poorer initial degree and stunted postoperative recovery of β-cell function. The current data in remitters vs nonremitters, and previous results (11, 12), are compatible with this conclusion.

The pathophysiological picture of BPD stands in contrast with that of gastric bypass surgery, where we (9) and others (8) have found that the long-term improvement in insulin action is essentially proportional to the degree of weight loss. Taken together, these lines of evidence confirm what we had argued from a review of the available literature (21) and from a comparison of historical data (31), that BPD, but not gastric bypass, exerts a specific, weight-independent effect on insulin resistance.

The new, and somewhat unexpected, finding in the present series is that hepatic insulin sensitivity (as the EGP), if enhanced by surgery, was still impaired at 1 year, ie, at a time when insulin-mediated glucose disposal by peripheral tissues was fully restored (Table 2). This dissociation could be causally linked with the deterioration of the prehepatic insulin

to glucagon ratio during the meal observed after surgery (Table 1 and Supplemental Figure 1). Before the operation, both fasting and insulin-mediated EGP were abnormally high in the patients despite a significantly higher insulin to glucagon ratio, thereby signaling a severe degree of hepatic insulin resistance. After surgery, EGP was improved but still abnormal, likely on account of the reduced insulin to glucagon ratio.

With regard to the hormonal changes, the large and persistent rise in the GLP-1 response to the meal is consistent with previous reports (32, 33) and may be consequent upon the rapid delivery of food to the distal parts of the gastrointestinal tract. Enhanced GLP-1 release very probably contributed to the observed improvement in β-cell function, although abatement of glucose toxicity must have played an additional part. The stimuli for the heightened glucagon response, however, are more uncertain. Higher GLP-1 concentrations should suppress glucagon release, so one potential explanation is the drop in insulinemia with the attendant weakening of its restraint on α-cell activity.

In summary, in nonobese patients with long-standing T2D biliopancreatic diversion improved metabolic control in all but induced remission in only one third of them. Although modest weight loss contributed to the outcome, increases in both insulin sensitivity and β-cell function were primarily responsible for bringing down glucose and HbA_{1c} levels. Peripheral insulin sensitivity was restored to control levels already 2 months after surgery and similarly in remitters and nonremitters, implying a weight-independent effect of the operation. Hepatic insulin sensitivity, however, was still compromised 1 year after surgery, possibly because of an unfavorable prehepatic insulin to glucagon concentration ratio. In contrast, β-cell dysfunction was still prevailing at 1 year, especially in nonremitters. Thus, the initial extent of β-cell incompetence emerges an important predictor of the metabolic outcome of BPD.

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Soluble Human Leukocyte Antigen-G Expression and Glucose Tolerance in Subjects with Different Degrees of Adiposity

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Context: Type 2 diabetes mellitus (T2DM) and obesity are characterized by a low-grade inflammation, which might be related to the development of insulin resistance. Human leukocyte antigen-G (HLA-G) shows antiinflammatory and tolerogenic properties, including the modulation of CD8+ T-cell cytotoxicity and regulation of CD4+ T-lymphocyte function. These functions are partially shared with IL-10, whose levels are reduced in insulin-resistant states.

Objective: The aim was to explore the relationship between HLA-G and the metabolic and inflammatory pattern of obesity or T2DM.

Patients and Main Outcome Measures: Soluble HLA-G, IL-6, and IL-10 were measured and related with metabolic and biochemical parameters in 230 volunteers with normal glucose tolerance, impaired glucose tolerance, or T2DM by oral glucose tolerance test.

Results: sHLA-G, detected in 144 subjects (sHLA-G positive), was more frequent in T2DM or impaired glucose tolerance subjects than in normal glucose tolerance ($\chi^2 = 18.6$; $P < 0.0001$), and its plasma levels increased progressively across the classes of glucose tolerance. sHLA-G-positive individuals had higher body mass index, systolic blood pressure, and cholesterol levels; a reduced degree of insulin sensitivity; and almost 2-fold higher levels of IL-6, a cytokine related to insulin sensitivity, whereas IL-10 was similar. In the sHLA-G-positive subgroup, by a multivariate regression model, sHLA-G was significantly related to 2-h glucose, the area under insulin curve, and IL-6 levels (multiple $r^2 = 0.14$; $P < 0.001$), independently of age, gender, and body mass index.

Conclusions: A frequent expression of sHLA-G, linked to a typical biomarker of insulin resistance like IL-6, seems to characterize subjects with an impaired glucose metabolism. **(*J Clin Endocrinol Metab* 95: 3342–3346, 2010)**

Human leukocyte antigen-G (HLA-G) is a member of the class of Ib-HLA molecules characterized by low allelic polymorphism and restricted tissue expression. In contrast to classical HLA-I antigens, HLA-G has different protein isoforms: HLA-G1 to -G4 as membrane-bound, and HLA-G5 to -G7 as soluble molecules. The membrane-bound HLA-G1, its soluble counterpart HLA-G5, and the soluble HLA-G1 isoform, produced by the proteolytic release of the membrane-bound HLA-G1, are the most investigated isoforms.

HLA-G modulation was first evident on the surface of cytothrophoblast cells and associated with the tolerogenic microenvironment at the fetal-maternal interface (1). Further research has detected HLA-G antigen expression in

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Abbreviations: AUGC, Area under the glucose curve; AUIC, area under the insulin curve; BMI, body mass index; BP, blood pressure; CV, coefficient of variation; HLA-G, human leukocyte antigen-G; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; ns, not significant; OGIS, oral glucose-derived insulin sensitivity index; sHLA-G, soluble HLA-G; T2DM, type 2 diabetes mellitus.

thymic epithelial cells and cornea. The interest in HLA-G antigens rapidly increased because of its ability to modulate natural killer and CD8+ T-cell cytotoxicity and regulate the functions of CD4+ T-lymphocytes and dendritic cells. Moreover, the induction of regulatory T cells via HLA-G has been recently reported, confirming their tolerogenic function in innate and adaptive responses (2–4). Recently, the expression of HLA-G molecules has been associated with several autoimmune diseases, like Crohn's disease and rheumatoid arthritis, viral infections, tumors, and organ transplantations (5–8). Overall, these results confirm the role of HLA-G molecules in the induction and maintenance of antiinflammatory environments.

HLA-G expression is up-modulated by different biological molecules as interferons and IL-10. IL-10, a pleiotropic cytokine secreted by different cells, plays an important role in the regulation of the immune response, mainly inhibiting proinflammatory molecules, altering antigen presentation and T-cell activation pathways, and stimulating B-cell proliferation and differentiation. IL-10 shares with HLA-G immunoregulative properties and, possibly, the induction of immune tolerance; furthermore, IL-10 inhibits cellular responses through expression of HLA-G (9, 10).

The relationships between IL-10 and metabolic abnormalities are not fully elucidated. Cotreatment with IL-10 prevents IL-6-induced defects in both hepatic and skeletal muscle insulin action in rats (11), and low IL-10 circulating levels are described in obese individuals with metabolic syndrome and in type 2 diabetic patients (12, 13). The negative associations of IL-10 with insulin resistance, type 2 diabetes mellitus (T2DM), and the metabolic syndrome (12) suggest its role in contrasting the inflammatory state of these patients.

T2DM patients are frequently obese, obesity being a major risk factor for diabetes. Both conditions are characterized by a low-grade inflammation, possibly related to the development of insulin resistance (14). Cross-sectional studies show positive correlations between inflammatory molecules and markers of insulin resistance, and several prospective studies reported inflammatory markers predicting T2DM; among these, IL-6 is recognized as an important correlate of insulin resistance (15). No studies have so far explored the relationship between HLA-G and either the metabolic and inflammatory pattern of obesity and diabetes or the different degree of glucose tolerance; therefore, we designed a study to specifically address these issues.

Subjects and Methods

A total of 230 voluntary participants were consecutively recruited among those attending our outpatient clinic for metabolic diseases. Exclusion criteria were age higher than 70 yr, chronic heart or lung disease, cancer, systemic inflammatory disease, and kidney failure. All subjects gave their informed consent. Height and weight were recorded, sitting blood pressure (BP) was measured three times, and a fasting venous blood sample was obtained. Subjects underwent a 75-g oral glucose tolerance test, classifying them into three groups according to American Diabetes Association criteria: normal glucose tolerance (NGT), n = 118 (49 males/69 females); impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), n = 77 (34 males/43 females); and T2DM, n = 35 (15 males/20 females).

Biochemical parameters

Plasma glucose was measured by glucose oxidase technique and plasma insulin by RIA (Linco Research, St. Louis, MO). Plasma total and high-density lipoprotein cholesterol and triglycerides were assayed spectrophotometrically by an automatic colorimetric system (Cobas Miras; Roche, Basel, Switzerland). Insulin sensitivity was evaluated using the oral glucose-derived insulin sensitivity index (OGIS) (16), which provides a validated estimation of glucose clearance during the euglycemic hyperglycemic clamp. Areas under time-concentration curves were calculated by the trapezoidal rule.

Cytokine and soluble HLA-G (sHLA-G) assay

Highly sensitive IL-6 and IL-10 concentrations were analyzed in duplicate using commercially available ELISAs (R&D Systems, Minneapolis, MN). The intraassay and interassay coefficients of variation (CVs) for IL-6 were 4.0 and 6.9%, respectively. The detection limit for IL-10 was less than 3.9 pg/ml.

HLA-G determination

Total sHLA-G antigen concentrations (sHLA-G1 from proteolytic degradation of membrane HLA-G1 antigens and HLA-G5 from alternative splicing) were investigated by ELISA. Then 20 μg/ml of MEM-G9 monoclonal antibody (EXBIO Praha, Vestec, Czech Republic) was used as capture antibody, and anti-β2 microglobulin-horseradish peroxidase-conjugated monoclonal antibody (Dako, Glostrup, Denmark) as detection antibody. sHLA-G concentration (mean of triplicate plasma samples) was estimated by absorbance at 450 nm on a microplate reader (Wallac Victor-3; PerkinElmer, Waltham, MA). The intraassay CV was 1.4%, and the interassay CV was 4.0%. The limit of sensitivity was 1.0 ng/ml. Samples with sHLA-G level below the detection limit were indicated as 0 ng/ml.

Statistical analysis

Data are given as mean ± SEM. Group comparisons were carried out by Mann-Whitney or ANOVA and *post hoc* Bonferroni/Dunn test for normally or nonnormally distributed variables. Associations between categorical variables were examined by χ^2 tests. Relationships between variables were tested by Spearman correlation and multiple regression models. Adjustment for covariates was carried out by ANCOVA. Analyses were performed using Statview software (SAS Institute Inc., Cary, NC).

TABLE 1. Anthropometric and metabolic characteristics of the study subjects by sHLA-G presence

	sHLA-G negative	sHLA-G positive	*P*	Adjust *P*
n (males/females)	86 (41/45)	144 (62/82)	ns[a]	
NGT/(IFG/IGT)/T2DM (n)	57/14/15	61/63/20	<0.0001[a]	
Lean/overweight/obese (n)	28/29/29	15/97/32	<0.0001[a]	
BMI (kg/m^2)	29.0 ± 0.8	34.5 ± 0.7	<0.0001	
Age (yr)	46 ± 1	45 ± 1	ns	
Systolic BP (mm Hg)	129 ± 1	133 ± 1	0.02	0.05
Diastolic BP (mm Hg)	80 ± 1	82 ± 1	0.02	ns
Total cholesterol (mmol/liter)	4.92 ± 0.11	5.24 ± 0.09	0.01	0.004
HDL cholesterol (mmol/liter)	1.30 ± 0.04	1.27 ± 0.03	ns	ns
LDL cholesterol (mmol/liter)	2.74 ± 0.12	3.14 ± 0.09	0.01	0.06
Triglycerides (mmol/liter)	1.34 ± 0.10	1.36 ± 0.06	ns	ns
Uric acid (μmol/liter)	304 ± 11	321 ± 7	ns	ns
Fasting glucose (mmol/liter)	5.73 ± 0.16	5.63 ± 0.10	ns	ns
2-h glucose (mmol/liter)	7.21 ± 0.32	7.92 ± 0.21	0.0001	0.01
Fasting insulin (pmol/liter)	45 ± 3	89 ± 4	<0.0001	ns
AUIC (nmol · liter^{-1} · 2 h^{-1})	32.27 ± 2.52	54.13 ± 3.25	<0.0001	ns
OGIS (ml · min^{-1} · m^{-2})	417 ± 7	374 ± 6	<0.0001	ns

Data are expressed as mean ± SEM. *P*, By Mann-Whitney *U* test; adjusted *P*, *P* value adjusted for BMI. HDL, High-density lipoprotein; LDL, low-density lipoprotein.

[a] By χ^2 test.

Results

T2DM subjects were significantly heavier and older than NGT and IFG/IGT (both $P < 0.0001$). Gender distribution was similar; systolic BP increased progressively from NGT to IFG/IGT and T2DM. Insulin sensitivity was reduced in both IFG/IGT and T2DM, whereas the plasma glucose and insulin responses to oral glucose [area under the insulin curve (AUIC)] were significantly higher (both $P < 0.0001$) in IFG/IGT and T2DM.

sHLA-G was detected in 144 subjects (sHLA-G positive), being less frequent in NGT (negative/positive subjects, NGT = 57/61; IFG/IGT = 14/63; and T2DM = 15/20; $\chi^2 = 18.6$; $P < 0.0001$). sHLA-G positivity was similar between males and females and more frequent in overweight and obese than in lean subjects (52.4 and 77% *vs.* 34.9; $\chi^2 = 27.9$; $P < 0.0001$).

When the study group was stratified according to sHLA-G presence, sHLA-G-positive individuals had higher body mass index (BMI), systolic BP, and plasma cholesterol (Table 1); they also displayed higher areas under the curve of both glucose and insulin, and reduced insulin sensitivity (OGIS value). Because these variables are related to obesity, the results were adjusted for the BMI: systolic BP, total cholesterol, and both 2-h glucose and area under the glucose curve (AUGC) remained significantly higher in sHLA-G-positive individuals. Moreover, in sHLA-G-positive subjects, there was a progressive, significant increment of sHLA-G plasma levels from NGT to IFG/IGT ($P = 0.05$) and T2DM ($P = 0.007$) (Fig. 1A).

IL-6, a cytokine related to insulin sensitivity and increased in subjects with impaired glucose metabolism ($P = 0.0003$ by ANOVA), was almost 2-fold higher in sHLA-G-positive than in sHLA-G-negative subjects (3.15 ± 0.29 *vs.* 1.72 ± 0.14 pg/ml; $P = 0.004$ by ANCOVA), irrespective of BMI. However, IL-10 levels, frequently associated with the presence of sHLA-G, were similar [6.45 ± 0.42 *vs.* 6.44 ± 0.42 pg/ml; P = not significant (ns)].

In the whole study group, AUGC was directly associated to BMI, fasting insulin, and AUIC (rho = 0.38, 0.71, and 0.62; all $P < 0.0001$) and inversely to OGIS (rho = −0.61; $P < 0.0001$).

In the sHLA-G-positive subgroup, after adjustment for BMI, sHLA-G concentrations were directly related to systolic BP (r = 0.32; $P = 0.05$), total cholesterol (r = 0.16; $P = 0.04$), 2-h glucose (r = 0.38; $P = 0.002$), AUGC (Fig. 1B), and IL-6 plasma levels (Fig. 1C).

In a multivariate regression model, sHLA-G was significantly related to 2-h glucose (partial r = 0.22; $P = 0.03$), AUIC (partial r = −0.27; $P = 0.01$), and IL-6 (partial r = 0.22; $P = 0.04$) (multiple r^2 = 0.14; $P = 0.001$). In this model, age, gender, and BMI were not significant covariates; furthermore, replacing 2-h glucose with AUGC did not change the results. In the sHLA-G-positive subgroup, by a multiple regression model, AUGC was associated with age, AUIC, and OGIS, and with sHLA-G (multiple r^2 = 0.48; $P < 0.0001$).

Discussion

This study shows for the first time an association between degree of glucose tolerance and either presence and increased plasma levels of sHLA-G; moreover, HLA-G is directly related with IL-6, a cytokine involved in the sub-

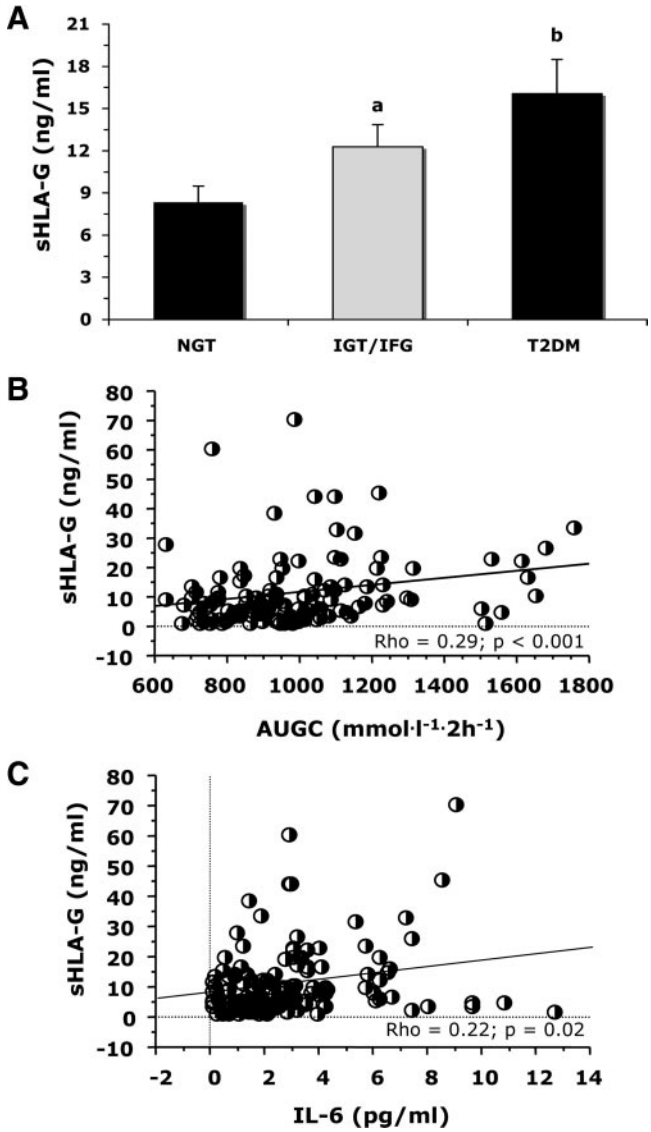

FIG. 1. A, sHLA-G levels, expressed as mean ± SEM, according to glucose tolerance status. a, $P = 0.05$; and b, $P = 0.007$ *vs.* NGT group by ANOVA and *post hoc* Bonferroni/Dunn test. B and C, Relationship between sHLA-G concentration and AUGC (B) (rho = 0.29; $P < 0.001$) and IL-6 (C) levels (rho = 0.22; $P = 0.02$).

clinical inflammatory status described during the course of obesity and T2DM.

Several reports have evaluated the influence of sHLA-G levels in different autoimmune diseases; despite some contrasting results, a positive function for increased sHLA-G levels has been reported in skin and neurological diseases, allergy, rheumatological and gastrointestinal diseases (5, 6, 17). Although T2DM and obesity are not autoimmune diseases, we found increased plasma concentrations of sHLA-G molecules in patients carrying these metabolic abnormalities. In comparison with controls, higher sHLA-G levels are present even in early stages of altered glucose tolerance, like IFG and IGT, and progressively increase across the different stages of glucose tolerance. These observations are further confirmed by age-, gender-, and BMI-independent correlations with metabolic parameters like glycemia and insulinemia in response to glucose ingestion. In addition, the correlation with blood pressure and cholesterol levels suggests that sHLA-G might also be involved in other components of the metabolic syndrome.

sHLA-G is of special interest because it contributes to control $CD4^+$ and $CD8^+$-T-cell activities, dendritic cell maturation, and regulatory T-cell production, thus playing an important role in innate and adaptive immunity. Although a major impairment of the humoral innate immunity has not been described in T2DM, studies have shown decreased functions (chemotaxis, phagocytosis, killing) of diabetic polymorphonuclear cells and monocytes/macrophages compared with controls (18). Our observation of higher sHLA-G levels in the incipient alterations of glucose metabolism reinforces the involvement of the immune system in the inflammatory condition that characterizes the development or progression of diabetes.

A possible link between obesity, T2DM, and HLA-G could be the chronic local inflammation in adipose tissue, where cells of the innate immune system, particularly macrophages, are crucially involved (19). An important marker of the adipose tissue inflammation is the increase of IL-6. In accordance with this hypothesis, we found higher IL-6 levels in sHLA-G-positive subjects, with a direct relationship between the concentrations of the two molecules, thus suggesting sHLA-G as a further putative marker of the subclinical inflammation accompanying glucose metabolism alterations.

In physiological condition, activated CD14+ monocyte cells are the main responsibility for sHLA-G secretion in plasma. This production is strictly dependent on IL-10 cytokine, secreted in the microenvironment in response to inflammatory stimuli (20), and a positive relationship exists between IL-10 and sHLA-G levels (21). We found similar IL-10 concentrations in sHLA-G-positive and -negative subjects, with no correlation between these molecules. IL-10 has been recently suggested as a protecting factor toward deterioration of insulin sensitivity (22), with lower circulating levels in obesity and T2DM (12, 13), as observed as a trend also in our study group (data not shown). We may speculate that the lack of such correlation, found in other diseases like psoriasis and asthma (17, 21), could be the consequence of a certain degree of insulin resistance described in early stages of glucose tolerance impairment.

In conclusion, we show for the first time that HLA-G is frequently expressed in subjects with an impaired glucose metabolism and is linked to a typical biomarker of insulin resistance like IL-6. Further studies in a larger study cohort are needed to confirm these observations, to clarify the precise origin of sHLA-G molecules, and to investigate

their potential role in the pathogenesis of the metabolic disturbances during the course of obesity and T2DM.

Acknowledgments

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Acute Effect of Roux-En-Y Gastric Bypass on Whole-Body Insulin Sensitivity: A Study with the Euglycemic-Hyperinsulinemic Clamp

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Context: Insulin resistance ameliorates after bariatric surgery, yet there is still a need for data on the acute effect of Roux-en-Y gastric bypass (RYGBP) on insulin sensitivity.

Objective: The objective of the study was to describe the acute effect of RYGBP on insulin sensitivity, measured by both the euglycemic-hyperinsulinemic clamp and homeostasis model assessment insulin resistance index (HOMA-IR).

Design and Setting: Evaluations were conducted before and 1 month after RYGBP at State University of Campinas (São Paulo, Brazil).

Patients: Patients included 19 premenopausal women with metabolic syndrome aged 35.3 (6.7) yr, body mass index 45.50 (3.74) kg/m^2 [mean (SD)]. Six had mild type 2 diabetes, seven impaired glucose tolerance, and six normal glucose tolerance.

Interventions and Main Outcome Measures: The volunteers underwent RYGBP either alone or combined with omentectomy. Euglycemic-hyperinsulinemic clamp, HOMA-IR, nonesterified fatty acids, leptin, ultrasensitive C-reactive protein, adiponectin, and IL-6 were assessed at baseline and 4.5 (0.9) wk postoperatively.

Results: Fasting glucose decreased [99.2 (13.1) to 83.6 (8.1) mg/dl, $P < 0.01$] along with a reduction in fasting insulin [30.4 (17.0) to 11.4 (6.3) mU/liter, $P < 0.01$]. M value did not improve postoperatively [25.82 (6.30) to 22.02 (6.05) μmol/kg$_{FFM}$ · min] despite of a decrease in body weight [114.8 (14.5) to 102.3 (14.5) kg, $P < 0.001$]. This finding was discordant to the observation of an improvement in HOMA-IR [3.85 (2.10) to 1.42 (0.76), $P < 0.01$]. Nonesterified fatty acids increased. Leptin and C-reactive protein decreased. IL-6 and adiponectin remained unchanged.

Conclusions: A month after RYGBP, fasting glucose metabolism improves independent of a change in peripheral insulin sensitivity. **(*J Clin Endocrinol Metab* 95: 3871–3875, 2010)**

Insulin resistance is related to the degree of obesity and decreases after weight loss (1, 2). Long-term, massive weight loss after bariatric surgery generally promotes a proportional increase in insulin sensitivity (IS) (1). Moreover, the short-term postoperative improvements in IS and metabolic syndrome components, especially glucose tolerance, are greater than expected for the body mass index (BMI) change and have been pre-

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Abbreviations: BMI, Body mass index; BPD, biliopancreatic diversion; FFM, fat-free mass; GIR, glucose infusion rate; IS, insulin sensitivity; HOMA-IR, homeostasis model assessment insulin resistance index; MCRg, glucose metabolic clearance rate; M/I, M adjusted for steady-state insulin; NEFA, nonesterified fatty acid; RYGBP, Roux-en-Y gastric bypass; us-CRP, ultrasensitive C-reactive protein.

sumed to be due to caloric restriction and an enhanced incretin effect (1).

The homeostasis model assessment insulin resistance index (HOMA-IR), a surrogate marker of IS, improves within the first month after different bariatric procedures (1, 3). In fact, nearly all of the long-term improvement in HOMA-IR observed after bariatric surgery occurs before clinically meaningful weight loss. Studies using the euglycemic-hyperinsulinemic clamp have demonstrated long-term (3 to >24 months) improvements in IS after Roux-en-Y gastric bypass (RYGBP) and biliopancreatic diversion (BPD) (4–6). However, few studies have assessed the shorter-term effects using the clamp technique after either BPD (7–9) or RYGBP (10).

In a 2-yr follow-up, our group previously showed a greater enhancement of IS after BPD compared with RYGBP (3). There is also evidence for even more dramatic differences between these two operations in the short term. After BPD, most of the long-term improvement was achieved within 7–10 d, before significant weight loss, in subjects with glucose tolerance ranging from normal to type 2 diabetes (7, 8). In contrast, it has been shown in nondiabetic subjects that HOMA-IR reduction was not accompanied by a change in IS measured using the clamp technique 14 d after RYGBP (10) or in subjects with glucose tolerance ranging from normal to diabetes using the frequently sampled iv glucose tolerance test 1 month after RYGBP (11). The authors of these studies hypothesized that hepatic IS (represented by HOMA-IR) improves earlier than peripheral IS (the main aspect of whole-body IS measured by the iv glucose tolerance test and clamp) after RYGBP.

RYGBP is the most performed obesity surgery worldwide, yet there is a need for specific data on the short-term effects of this technique on whole-body IS. The present study assessed the acute effects of RYGBP on IS, measured by both the euglycemic-hyperinsulinemic clamp and HOMA-IR, 1 month postoperatively across a range of glucose tolerance including type 2 diabetes. This work is a portion of a randomized trial on the long-term metabolic effects of total greater omentectomy, a potential ancillary procedure to obesity surgery.

Subjects and Methods

Nineteen grade III obese (BMI ≥40 kg/m^2), premenopausal women, aged 35.3 (6.7) yr [mean (SD)], with the metabolic syndrome based on International Diabetes Federation criteria (12), participated in a prospective trial that had been approved by the Institutional Ethics Review Board at State University of Campinas. All participants provided written informed consent before participation. They underwent a RYGBP between March 2006 and February 2008 and were randomized to simultaneously receive a total greater omentectomy (n = 10 of 19) to study its long-term metabolic effects (1, 6–8, and 12–18 months after surgery).

Subjects were classified by a standard oral glucose tolerance test as normal glucose tolerance (n = 6), impaired fasting glucose and impaired glucose tolerance (n = 7), and type 2 diabetes (n = 6), of whom three, four, and three, respectively, were randomized to omentectomy. Four diabetic subjects had been diagnosed within 1–3 yr, were not taking any glucose-lowering agent, and had a glycosylated hemoglobin less than 7%.

Evaluations were performed at baseline and 4.5 (0.9) wk after surgery for IS, biochemistry, anthropometric measures, and body composition, the latter by electric bioimpedance (Biodynamics Corp., Seattle, WA).

To quantify IS, a 180-min euglycemic-hyperinsulinemic clamp (4) was performed. For this, a primed continuous iv insulin infusion (40 mU/m^2 · min) was administered. Fasting glycemia was maintained (variation < 5%) by a variable rate glucose infusion and blood glucose determination (glucose oxidase) every 5 min by YSI 2700 biochemistry analyzer (Yellow Springs Inc., Yellow Springs, OH). If fasting hyperglycemia was present, it was corrected to a target of 100 mg/dl by the initial iv insulin infusion. IS was calculated as the glucose infusion rate (GIR) in the last 60 min (steady state), corrected for the glucose distribution space and adjusted to fat-free mass (FFM), resulting in the M value. Other indexes of IS were calculated from the M value: glucose metabolic clearance rate (MCRg = M/steady state glycemia) and M adjusted for steady-state insulin (M/I).

HOMA-IR was calculated using the HOMA Calculator (version 2.2.2, http://www.dtu.ox.ac.uk) (13).

Serum samples were analyzed for basal and steady-state insulin (ELISA; Bayer Corp., Tarrytown, NY) as well as basal levels of nonesterified fatty acids (NEFAs; ELISA; Wako Chemicals, Richmond, VA), leptin, ultrasensitive C-reactive protein (us-CRP), IL-6, and adiponectin (ELISA; R&D Systems Inc., Minneapolis, MN).

SPSS (version 12; SPSS Inc., Chicago, IL) was used for statistical analysis. Wilcoxon signed-rank tests were used to compare baseline to postsurgery data; Mann-Whitney *U* tests to compare omentectomy subgroups and Kruskal-Wallis tests to compare glucose tolerance subgroups. Pearson correlation coefficient was used to assess bivariate correlations. Data are presented as mean and SD [mean (SD)]. Postsurgery change in the variables was calculated as the percent difference from baseline values. Statistical significance was assumed if $P < 0.05$.

Results

The anthropometric and metabolic variables at baseline and a month after surgery are presented in Table 1. The subgroups based on omentectomy had similar changes in the variables aside from omentectomy being associated with slightly greater weight loss [−12.6% (2.5%) *vs.* −9.3% (2.5%), $P < 0.05$]. Thus, we analyzed the pooled data of the whole group of operated subjects.

Both fasting glucose and insulin decreased so that there was a significant reduction in HOMA-IR [−55.0% (30.6%), $P = 0.01$] in all subjects except two, in whom it remained unchanged (one had normal glucose tolerance

TABLE 1. Anthropometric and metabolic variables at baseline and 1 month after RYGBP in the whole group or based on omentectomy

Variables	Whole group (n = 19) [mean (SD)]		Omentectomy (n = 10) [mean (SD)]		Control (n = 9) [mean (SD)]	
	Baseline	After surgery	Baseline	After surgery	Baseline	After surgery
Body weight (kg)	114.8 (14.5)	102.3 (14.5)[a]	115.0 (14.0)	100.8 (14.5)[a]	114.6 (15.9)	104.0 (15.1)[a]
BMI (kg/m^2)	45.5 (3.7)	40.5 (4.2)[a]	45.0 (4.4)	39.4 (4.7)[a]	46.1 (3.0)	41.8 (3.2)[a]
FFM (kg)	64.1 (7.7)	58.9 (7.3)[a]	64.2 (7.1)	58.8 (7.4)[a]	64.1 (8.7)	59.0 (7.6)[a]
Fat mass (kg)	50.3 (7.8)	43.4 (7.9)[a]	50.4 (8.2)	42.0 (7.8)[a]	50.1 (7.7)	44.9 (8.2)[b]
Fat mass (%)	43.7 (2.2)	42.3 (2.7)[b]	43.7 (2.6)	41.5 (2.5)[b]	43.7 (1.8)	43.0 (2.8)
Fasting glucose (mg/dl)	99.2 (13.1)	83.6 (8.1)[a]	101.1 (18.0)	83.7 (11.2)[c]	100.3 (13.3)	84.9 (6.5)[a]
Fasting insulin (mU/liter)	30.4 (17.0)	11.4 (6.3)[a]	26.9 (13.5)	11.6 (7.5)[a]	34.2 (20.3)	11.3 (5.0)[b]
HOMA-IR	3.85 (2.10)	1.42 (0.76)[a]	3.64 (1.93)	1.59 (0.82)[a]	4.09 (2.48)	1.44 (0.67)[a]
M (μmol/kg$_{FFM}$ · min)	25.8 (6.3)	22.0 (6.1)	28.2 (6.5)	22.4 (8.3)	23.2 (5.3)	22.7 (3.5)
GIR (μmol/kg · min)	16.2 (4.5)	13.7 (5.0)	15.8 (3.7)	12.5 (4.9)	13.0 (3.0)	12.9 (2.2)
MCRg (ml/kg$_{FFM}$ · min)	4.9 (1.4)	4.7 (1.5)	5.5 (3.5)	4.8 (2.5)	4.3 (2.7)	4.7 (3.8)
M/I [(μmol/kg$_{FFM}$ · min · pmol · liter) · 10^{-3}]	34.0 (15.1)	34.6 (14.0)	34.9 (15.3)	32.7 (13.8)	27.8 (11.1)	32.0 (10.3)
Steady-state glucose (mg/dl)	94.4 (8.8)	84.7 (8.2)[b]	92.7 (7.8)	82.7 (10.6)[b]	97.7 (10.1)	86.5 (3.7)[b]
Steady-state insulin (mU/liter)	151.6 (48.7)	121.9 (33.0)[b]	148.7 (51.9)	119 (39.1)[b]	154.8 (47.8)	124.8 (27.7)
Adiponectin (μg/ml)	5.22 (3.33)	6.04 (3.64)	6.46 (3.67)	6.12 (3.34)	4.08 (2.68)	6.29 (4.22)
Leptin (ng/ml)[d]	73.39 (39.00)	40.52 (30.88)[a]	54.70 (25.9)	28.7 (24.23)[b]	97.7 (40.3)	54.02 (34.97)[c]
IL-6 (pg/ml)	3.10 (2.20)	4.25 (5.72)	2.50 (1.43)	4.63 (7.85)	4.00 (4.16)	4.16 (2.69)
us-CRP (mg/dl)	1.37 (0.67)	0.77 (0.63)[a]	1.16 (0.63)	0.76 (0.81)	1.64 (0.69)	0.83 (0.42)[b]
NEFAs (mg/dl)	67.96 (25.66)	98.98 (43.43)[a]	71.63 (20.87)	106.88 (42.30)[b]	56.40 (25.94)	96.16 (45.40)

Control, RYGBP without omentectomy; M, clamp insulin sensitivity index (GIR adjusted to FFM).

[a] $P < 0.01$, Wilcoxon test (after surgery *vs.* baseline).

[b] $P < 0.05$, Wilcoxon test (after surgery *vs.* baseline).

[c] $P = 0.05$, Wilcoxon test (after surgery *vs.* baseline).

[d] There was a difference in the leptin values between the subgroups ($P < 0.05$, Mann-Whitney *U* test) both at baseline and after surgery but not in its change.

and the other diabetes). In contrast, IS (M value) did not improve postoperatively, despite a significant decrease in body weight (Table 1). Adjustments of the M value for the steady-state insulin or steady-state glucose, expressed respectively as M/I or MCRg (Table 1), did not alter the findings and even increased the spread of the results. There was no relationship between the postoperative change in steady-state insulin and change in M value. Despite no change in the average M value, it did improve in four subjects (+27 to +58%), decreased in nine (−19 to −68%), and was considered stable in six (−11 to +7%) based on the intrasubject variability of approximately 10% found in the literature (14).

Table 2 presents the data based on glucose tolerance subgroups. The changes in the variables were not different among subgroups with the exception of fasting glucose ($P < 0.05$). Type 2 diabetes remitted within the first month after surgery in all cases based on the fasting glucose, and 3 months after surgery, all had a glycosylated hemoglobin less than 6%.

IS (M value and HOMA-IR) did not correlate with body weight, BMI, FFM, fat mass, or percentage of body fat either before or after surgery. Also, the change in IS did not correlate with the change in these variables.

After surgery, NEFAs increased, leptin and us-CRP decreased, and there was no significant change in IL-6 and adiponectin (Table 1). Leptin was lower in the omentectomy subgroup both at baseline and after surgery but the change was similar between the subgroups (Table 1). None of these variables correlated with each other or the M value, HOMA-IR, fasting insulin, and glucose, either before or after surgery. Their baseline and postsurgery values and change did not correlate with the change in IS.

Discussion

This study represents the first description of the acute effect of RYGBP on whole-body IS using the gold standard method, the euglycemic-hyperinsulinemic clamp, across a range of glucose tolerance including type 2 diabetes. IS measured by the clamp did not increase within a month after surgery among premenopausal women. This finding was discordant to the observation of an improvement in HOMA-IR. Because HOMA represents essentially hepatic IS, whereas M is more a measure of peripheral IS, this suggests differential effects in the short period after

TABLE 2. Main anthropometric and metabolic variables at baseline and 1 month after RYGBP based on glucose tolerance category

	Normal tolerance (n = 6) [mean (SD)]		Impaired tolerance (n = 7) [mean (SD)]		Type 2 diabetes (n = 6) [mean (SD)]	
Variables	**Baseline**	**After surgery**	**Baseline**	**After surgery**	**Baseline**	**After surgery**
Body weight (kg)	113.1 (9.7)	100.3 (11.4)[a]	119.8 (12.0)	107.3 (10.7)[a]	110.7 (20.8)	98.5 (20.7)[a]
BMI (kg/m^2)	43.6 (3.0)	38.7 (4.0)[a]	47.7 (4.0)	42.8 (4.1)[a]	44.9 (3.2)	39.8 (3.8)[a]
Fasting glucose (mg/dl)	86.2 (4.4)	85.0 (5.2)	100.9 (7.1)	86.8 (8.5)[a]	115.0 (16.9)	80.7 (12.4)[a]
Fasting insulin (mU/liter)	23.8 (7.7)	12.5 (8.7)[a]	29.9 (15.2)	12.6 (6.2)[a]	37.5 (24.3)	8.9 (2.9)[a]
HOMA-IR	2.89 (1.05)	1.57 (0.99)[a]	4.25 (2.14)	1.79 (0.70)[a]	4.36 (2.9)	1.16 (0.34)[a]
GIR (μmol/kg · min)	16.5 (3.0)	12.7 (5.4)	12.4 (2.9)	11.7 (2.9)	14.8 (4.0)	14.2 (2.0)
M (μmol/kg$_{FFM}$ · min)	28.9 (5.4)	21.5 (9.1)	22.6 (5.5)	20.8 (4.5)	26.5 (7.3)	25.7 (4.4)
MCRg (ml/kg$_{FFM}$ · min)	6.0 (1.3)	4.5 (1.8)[a]	4.1 (0.8)	4.3 (1.1)	4.8 (1.4)	5.7 (1.3)
M/I [(μmol/kg$_{FFM}$ · min · pmol · liter) · 10^{-3}]	36.4 (6.1)	28.2 (12.2)[a]	26.7 (9.3)	32.2 (12.3)	32.4 (21.5)	37.5 (11.0)
Steady-state glucose (mg/dl)	87.4 (6.7)	85.3 (3.3)	98.2 (10.7)	87.7 (7.4)	99.2 (3.5)	79.3 (11.0)[a]
Steady-state insulin (mU/liter)	133.5 (24.7)	134.1 (34.8)	155.3 (57.9)	116.2 (34.6)[a]	165.3 (57.2)	115.2 (31.2)

M, Clamp insulin sensitivity index (GIR adjusted for FFM).

[a] $P < 0.05$, Wilcoxon test (after surgery *vs.* baseline).

RYGBP and is in keeping with the known differential IS of the liver and peripheral tissues (13).

The reason(s) for our observation of no change in IS with the clamp study is unclear. One possible explanation relates to body composition. Both fat and FFM loss contributed largely to the initial weight reduction and the percentage of body fat remaining was still high. Moreover, obesity indexes did not correlate with IS, indicating that other factors likely determine IS in individuals with such extreme fat mass and with limited postsurgery change.

HOMA-IR mainly reflects the interaction between hepatic glucose output and insulin secretion in the fasting state (15). During the clamp, in the insulin-stimulated state, peripheral tissues account for approximately 90% of glucose disposal, more than half occurring in skeletal muscle (14). Caloric restriction has been proposed as a major contributor to the acute decrease in HOMA-IR (1, 3). Under conditions of negative energy balance, glucose disposal (mostly insulin independent) exceeds carbohydrate intake, thereby restricting the glucose pool and more specifically hepatic glycogen (16). Peripheral insulin-mediated glucose disposal might eventually decrease due to preferential fatty acid oxidation in skeletal muscles (16).

Selective improvement of hepatic IS regardless of peripheral change after a very low-calorie diet has been demonstrated in studies using the euglycemic-hyperinsulinemic clamp (17). Variability in intramyocellular lipid depletion, in contrast to a more uniform intrahepatic lipid reduction (17, 18), could account for the divergences observed in the present study. Major lipid malabsorption and intramyocellular lipid depletion are distinct effects of BPD (1, 19), which might enhance IS in skeletal muscle earlier than occurs with RYGBP.

The design of our study has some limitations, although none bear on our findings. First, we did not perform radioisotope-labeled glucose clamp studies that can differentiate changes in hepatic *vs.* peripheral IS and studies that assess substrate use. Second, we did not include a BDP arm for a direct comparison with RYGBP. Third, we studied only premenopausal women so cannot be sure that our results apply to postmenopausal women and men, although there is no reason to believe that would be the case. Lastly, the small number of subjects within subgroups by glucose tolerance make across-group comparisons unreliable and will require further study.

In summary, our data suggest that RYGBP affects hepatic glucose metabolism earlier than peripheral insulin action, which differs from observations after BPD. These findings underscore that the outcomes of the various bariatric techniques differ, and there are likely many that have not yet been identified, some of which could provide insights and new approaches to the treatment of obesity, diabetes, and related disorders.

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Mechanisms for the Antihyperglycemic Effect of Sitagliptin in Patients with Type 2 Diabetes

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Context: Dipeptidyl peptidase IV (DPP-4) inhibitors improve glycemic control in patients with type 2 diabetes. The underlying mechanisms (incretin effect, β-cell function, endogenous glucose production) are not well known.

Objective: The aim of the study was to examine mechanisms of the antihyperglycemic effect of DPP-4 inhibitors.

Design, Setting, and Patients: We administered a mixed meal with glucose tracers ([6,6-$^2H_2$]-glucose infused, [1-^{2}H]-glucose ingested), and on a separate day, a glucose infusion matched the glucose responses to the meal (isoglycemic test) in 50 type 2 diabetes patients (hemoglobin A_{1c} = 7.4 $\pm$ 0.8%) and seven controls; 47 diabetic completers were restudied after 6 wk. Glucose fluxes were calculated, and β-cell function was assessed by mathematical modeling. The incretin effect was calculated as the ratio of oral to iv insulin secretion.

Intervention: We conducted a 6-wk, double-blind, randomized treatment with sitagliptin (100 mg/d; n = 25) or placebo (n = 22).

Results: Relative to placebo, meal-induced changes in fasting glucose and glucose area under the curve (AUC) were greater with sitagliptin, in parallel with a lower appearance of oral glucose [difference (post-pre) AUC = -353 ± 915 *vs.* $+146 \pm 601$ μmol $\cdot$ kg^{-1} $\cdot$ 5 h] and greater suppression of endogenous glucose production. Insulin sensitivity improved 10%, whereas total insulin secretion was unchanged. During the meal, β-cell glucose sensitivity improved (+19[29] *vs.* 5[21] pmol $\cdot$ min^{-1} $\cdot$ m^{-2} $\cdot$ mM^{-1}; median [interquartile range]) and glucagon AUC decreased (19.6 $\pm$ 7.5 to 17.3 $\pm$ 7.1 ng $\cdot$ ml^{-1} $\cdot$ 5 h), whereas intact glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 AUC increased with sitagliptin *vs*. placebo. The incretin effect was unchanged because sitagliptin increased β-cell glucose sensitivity also during the isoglycemic test.

Conclusions: Chronic sitagliptin treatment improves glycemic control by lowering the appearance of oral glucose, postprandial endogenous glucose release, and glucagon response, and by improving insulin sensitivity and β-cell glucose sensing in response to both oral and iv glucose. (***J Clin Endocrinol Metab*** **97: 2818–2826, 2012)**

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Abbreviations: AUC, Area under time-concentration curve; BMI, body mass index; DPP-4, dipeptidyl peptidase IV; EGP, endogenous glucose production; FFM, fat-free mass; FPG, fasting plasma glucose; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; β-GS, β-cell glucose sensitivity; HbA_{1c}, hemoglobin A_{1c}; Iso-G, isoglycemic test; MCR, metabolic clearance rate of glucose; MTT, meal tolerance test; O/IV, ratio of oral to iv; RaO, rate of appearance of oral glucose; RaT, total rate of glucose appearance; Rd, rate of glucose disappearance; T2D, type 2 diabetes mellitus; TTR, tracer-to-tracee ratio.

β-Cell dysfunction is a key pathogenetic defect in patients with type 2 diabetes mellitus (T2D); worsening of β-cell function marks the progression of the disease and is a main target for treatment (1).

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) potentiate glucose-induced insulin release; in addition, GLP-1 also suppresses glucagon release and slows down gastric emptying (2). Enhanced insulin release, in concert with a reduction in glucagon concentration, reduces the postprandial rise in glucose concentrations. Although it is still somewhat uncertain whether and to what extent the GLP-1 response is reduced in T2D patients (3), the incretin effect—operationally defined as the potentiation of insulin release by oral *vs.* iv glucose—is compromised in T2D (4, 5). Although the secretion of GLP-1 is often reduced, the insulinotropic effect of both GLP-1 and GIP is severely compromised (6).

Infusion of GLP-1 in T2D patients augments insulin release, leading to normalization of glucose concentrations (7, 8). In rodents, GLP-1 also regulates islet-cell mass and morphology (9). Both GLP-1 and GIP have very short half-lives because several peptidases clear them from plasma in a few minutes; among them, dipeptidyl peptidase IV (DPP-4) plays a quantitatively important role. Specific inhibition of DPP-4 delays clearance of GLP-1 and GIP, thereby augmenting active incretin levels. Sitagliptin, an orally active DPP-4 inhibitor, has been shown to improve glycemic control in T2D patients (10). Although the increase in intact GLP-1 by DPP-4 inhibition is likely both to improve glucose-induced insulin secretion (11, 12) and maximal insulin response (13) and to decrease glucagon levels (12, 14, 15), it is not known whether chronic DPP-4 inhibition with sitagliptin restores the incretin effect in T2D patients. Improved insulin secretion in response to oral and iv glucose without changes in incretin effect was demonstrated for the DDP-4 inhibitor vildagliptin (16). Balas *et al.* (12) reported a decrease in endogenous glucose production (EGP) and unchanged rates of plasma glucose appearance and disappearance after vildagliptin. However, DPP-4 inhibitors differ in their structure and pharmacokinetic and pharmacodynamic properties (17), and it is not clear whether effects are a class effect or unique. The downstream action of sitagliptin on peripheral glucose utilization, EGP, and oral glucose handling in diabetic patients has not been examined.

The aim of this study was to assess the mechanism of action of chronic DPP-4 inhibition by measuring the integrated metabolic response to sitagliptin in T2D patients.

Subjects and Methods

Study population

Fifty patients with T2D diagnosed within the past 5 yr were recruited into the study. Inclusion criteria were either sex, age between 30 and 70 yr, and body mass index (BMI) between 20 and 40 kg/m^2. At screening, antihyperglycemic therapy-naive patients had to have hemoglobin A_{1c} (HbA_{1c}) between 7 and 10%, whereas patients on treatment (monotherapy or low-dose oral combination therapy) had to have HbA_{1c} between 6.5 and 10%. After 4 wk of washout and before randomization, fasting plasma glucose (FPG) was required to range from 7.2–14.4 mmol/liter. Exclusion criteria were: unstable body weight; history of malignancy in the last 5 yr; significant cardiovascular disorder within the last 6 months; treatment with more than 12.5 mg daily of thiazide, β-blocker therapy; monotherapy or combination with peroxisome proliferator-activated receptor-γ agonists in the last 12 wk; pregnant women or women expecting to conceive within the study duration; plasma creatinine of 1.5 mg/dl or greater; alanine aminotransferase and aspartate aminotransferase greater than 2.0 × upper limit of normal; abnormal TSH levels; triglycerides greater than 3.39 mmol/liter; creatinine clearance less than 60 ml/min; and blood pressure of 160/95 mm Hg or greater.

Seven subjects with FPG less than 5.6 mmol/liter participated as controls in all baseline experiments.

Study design and protocol

T2D patients were randomized to sitagliptin (100 mg/d) or placebo for 6 wk in a double-blind design with an equal fraction of drug-naive and treated patients in each group.

Each subject underwent two studies within 2–7 d, both before and after treatment. The first study was a meal tolerance test (MTT; 53% carbohydrate, 30% fat, 17% protein, 560 kcal), and the second was an isoglycemic iv glucose infusion [isoglycemic test (Iso-G)]. For the MTT, a primed-constant infusion of [6,6-$^2H_2$]glucose (0.28 $\mu mol \cdot min^{-1} \cdot kg^{-1}$; prime 28 $\mu mol \cdot kg^{-1}$ ×[FPG]/5) was administered throughout a 3-h basal period and during the 5-h meal test. At time 0, subjects ingested (in <10 min) the meal consisting of one egg, 50 g of parmesan cheese, and 75 g of an aqueous solution of D-glucose labeled with [1-^{2}H]glucose. On the Iso-G, tracers were not used, and the plasma glucose profile of the MTT was reproduced by a variable iv glucose infusion, using an *ad hoc*-developed algorithm. Blood was collected at the same time-points of the MTT.

Measurements

Fat-free mass (FFM) was measured by electrical bioimpedance using a Body Composition Analyzer model TB-300 (Tanita, Tokyo, Japan); fat mass was then obtained as the difference between body weight and FFM.

Tracer enrichment of [6,6-$^2H_2$]-glucose and [1-^{2}H]-glucose was measured by gas chromatography/mass spectrometry (model 5985B; Hewlett-Packard, Fullerton, CA) using electron impact ionization and selective ion monitoring at mass-to-charge ratio 202/200 and 205/200.

Plasma glucose was measured by the glucose-oxidase technique (Beckman Glucose Analyzers; Beckman, Fullerton, CA), plasma insulin and C-peptide by electro-chemiluminescence (on a COBASe411; Roche, Indianapolis, IN), and glucagon by RIA (Millipore, Billerica, MA). Samples were assayed for total GLP-1 immunoreactivity using an antiserum (no. 89390) specific for the

TABLE 1. Anthropometric and metabolic characteristics of the study subjects

	Controls	T2D	P^a
n (males/females)	7 (4/3)	47 (14/33)	
Age (yr)	59.6 ± 6.0	56.1 ± 7.3	ns
BMI ($kg \cdot m^{-2}$)	28.8 ± 2.3	29.9 ± 4.2	ns
HbA_{1c} (%)	5.7 ± 0.2	7.4 ± 0.8	<0.0001
FPG (mmol/liter)	5.56 ± 0.33	9.28 ± 1.89	<0.0001
Fasting plasma insulin (pmol/liter)	41 [22]	67 [53]	0.03
Fasting plasma glucagon (pg/ml)	48 ± 15	53 ± 21	ns
OGIS ($ml \cdot min^{-1} \cdot kg_{FFM}^{-1}$)	9.47 ± 1.22	6.33 ± 1.15	<0.0001

Data are expressed as mean ± SD or median [interquartile range]. ns, Nonsignificant.

[a] For the comparison of controls *vs.* T2D patients, by Mann-Whitney test.

C terminal, which reacts equally with intact GLP-1 and the primary (N-terminally truncated) metabolite (18). Intact GLP-1 was measured using an ELISA (19). The assay is a two-site sandwich assay involving two monoclonal antibodies—GLP-1F5 as a catching antibody (C-terminally directed), and Mab26.1 as a detecting antibody (N-terminally directed) (20). For both assays, the detection limit was below 1 pmol/liter, and the intraassay coefficient of variation was below 5% at 20 pmol/liter. Active (N terminal) GIP was assayed by RIA using a polyclonal antiserum 98171, raised in rabbits, that is N-terminally directed and does not recognize N-terminally truncated peptides. It has a cross-reactivity of 100% with human GIP1–42 and less than 0.1 with human GIP3–42, GLP-1 (7–36) amide, GLP-1 (9–36) amide, GLP-2 (1–33), GLP-2 (3–33), and glucagon. The detection limit is approximately 5 pmol/liter, with an ED_{50} of 48 pmol/liter. Intra- and interassay coefficients of variation are less than 6% and less than 15%, respectively (21).

Calculations

Glucose fluxes were expressed per kilogram of FFM because this normalization minimizes differences due to sex, obesity, and age (22). During the last 20 min of the tracer equilibration period, glucose concentrations and [6,6-$^2H_2$]-glucose enrichment were stable in all subjects. Therefore, EGP was calculated as the ratio of [6,6-$^2H_2$]-glucose infusion rate to the plasma tracer enrichment (tracer-to-tracee ratio, $TTR_{6,6}$; mean of three determinations). After glucose ingestion, the total glucose rate of appearance (RaT) was calculated from $TTR_{6,6}$ using Steele's equation (23). Before applying Steele's equation, plasma $TTR_{6,6}$ data were smoothed using a spline fitting to stabilize the calculation of derivatives. The plasma glucose concentration resulting from the absorption of ingested glucose (exogenous glucose concentration) was calculated from the product of total plasma glucose concentration and the ratio of plasma [1-^{2}H]-glucose TTR to the [1-^{2}H]-glucose TTR of the ingested glucose. The plasma glucose concentration resulting from endogenous glucose release was obtained as the difference between total and exogenous glucose concentration. TTR of endogenous glucose and oral glucose rate of appearance (RaO) were calculated as described (23). The tracer-determined rate of glucose disappearance (Rd) provided a measure of insulin-mediated total-body glucose disposal, whereas the metabolic clearance rate of glucose (MCR) provided another index of insulin sensitivity.

β-Cell function was assessed with the use a previously described mathematical model (24). Briefly, the model consists of a model fitting the glucose concentration profile (to smooth and interpolate plasma glucose concentrations), a model describing the dependence of insulin (or C-peptide) secretion on glucose concentration, and a model of C-peptide kinetics, *i.e.* the two-exponential model proposed by Van Cauter *et al.* (25), in which the model parameters are individually adjusted to the subject's anthropometric data. For the purpose of the present analysis, we report the most important parameter, namely, the dose-response relationship between insulin release and plasma glucose concentrations; the mean slope of the dose-response function is taken to represent β-cell glucose sensitivity.

Insulin sensitivity was estimated from the plasma glucose and insulin responses to MTT between times 0 and 120 min by the Oral Glucose Insulin Sensitivity (OGIS), a validated index (26). Areas under time-concentration curves (AUC) were calculated by the trapezium rule.

The incretin effect was operationally defined as the ratio of oral to iv (O/IV) for all insulin secretion measures (4). This cal-

TABLE 2. Glucose concentrations and fluxes during the meal test (Meal) and the isoglycemic test (Iso-G)

			Placebo		Sitagliptin		
	Study	Controls	Pre	Post	Pre	Post	P^a
Glucose AUC ($g \cdot dl^{-1} \cdot 5$ h)	Meal	35.0 ± 1.7[b]	63.0 ± 12.6	64.7 ± 16.3	59.5 ± 11.3	48.8 ± 9.0	<0.0001
	Iso-G	35.8 ± 1.6	64.9 ± 13.1	66.3 ± 16.1	60.9 ± 11.0	50.0 ± 9.1	<0.0001
Glucose incremental AUC ($g \cdot dl^{-1} \cdot 5$ h)	Meal	6.1 ± 1.5[b]	16.4 ± 5.8	17.8 ± 7.1	16.3 ± 7.0	10.8 ± 5.6	<0.0001
	Iso-G	6.6 ± 2.2[b]	15.5 ± 6.9	18.0 ± 7.5	15.8 ± 7.1	10.0 ± 5.5	<0.0001
RaO AUC ($g \cdot 5$ h)	Meal	55 ± 6	53 ± 5	56 ± 9	55 ± 11	50 ± 13	0.017
Intravenous glucose AUC ($g \cdot 5$ h)	Iso-G	51 ± 16	50 ± 15	53 ± 12	48 ± 11	43 ± 13	0.015
Fasting EGP ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)	Meal	9.0 ± 1.6	9.2 ± 1.6	9.0 ± 1.6	9.1 ± 1.3	8.7 ± 1.2	0.02
EGP AUC ($g \cdot 5$ h)	Meal	21 ± 8	22 ± 8	21 ± 8	22 ± 6	19 ± 6	0.16
RaT AUC ($g \cdot 5$ h)	Meal	76 ± 9	74 ± 8	77 ± 13	77 ± 11	69 ± 13	0.005
Rd AUC ($g \cdot 5$ h)	Meal	77 ± 10	72 ± 9	74 ± 17	74 ± 11	68 ± 14	0.043
Fasting MCR ($ml \cdot min^{-1} \cdot kg^{-1}$)	Meal	1.67 ± 0.32[b]	1.10 ± 0.19	1.08 ± 0.25	1.17 ± 0.23	1.24 ± 0.18	ns
MCR AUC ($ml \cdot kg^{-1} \cdot 5$ h)	Meal	783 ± 130[b]	438 ± 117	432 ± 116	460 ± 134	505 ± 138	0.08

Data are expressed as mean ± SD. Pre, Before treatment; post, after treatment; ns, nonsignificant.

[a] For the placebo-adjusted difference between baseline and 6 wk of sitagliptin treatment.

[b] $P < 0.0001$ for the comparison of controls *vs.* all diabetic patients by Mann-Whitney test.

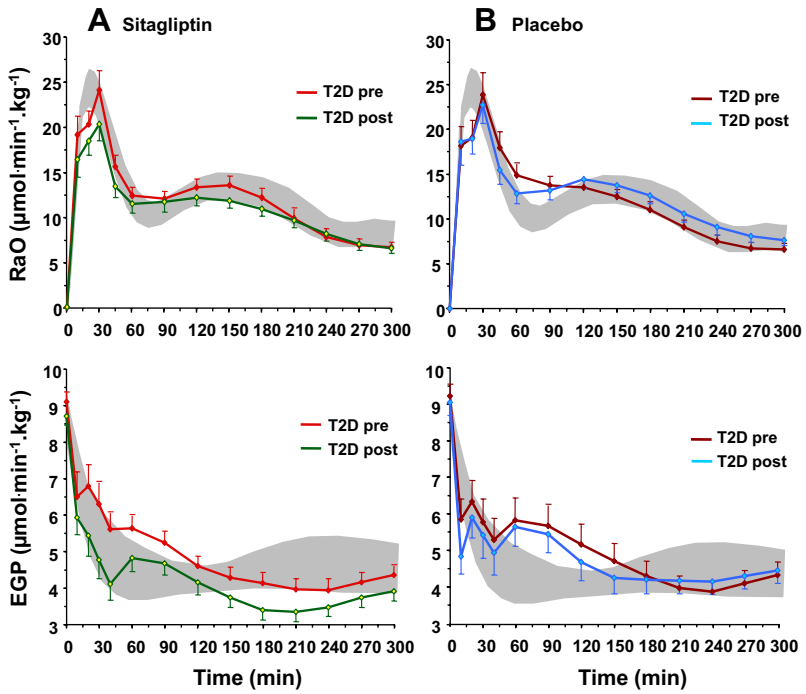

FIG. 1. RaO and EGP in T2D patients before (*pre*) and after (*post*) 6 wk of treatment with sitagliptin (A) or placebo (B). The *shaded areas* are the mean ± SE for the control group.

culation cancels the impact of glucose levels *per se*, which were matched by protocol.

Statistical analysis

Data are given as mean ± SD or median [interquartile range] for non-normally distributed variables. The latter were transformed into their natural logarithms for use in statistical testing. Differences between control and T2D subjects were analyzed by Mann-Whitney test. Treatment responses were analyzed by an analysis of covariance model with change from baseline as the outcome variable and baseline values and prior antidiabetic treatment as covariates. The time-course of glucose fluxes was analyzed by repeated measures ANOVA, modeling treatment (sitagliptin *vs.* placebo), week (baseline and end of study), and MTT time as factors. Univariate associations were tested by Spearman regression (*rho* value). A P value ≤ 0.05 was considered statistically significant.

Results

Three of the 50 patients dropped out after randomization (two from the placebo group—one due to hyperglycemia, the other due to personal reasons; one from the sitagliptin group after an adverse event, *i.e.* canalithiasis, not drug-related). The other 47 patients completed all studies. Age and BMI were similar between patients and controls, whereas baseline HbA_{1c}, fasting glucose, and insulin concentrations were higher in patients; all baseline characteristics were similar between patients randomized to sitagliptin or placebo. Insulin sensitivity was markedly reduced in patients (Table 1).

Both in the fasting state (from 8.94 ± 1.67 to 8.08 ± 1.31 mmol/liter; $P < 0.01$ for the placebo-adjusted change) and in response to the meal ($P < 0.0001$ for placebo-adjusted total and incremental AUC), plasma glucose concentrations were lower with sitagliptin than placebo (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org; and Table 2).

Glucose fluxes

At baseline, RaO peaked at 30 min, with a smaller secondary rise at 120–180 min, in similar amounts and time-course in controls and patients. At the end of 5 h, oral glucose was still appearing in the peripheral circulation at rates significantly different from zero in all groups; RaO totaled three fourths of the load, on average, in patients and controls alike. Sitagliptin treatment was associated with a lower RaO (change from pretreatment, difference = −353 ± 915 *vs.* +146 ± 601 μmol · kg^{-1} · 5 h of placebo; $P = 0.017$), but without distortion in the time pattern (Fig. 1). Baseline fasting EGP was similar between placebo and sitagliptin; at wk 6, it was lower after sitagliptin than placebo (Table 2). During the meal, EGP was suppressed by approximately 50% on average in both controls and patients. In the sitagliptin group, EGP suppression after treatment was greater than in the placebo group (difference AUC = −181 ± 448 *vs.* −41 ± 409 μmol · kg^{-1} · 5 h); although short of statistical significance as AUC, the difference was significant ($P = 0.006$) when analyzed as time-course (by repeated measures ANOVA) (Fig. 2). Consequently, total rate of glucose appearance (RaT = RaO + EGP) and disappearance (Rd) were both reduced by sitagliptin treatment (Table 2). The total amount of glucose infused over the 5 h of the Iso-G was similar in patients and controls; after sitagliptin but not placebo, this amount was significantly reduced (by 9%) because of the lower glucose levels achieved. The baseline glucose MCR was reduced in patients compared with controls in the fasting state; during the meal, MCR increased by approximately 50% in controls and by only approximately 30% in the patients. At 6 wk, meal-induced MCR tended to be higher in the sitagliptin group than the placebo group ($P = 0.08$ for the placebo-adjusted change) (Table 2). A similar approximately 10% improvement in OGIS (6.31 ± 1.09 *vs.* 7.13 ±

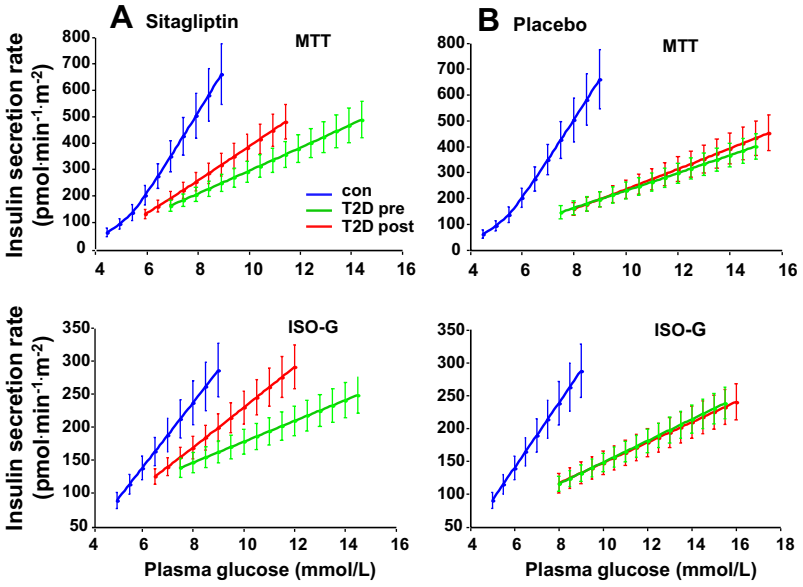

FIG. 2. Insulin secretion rates against concomitant plasma glucose concentrations during the MTT and the isoglycemic glucose infusion in controls (*con*) and in T2D patients before (*pre*) and after (*post*) 6 wk of treatment with sitagliptin (A) or placebo (B).

$1.28\ ml \cdot kg_{FFM}^{-1} \cdot min^{-2}$; $P = 0.004$ for the placebo-adjusted change) supports improved insulin sensitivity.

β-Cell function

The insulin response to the meal was assessed as the insulin AUC, incremental AUC, and total output. For none of these measures was there any difference between controls and patients at baseline; furthermore, neither sitagliptin nor placebo changed these parameters significantly, except for the incremental insulin AUC, which was lower after sitagliptin *vs.* placebo. Likewise, when these parameters were calculated for the isoglycemic experiment, they were all systematically lower than seen with the meal, but without differences between controls and patients or between treatments. Fasting insulin secretion rates were comparable throughout (Table 3).

In contrast, when β-cell function was analyzed as model-derived β-cell glucose sensitivity (β-GS), this was markedly lower at baseline in patients compared with controls (Table 3). Moreover, sitagliptin treatment was associated with a significant improvement in β-GS compared with placebo during both the meal and the isoglycemic study (Fig. 2).

Because of the matched glucose levels during the isoglycemic study and meal test, the incretin effect could be quantified as the O/IV of the insulin secretory responses. As shown in Table 3, for all concentration and secretion parameters, the incretin effect was only marginally (and not significantly) reduced in patients as compared with controls at baseline and was not significantly changed by treatment. The same was true of the incretin effect on β-GS (Table 3).

Hormones

The glucagon response to the meal was higher in patients as compared with controls (Fig. 3). After sitagliptin, the glucagon response decreased (AUC from 19.6 ± 7.5 to 17.3 ± 7.1 $ng \cdot ml^{-1} \cdot 5\ h$; $P = 0.04$ for the placebo-adjusted change). On the isoglycemic study, glucagon levels were suppressed in both controls and patients; sitagliptin induced a nonsignificant decrease in this response.

TABLE 3. Insulin concentrations, β -cell function parameters and incretin effect during the MTT and Iso-G

			Placebo		Sitagliptin		
	Study	Controls	Pre	Post	Pre	Post	P[a]
$AUC_{Insulin}$ ($U \cdot liter^{-1} \cdot 5\ h$)	Meal	10.6 [16.3]	11.9 [7.9]	12.1 [7.8]	11.3 [12.0]	10.3 [10.5]	ns
	Iso-G	4.7 [3.4]	5.0 [4.2]	5.0 [4.5]	6.0 [4.1]	6.0 [3.9]	ns
	O/IV	2.4 [1.0]	2.3 [0.9]	2.3 [0.9]	2.2 [0.6]	2.0 [0.7]	ns
Incremental $AUC_{Insulin}$ ($U \cdot liter^{-1} \cdot 5\ h$)	Meal	8.7 [14.7]	7.4 [5.1]	7.1 [5.6]	8.4 [10.5]	7.6 [9.3]	0.01
	Iso-G	3.0 [1.5]	1.7 [1.3]	1.6 [1.8]	2.2 [2.4]	2.4 [2.2]	ns
	O/IV	3.4 [3.7]	4.4 [5.0]	5.0 [3.0]	4.1 [2.2]	3.4 [1.5]	ns
Fasting ISR ($nmol \cdot m^{-2} \cdot 5\ h$)	Meal	33 [9]	32 [17]	29 [18]	33 [13]	30 [19]	ns
	Iso-G	36 [23]	35 [17]	30 [20]	32 [13]	32 [8]	ns
Total insulin output ($nmol \cdot m^{-2} \cdot 5\ h$)	Meal	82 [59]	74 [35]	77 [36]	74 [45]	82 [46]	ns
	Iso-G	45 [26]	50 [19]	48 [22]	51 [26]	52 [14]	ns
	O/IV	2.0 [0.5]	1.7 [0.6]	1.7 [0.5]	1.7 [0.4]	1.6 [0.6]	ns
β-GS ($pmol \cdot min^{-1} \cdot m^{-2} \cdot mM^{-1}$)	Meal	98 [115][b]	33 [30]	35 [33]	31 [36]	53 [52]	0.01
	Iso-G	45 [35][b]	15 [12]	14 [11]	13 [10]	24 [27]	0.002
	O/IV	2.9 [1.2]	2.1 [1.4]	2.5 [2.2]	2.6 [1.4]	2.1 [1.5]	ns

Data are expressed as median [interquartile range]. Pre, Before treatment; post, after treatment; ISR, insulin secretion rate; ns, nonsignificant.

[a] For the placebo-adjusted difference between baseline and 6 wk of sitagliptin treatment.

[b] $P < 0.0001$ for the comparison of controls *vs.* all diabetic patients by Mann-Whitney test.

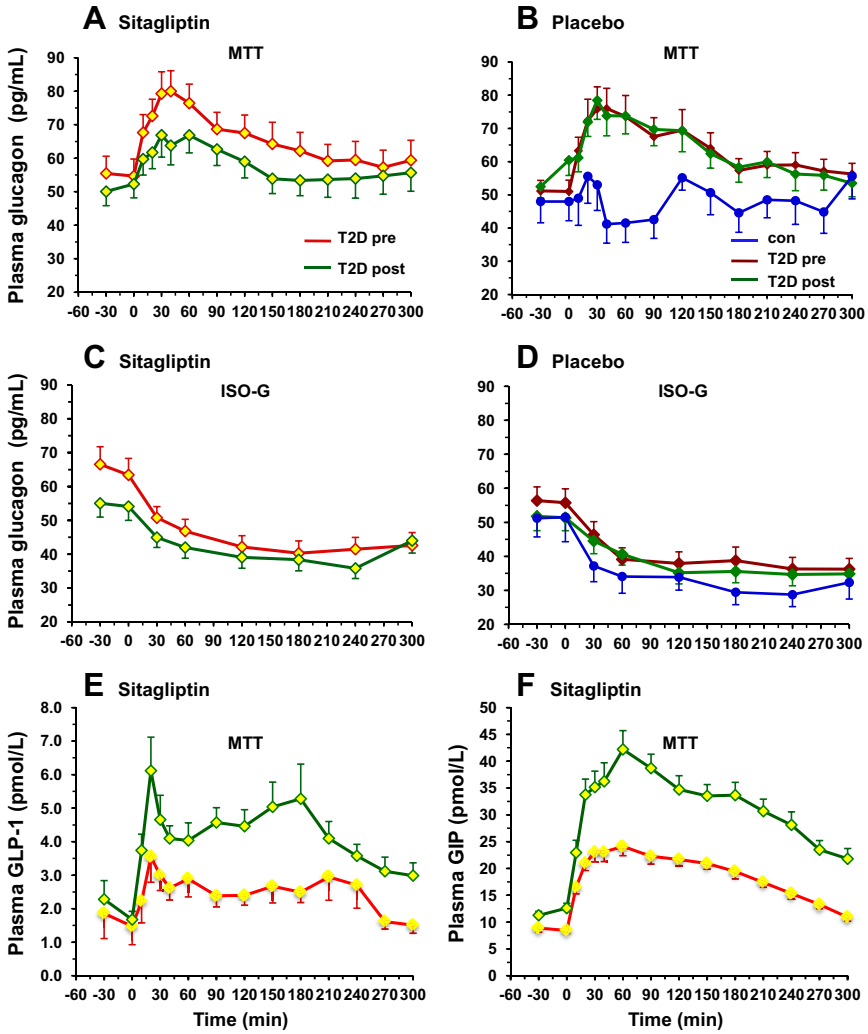

FIG. 3. Plasma glucagon concentrations during the MTT and the isoglycemic glucose infusion in controls and in T2D patients before (*pre*) and after (*post*) 6 wk of treatment with sitagliptin (A and C) or placebo (B and D). Time-course of intact GLP-1 and GIP concentrations in response to the meal (E and F).

At baseline, total GLP-1 response was blunted in T2D (AUC = 4.1 [1.6] *vs.* 8.1 [6.6] nmol · liter^{-1} · 5 h of controls, $P = 0.003$; incremental AUC = 1.6 [1.5] *vs.* 3.6 [4.5] nmol · liter^{-1} · 5 h, $P < 0.01$), as was the GIP response (incremental AUC = 9.3 [4.8] *vs.* 14.5 [3.9] nmol · liter^{-1} · 5 h; $P < 0.01$). At 6 wk, the GIP response was decreased with sitagliptin (−3.82 ± 3.10 *vs.* 1.11 ± 3.37 nmol · liter^{-1} · 5 h of placebo; $P = 0.004$), whereas the total GLP-1 response was unchanged (−0.94 ± 1.65 *vs.* −0.56 ± 2.15 nmol · liter^{-1} · 5 h; P = nonsignificant).

Intact GLP-1 response was lower in controls (0.50 [0.30] nmol · liter^{-1} · 5 h) than in patients (0.65 [0.49] nmol · liter^{-1} · 5 h; $P = 0.05$). After treatment, intact GLP-1 increased with sitagliptin (from 0.74 [0.66] to 1.18 [0.70] nmol · liter^{-1} · 5 h) but not placebo (from 0.55 [0.43] to 0.49 [0.58] nmol · liter^{-1} · 5 h; $P = 0.005$, placebo-adjusted). Intact GIP response was marginally smaller ($P = 0.06$) in patients than controls (5.4 [1.8] *vs.* 7.0 [2.1] nmol · liter^{-1} · 5 h). After treatment, intact GIP increased with sitagliptin (from 5.6 [1.9] to 8.9 [3.6] nmol · liter^{-1} · 5 h) but not placebo (from 4.8 [1.4] to 5.5 [1.3] nmol · liter^{-1} · 5 h; $P < 0.0001$, placebo-adjusted) (Fig. 3).

Correlations

In the whole diabetic cohort, the changes in fasting glycemia at 6 wk were reciprocally related to the changes in OGIS (*rho* = −0.74; $P < 0.0001$) and β-GS (*rho* = −0.39; $P = 0.008$). Likewise, the treatment-induced changes in the incremental glucose AUC on the meal test were reciprocally related to both OGIS (*rho* = −0.40; $P = 0.007$) and β-GS (*rho* = −0.29; $P = 0.05$).

Discussion

The T2D patients recruited for this study were middle-aged, overweight/obese, and in fair glycemic control. They showed the typical phenotype of insulin resistance and β-cell dysfunction but were not insulinopenic. After a 4-wk pharmacological washout, 6 wk of sitagliptin monotherapy lowered both fasting and postmeal plasma glucose levels.

The mechanisms underlying the improved glucose control were multiple: 1) the systemic recovery of the glucose present in the meal was lower, by roughly 7 g over 5 h; 2) EGP was more effectively suppressed during meal absorption; 3) β-cell glucose sensitivity was 50% improved, and insulin sensitivity was 10% improved; and 4) the glucagon response to the meal was suppressed, whereas the intact GLP-1 and GIP fractions were increased, and total GIP levels were decreased. All these effects were significantly different from the placebo-induced changes and were observed in the absence of body weight changes.

The reduced appearance of oral glucose was unexpected. DPP-4 inhibitors are known not to slow down gastric emptying to any meaningful extent (10, 27), and even the inhibitory effect of GLP-1 on gastric motility is subject to tachyphylaxis (28). Accordingly, in our sitagliptin-treated patients the time-course of oral glucose appearance was not distorted—as would most likely be the

case with slower gastric emptying— but only shifted downward (Fig. 2). The missing glucose might eventually appear in the circulation, albeit at later times; however, the absorptive capacity of the intestine is very large (29), and there is no evidence that it is reduced in diabetes. Therefore, a potential explanation for this finding is that after sitagliptin treatment, more ingested glucose was taken up in the splanchnic bed by enterocytes or hepatocytes. That blockade of endogenous GLP-1/GIP degradation increases hepatic glucose uptake, without changes in nonhepatic glucose uptake, insulin secretion/clearance, or glucagon inhibition, has been documented in the conscious dog using vildagliptin and GLP-1 infusion into the liver (30).

The improved suppression of EGP during the meal is plausibly linked with the reduced rise in glucagon levels in the sitagliptin group, confirming that the ability of GLP-1 to restrain α-cell hyperactivity in diabetes is an important component of its antihyperglycemic effect [as also observed after vildagliptin (12, 31)]. Moreover, higher levels of intact GLP-1 might directly suppress EGP, as suggested by a study in normal subjects (32). This is of special importance under conditions, such as a mixed meal, where glucagon release is stimulated rather than suppressed (as occurs with oral glucose alone), especially in diabetic patients.

With regard to insulin secretion, fasting secretion rate and total output in response to the meal were similar in patients and controls, as was the integrated insulin release during the 5 h of isoglycemic glucose infusion. Furthermore, neither sitagliptin nor placebo changed these parameters (Table 3). However, when insulin secretion was related to the concomitant plasma glucose concentrations, the resulting β-cell glucose sensitivity parameter was significantly improved by sitagliptin treatment to levels reaching 50% of those of controls (Table 3). Similar results have been observed with sitagliptin and vildagliptin using the iv glucose tolerance test-minimal model, MTT, or the hyperglycemic clamp technique (13, 33, 34).

In controls, the incretin effect averaged 2 when calculated as the O/IV glucose-induced insulin secretion, which corresponds to 45 [17]% (median [interquartile range]) of orally related secretion being induced by mechanisms other than the glycemic excursions. In the diabetic group as a whole, the incretin effect was marginally reduced (36 [18] %) as compared with controls. The lack of statistical significance of this difference could be due to the small size of the control group and the large variability of the incretin effect. However, it should be mentioned that our controls were matched to the patients by age and BMI; because obesity independently weakens the incretin hormone response and incretin effect (5), the defect in well-controlled T2D may be smaller than previously thought. It is also possible that the incretin defect is attenuated by the use of a mixed meal as opposed to the habitual quantification based on oral glucose alone.

None of the parameters describing the classical incretin effect (insulin area, incremental insulin area, insulin output) were significantly modified by sitagliptin treatment, not even as a trend. This result confirms the findings obtained by Vardarli *et al.* (16) using the oral glucose tolerance test/Iso-G protocol in a small group of well-controlled T2D patients after 13 d of vildagliptin in a crossover design. The present results extend those findings by showing that β-cell glucose sensitivity was improved on both the mixed meal and the Iso-G (Fig. 3), such that their ratio, also an expression of the incretin effect, was unchanged. The ability of chronic sitagliptin therapy to specifically improve glucose sensing regardless of the stimulus is a novel observation whose clinical relevance stems from the fact that β-cell glucose sensitivity is the major determinant of glucose control (35) (*cf.*, the correlation between changes in β-GS and fasting and postmeal glycemia in the present dataset). Because iv glucose did not stimulate GLP-1 (or GIP) release (data not shown), it is difficult to ascribe the improved β-cell sensitivity to iv glucose to acute gastrointestinal hormone changes. Therefore, either the effects of GLP-1 on the β-cell are carried over from the stimulated state of 1 d to the fasting state of the next day (as suggested in Ref. 16), or the changes in β-cell function are the nonspecific consequence of partially removing glucose toxicity (or a combination of these two mechanisms). It should be noted that, in the absence of an equipoised comparator, it is impossible to discriminate an effect of reduced glucose toxicity from a pharmacological effect. With regard to this, it is of interest that D'Alessio *et al.* (13) have reported an improvement of β-cell function in the fasting state (*i.e.* in response to iv glucose) in parallel with unchanged incretin concentrations, suggesting metabolic benefits of DPP-4 inhibition beyond increased incretins levels.

The Rd was similar between patients and controls on account of the higher plasma glucose levels in the patients (Table 2). After sitagliptin, Rd was lower than after placebo, owing to the lower plasma glucose levels. In fact, when accounting for the mass-action effect of glycemia, the MCR tended to be higher with sitagliptin (Table 2). Because insulin secretion was not differentially affected by sitagliptin *vs.* placebo, this finding must be explained by enhanced insulin action. This is supported by the OGIS parameter, also indicating increased insulin sensitivity in sitagliptin-treated patients, as also observed after 6 wk of vildagliptin (31). Because the weight of evidence indicates that GLP-1 does not directly enhance insulin action on

peripheral glucose uptake (12, 36–40), the improved insulin sensitivity in our sitagliptin-treated patients may result from abatement of glucose toxicity.

In summary, chronic treatment with sitagliptin reproduces the acute inhibitory effect that a single dose of DPP-4 inhibitor induces during a meal ingestion on EGP, glucagon release, and improved β-cell glucose sensitivity, thereby restraining glucose excursions in patients with type 2 diabetes (12, 15). Additionally, chronic treatment lowers oral glucose appearance, probably by augmenting splanchnic glucose retention, improves insulin sensitivity, and potentiates β-cell function in response to iv glucose. Because the incretin effect is not altered by sitagliptin, these mechanisms of glucose control may be, at least in part, explained by removal of glucose toxicity.

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ARTICLE

Direct effect of GLP-1 infusion on endogenous glucose production in humans

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Abstract
Aims/hypothesis Glucagon-like peptide-1 (GLP-1) lowers glucose levels by potentiating glucose-induced insulin secretion and inhibiting glucagon release. The question of whether GLP-1 exerts direct effects on the liver, independently of the hormonal changes, is controversial. We tested whether an exogenous GLP-1 infusion, designed to achieve physiological postprandial levels, directly affects endogenous glucose production (EGP) under conditions mimicking the fasting state in diabetes.
Methods In 14 healthy volunteers, we applied the pancreatic clamp technique, whereby plasma insulin and glucagon levels are clamped using somatostatin and hormone replacement. The clamp was applied in paired, 4 h experiments, during which saline (control) or GLP-1(7–37)amide (0.4 pmol$min^{-1}kg^{-1}$) was infused.
Results During the control study, plasma insulin and glucagon were maintained at basal levels and plasma C-peptide was suppressed, such that plasma glucose rose to a plateau of ~10.5 mmol/l and tracer-determined EGP increased by ~60%. During GLP-1 infusion at matched plasma glucose levels, the rise of EGP from baseline was fully prevented. Lipolysis (as indexed by NEFA concentrations and tracer-determined glycerol rate of appearance) and substrate utilisation (by indirect calorimetry) were similar between control and GLP-1 infusion.
Conclusions/interpretation GLP-1 inhibits EGP under conditions where plasma insulin and glucagon are not allowed to change and glucose concentrations are matched, indicating either a direct effect on hepatocytes or neurally mediated inhibition.

Keywords Endogenous glucose production · GLP-1 · Liver glucose output · Pancreatic clamp

Abbreviations

EGP	Endogenous glucose production
FFM	Fat-free mass
GLP-1	Glucagon-like peptide-1
Ra	Rate of appearance
Rd	Rate of disappearance
RQ	Respiratory quotient
SRIF	Somatotropin release-inhibiting factor
TTR	Tracer:tracer ratio

Introduction

The classical physiological actions of glucagon-like peptide-1 (GLP-1) include potentiation of glucose-induced insulin secretion, suppression of glucagon release, inhibition of gastric emptying and enhancement of satiety [1]. The opposing effects on insulin and glucagon secretion result in reductions of endogenous glucose production (EGP) and blood glucose levels. The question of whether the hormone exerts direct actions on insulin target tissues, i.e. liver, adipose and skeletal muscle tissue, is controversial.

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GLP-1 receptors were originally not found in human liver [2]. However, the results of more recent in vitro studies are compatible with the presence of GLP-1 receptors in human hepatocytes [3, 4]. Additionally, GLP-1 has been reported to increase glucose transporter levels and insulin-mediated glucose uptake in 3T3-L1 adipocytes [5], and glucose transport in cultured human myocytes [6]. GLP-1 receptor mRNA has also been described in neurons in the hepatic portal region [7].

Studies in humans are scarce and inconsistent. Hvidberg et al [8] concluded that the decrease in EGP and increase in glucose rate of disappearance (Rd) during GLP-1 infusion in healthy volunteers could be entirely explained by the changes in insulin and glucagon concentrations. Likewise, others [9, 10] reported that the effects of GLP-1 on EGP and glucose disposal were abolished when co-infusing somatotropin release-inhibiting factor (SRIF), thereby blocking the insulin and glucagon response to GLP-1. The same conclusion was reached in experiments using somatostatin infusion during a high-dose hyperinsulinaemic–euglycaemic clamp [11]. In contrast, in uncontrolled studies in healthy volunteers, Prigeon et al used the pancreatic clamp technique to show that fasting EGP and plasma glucose concentrations declined ~20% upon adding a short-term (60 min), high-dose GLP-1 infusion [12].

With regard to the effects on whole-body glucose disposal, early studies [8, 11] found no direct effect of GLP-1, i.e. no effect that was independent of changes in insulin concentrations, on the potentiation of glucose disappearance. Subsequent work, however, reported an independent effect of GLP-1 on the promotion of glucose disposal in non-diabetic [13], obese [14] or diabetic participants [15].

Another potential extrapancreatic action of GLP-1 is on lipid metabolism. Although GLP-1 receptors are not produced in adipocytes, the peptide appeared to stimulate lipolysis in fat cells from obese participants [16]. In contrast, using in situ microdialysis and local GLP-1 perfusion, Bertin et al [17] detected no change in lipolysis or blood flow in adipose tissue or muscle. Finally, intracerebroventricular GLP-1 administration in mice [18] and peripheral GLP-1 infusions in man [19] increased sympathetic activity. It has not yet been determined whether this sympathoexcitatory action is mediated by insulin.

Here, we reassessed the in vivo direct effects of physiological GLP-1 elevations, created by exogenous administration of GLP-1(7-37)amide, on EGP, glucose disposal, lipolysis and indices of sympathetic activation in healthy volunteers.

Methods

Participants Healthy volunteers (n=14) aged 18 to 60 years and with a BMI <30 kg/m^2 participated in the study (Table 1). The nature and purpose of the study were carefully explained to all participants before they provided written consent to participate. The study procedures were approved by the Institutional Ethics Committee of Pisa University.

Study design and protocol Each participant underwent two studies within 7 to 14 days of each other. In each study, after an overnight (12 h) fast, catheters were inserted into an antecubital vein (for infusion of all test substances) and retrogradely into a vein on the dorsum of the hand for blood withdrawal. The hand was heated to 55°C to allow sampling of arterialised venous blood. At 09:00 hours primed continuous infusions of 6,6-[$^2H_2$]glucose (0.28 μmol min^{-1} kg^{-1}; prime 28.0 μmol/kg × [fasting plasma glucose/5]) and [$^2H_5$]glycerol (0.11 μmol min^{-1} kg^{-1}; prime 1.65 μmol/kg) were started and continued for the duration of the study (6 h). At time 0, constant infusions of SRIF (450 μg/h) and glucagon (1 ng min^{-1} kg^{-1}) were begun and continued for 4 h. At time 20 min, a primed continuous insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) infusion (12 pmol min^{-1} m^{-2}) was initiated, along with a saline drip. During the second study, from time 60 min onward, saline was replaced by a constant GLP-1(7–37)amide infusion (0.4 pmol min^{-1} kg^{-1}), while the plasma glucose profile of the first study was closely reproduced through a variable intravenous glucose infusion, using an algorithm developed ad hoc [20]. Plasma insulin, C-peptide, glycerol, glucagon and NEFA concentrations, as well as 6,6-[$^2H_2$]glucose and [$^2H_5$]glycerol enrichment were measured at pre-determined intervals.

In 13 of 14 participants, indirect calorimetry was used to measure the respiratory quotient (RQ) and substrate oxidation rates, using a continuous, open-circuit canopy system (Metabolic Measurement Cart Horizon; SensorMedics,

Table 1 Anthropometric and metabolic characteristics

Characteristic	Mean±SD	Range
n	14	–
Men (n)	11	–
Women (n)	3	–
Age (years)	26±2	21–30
Waist (cm)	84±5	75–91
Hip (cm)	105±7	96–114
BMI (kg/m^2)	25.5±3.7	19.0–30.0
Fasting glucose (mmol/l)	5.2±0.3	4.6–5.8
Total cholesterol (mmol/l)	4.1±0.5	3.5–5.5
HDL-cholesterol (mmol/l)	1.5±0.3	1.2–2.0
Triacylglycerol (mmol/l)	0.7±0.2	0.4–1.0
AST (μkat/l)	0.37±0.08	0.25–0.55
ALT (μkat/l)	0.28±0.07	0.15–0.43

AST, aspartate aminotransferase; ALT, alanine aminotransferase

Anaheim, CA, USA). These measurements were collected during the basal period (−40 to 0 min) and over the last 40 min of the study.

Fat-free mass (FFM) was evaluated using a body composition analyser (TB-300; Tanita, Tokyo, Japan); fat mass was then obtained as the difference between body weight and FFM.

Assays Plasma glucose was measured by the glucose oxidase technique (Beckman Glucose Analyzers; Beckman, Fullerton, CA, USA). Plasma insulin and C-peptide were measured by an electro-chemiluminescence assay on a COBAS e411 (both from Roche, Indianapolis, IN, USA). Glucagon was measured by radioimmunoassay (Millipore, Billerica, MA, USA). The tracer enrichment of 6,6-[$^2H_2$]glucose and [$^2H_5$]glycerol was measured by gas chromatography/mass spectrometry as previously described [21]. NEFA and glycerol were measured using an enzymatic colorimetric system (Syncron; Beckman).

Plasma samples were assayed for intact GLP-1 using a GLP-1 ELISA kit following the manufacturer's protocol (Millipore). The detection limit for this assay is 2 pmol/l in 100 μl plasma.

Calculations Glucose fluxes were expressed per kg of FFM. During the last 20 min of the basal tracer equilibration period, plasma glucose and glycerol concentrations, as well as 6,6-[^{2}H]glucose and [$^2H_5$]glycerol enrichment (expressed as tracer:tracer ratio [TTR]) were stable in all participants. Therefore, EGP and the glycerol rate of appearance (Ra) were calculated as the ratio of tracer infusion rate to the plasma TTR (mean of three determinations). After starting SRIF infusion, the total glucose and glycerol Ra were calculated using Steele's equation, as previously described [22]. Before applying Steele's equation, plasma TTR data for 6,6-[^{2}H]glucose were smoothed using a spline fitting approach to stabilise the calculation of the derivative of enrichment. The plasma glucose concentration resulting from EGP was obtained as the difference between total and exogenous glucose concentrations. The tracer-determined Rd provided a measure of insulin-mediated total-body glucose disposal.

Substrate oxidation rates were calculated from gas exchange measurements as described [23]. Areas under the time–concentration curve were calculated by the trapezium rule.

Statistical analysis Data are given as mean±SD. Differences between saline and GLP-1 infusion were analysed by Wilcoxon's signed-rank test. The time course of glucose fluxes was analysed by two-way, doubly repeated-measures ANOVA, modelling infusion (GLP-1 vs saline) and experimental time (and their interactions) as factors. A value of $p \leq 0.05$ was considered statistically significant.

Results

In the control study, glucose levels began to rise ~1 h into the SRIF infusion and levelled off at ~10.6 mmol/l during the last hour; this time course was reproduced in the GLP-1 study (Fig. 1a). Upon starting SRIF infusion, insulin concentrations initially dropped from baseline, then returned to the fasting value by ~60 min in the control and test study ($p=0.40$). During the last hour, however, plasma insulin levels were higher under GLP-1 infusion than under control conditions (38±18 vs 25±7 pmol/l, $p<0.002$), probably reflecting beta cell escape from SRIF blockade, as confirmed by the C-peptide time course (Fig. 1b, d). Plasma glucagon concentrations also decreased from baseline following the start of SRIF, then rose gradually and slightly until the end of the study, without significant ($p=0.18$) differences between saline and GLP-1 infusion (Fig. 1c).

The glucose Ra rose from baseline under saline and GLP-1 infusion, the time-pattern of the rise being similar in both (Fig. 2a). Exogenous glucose infusion rates, however, were higher with GLP-1 than saline infusion ($p<0.0001$); consequently, EGP was lower throughout the 3 h of GLP-1 infusion (Fig. 2b). Over the time-period when pancreatic hormones were closely superimposable between saline and GLP-1 (i.e. between 60 and 180 min), EGP was 27% lower (by 3.6 μmol $kg^{-1}min^{-1}$, 95% CI 2.4, 4.8, $p<0.0001$) with GLP-1 than with saline (Fig. 2c). The glucose Rd increased slightly only during the last hour of both studies ($p<0.01$ for saline and GLP-1), without differences between saline and GLP-1 (Fig. 2d).

Plasma NEFA increased from baseline until 40 min (from 0.53±0.10 and 0.54±0.03 to 0.70±0.09 and 0.72±0.04 mEq/l, respectively, for saline and GLP-1), subsequently dropping below the basal levels, with no difference between the two studies ($AUC_{0–240\ min}$ 115.5±13.2 vs 110.0±9.6 mEq/l×240 min, $p=0.65$) (Fig. 3a). The glycerol Ra averaged 2.72±0.24 and 3.11±0.22 μmol$min^{-1}kg^{-1}$ during the baseline period of the saline and GLP-1 studies, respectively. During the infusion period, after an initial slight increase, the glycerol Ra declined slowly over time and to similar degrees under saline and GLP-1, to reach values somewhat lower with the latter (2.01±0.92) than the former (2.43±2.01) during the final hour of the study (Fig. 3b).

The RQ did not change between baseline (0.75±0.02 vs 0.76±0.02, saline vs GLP-1, $p=0.84$) through to the final hour of the study (0.76±0.03 vs 0.78±0.01, $p=0.33$). Accordingly, baseline rates of carbohydrate and lipid oxidation were similar between the two study days and did not change significantly with either saline or GLP-1 infusion (Fig. 4a, b).

During saline infusion, there was no change in intact GLP-1, whereas GLP-1 infusion raised the plasma levels of intact hormone threefold ($AUC_{60–180\ min}$ 239±515 vs

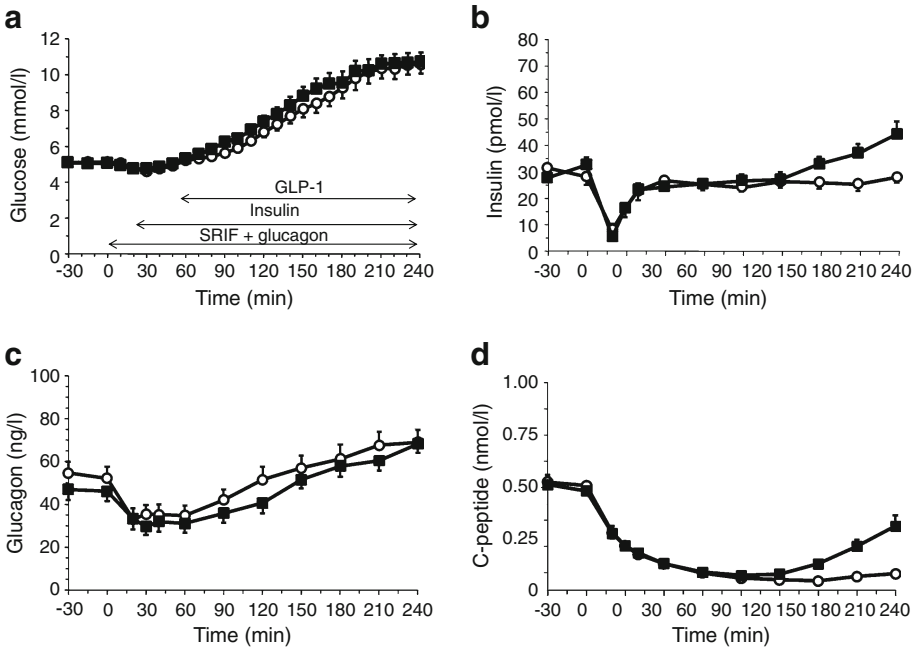

Fig. 1 Time course of glucose (**a**), insulin (**b**), glucagon (**c**) and C-peptide (**d**) during the saline study (white circles) and GLP-1 infusion (black squares). In the final hour, plasma insulin levels were higher under GLP-1 infusion than control conditions ($p<0.002$)

441±200 pmol/l×120 min, $p=0.001$). In the pooled saline and GLP-1 data, there was a significant, albeit weak, ($\rho=-0.49$, $p=0.01$) reciprocal relationship between EGP and intact GLP-1 concentrations measured over the 60–180 min time interval.

Discussion

The present studies demonstrate that exogenous GLP-1 inhibits EGP by mechanisms that are largely independent of changes in plasma glucose, insulin and glucagon levels. Our experimental settings mimicked a diabetic state, i.e. raised glucose concentrations and glucagon:insulin ratios. Under these conditions, EGP was increased by ~70% from baseline, with plasma glucose rising to a plateau of ~11.1 mmol/l. Replacing the saline with a GLP-1 infusion, at a rate producing steady-state plasma levels approximately in the postprandial range, caused a marked reduction of EGP, which remained close to the starting levels. Interestingly, insulin secretion during the 3rd hour of GLP-1 infusion tended to rise, reflecting an escape of the beta cells

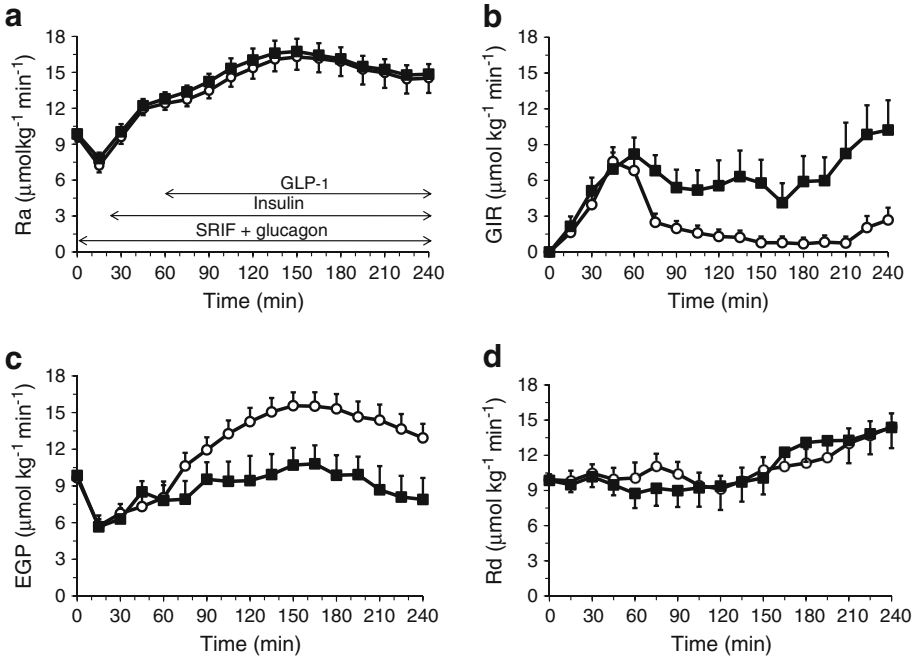

Fig. 2 Time course of glucose Ra (**a**), glucose infusion rate (GIR) (**b**), glucose Rd (**d**) and EGP (**c**) during the saline study (white circles) and GLP-1 infusion (black squares)

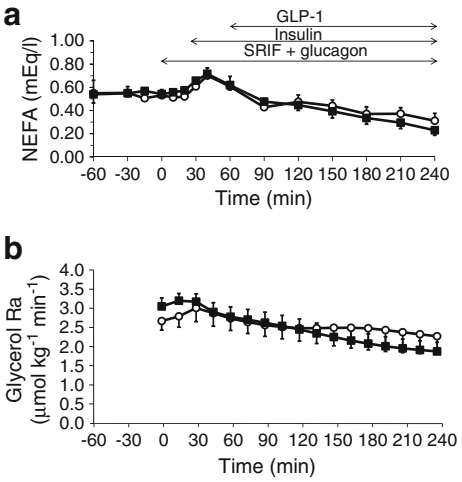

Fig. 3 Time course of plasma NEFA concentrations (**a**) and glycerol Ra (**b**) during saline (white circles) or GLP-1 infusion (black squares) in healthy volunteers

from SRIF blockade. Therefore, quantification of the GLP-1 effect was restricted to the 2 h during which plasma insulin and C-peptide levels were stable and superimposable between the two studies (Fig. 1).

Previous reports [11, 24] that failed to observe a direct inhibitory effect of GLP-1 on EGP under pancreatic clamp conditions can probably be explained by their use of high insulin replacement doses, which suppressed EGP completely [11] or by greater than 75% [24] in the control studies, thereby leaving little room for a further inhibitory action of GLP-1. In addition, we did not detect any effect on whole-body glucose disposal, in accordance with previous

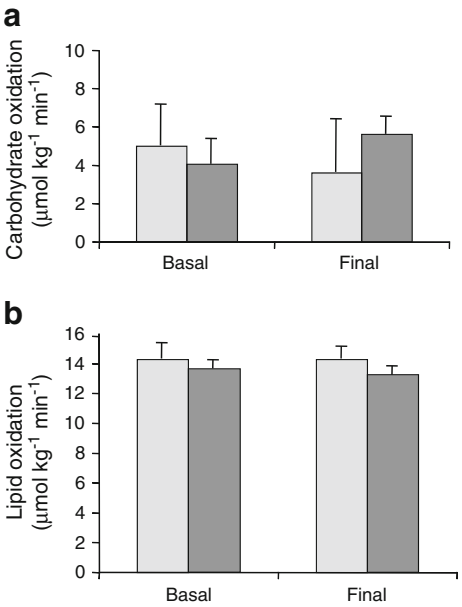

Fig. 4 Rates of carbohydrate (**a**) and lipid (**b**) oxidation before starting SRIF (basal) and at the end of the 4 h infusion period (final). Light grey, saline; dark grey, GLP-1 infusion

findings in healthy volunteers [9, 10]. However, we cannot rule out the possibility that pharmacological doses of GLP-1 such as those used in previous studies [14–25] may promote whole-body glucose uptake.

The only previous study that is indicative of a direct effect of GLP-1 on EGP [12] was carried out in eight healthy volunteers, who did not receive a saline infusion control study. Moreover, exogenous GLP-1 was infused, for a short time (60 min), at rates achieving total plasma GLP-1 concentrations that were twice as high as the steady-state levels created by us. More importantly, the insulin replacement (36 pmol$min^{-1}kg^{-1}$) was at least twice as high as ours (12 pmol$min^{-1}kg^{-1}$), achieving two to three times higher steady-state plasma insulin concentrations (thus raising plasma glucose clearance by ~50%). Thus, in Prigeon's protocol [12], the effect of short-lived, supraphysiological GLP-1 concentrations was tested under conditions of euglycaemia and hyperinsulinaemia. With the present protocol, we demonstrated that physiological GLP-1 increments prevent EGP from increasing under conditions simulating the fasting state in diabetes.

With regard to the mechanisms underlying the direct action of GLP-1 on EGP, we measured lipolysis, as indexed by glycerol Ra and plasma NEFA levels, and the pattern of substrate utilisation (using indirect calorimetry). As no differences, not even in trend, were observed between saline and GLP-1 infusion, we can rule out the possibility that the GLP-1-induced inhibition of EGP may have been due to a reduction of NEFA delivery to the liver, which would stimulate EGP via gluconeogenesis, or to an increase in sympathetic drive, which would stimulate lipolysis and shift the substrate oxidation pattern toward lipid oxidation.

In the present studies, the threefold elevated intact GLP-1 levels could have engaged hepatic GLP-1 receptors similar to those on beta cells [4]. Alternatively, the GLP-1(28–36)amide nonapeptide, which enters hepatocytes independently of the GLP-1 receptor, may have suppressed glucose production, as shown in mouse hepatocytes [26].

Experimental evidence for the possibility that GLP-1 may act on the liver by engaging sensors in the portal circulation or nerve endings in the intestinal wall comes from different animal species, but is convergent. Thus, in insulin clamp experiments in GLP-1 receptor knockout mice, insulin suppression of EGP was impaired and animals became hyperglycaemic during exercise [27]. Nakabayashi et al [28] measured changes in the impulse discharge rate of the hepatic afferent vagus, following a bolus intraportal GLP-1 injection in the rat. They found that the hormone dose-dependently increased the firing rate and that this effect could be cancelled by vagotomy. In catheterised dogs, Johnson et al [29] found that direct infusion of GLP-1 into the portal vein at matched plasma glucose, insulin and glucagon concentrations resulted in a more positive net

hepatic glucose balance, which is the net sum of EGP and hepatic glucose uptake.

In summary, the present studies provide conclusive evidence that a physiological action of GLP-1 inhibits glucose production under conditions where its major controlling signals, namely plasma insulin, glucagon and glucose concentrations, are not allowed to change. The effect is quantitatively significant and does not appear to be mediated by changes in substrate availability or sympathetic drive.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement MS, ER, BDA, AG, AP, AC, EB and EM acquired and analysed the data and drafted the article. EM, EF and MN reviewed the article and were responsible for the conception of the study. All authors approved the final version.

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Fig 6. Anthropometric and biochemical characteristics of T2DM subjects before and after BPD.

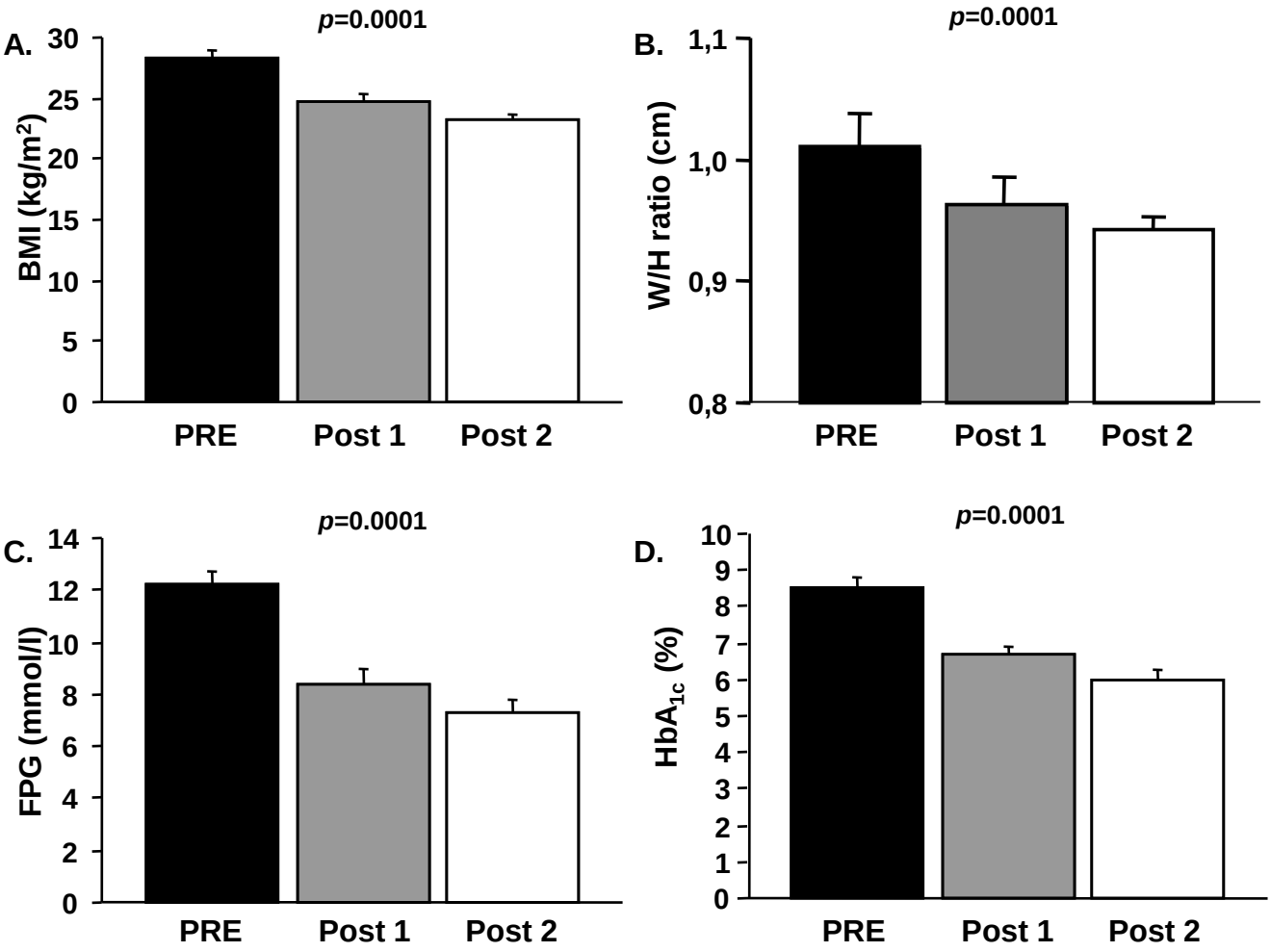

Figure. 6 Data are means ± SEM. Changes induced by BPD on **A.** Body mass index (BMI), **B.** waist and hip ratio (W/H ratio), **C.** fasting plasma glucose (FPG) and **D.** glycosylated haemoglobin (HbA_{1c}). *p* = ANOVA for repetitive measures.

Fig 7. Changes in plasma glucose concentration during OGTT.

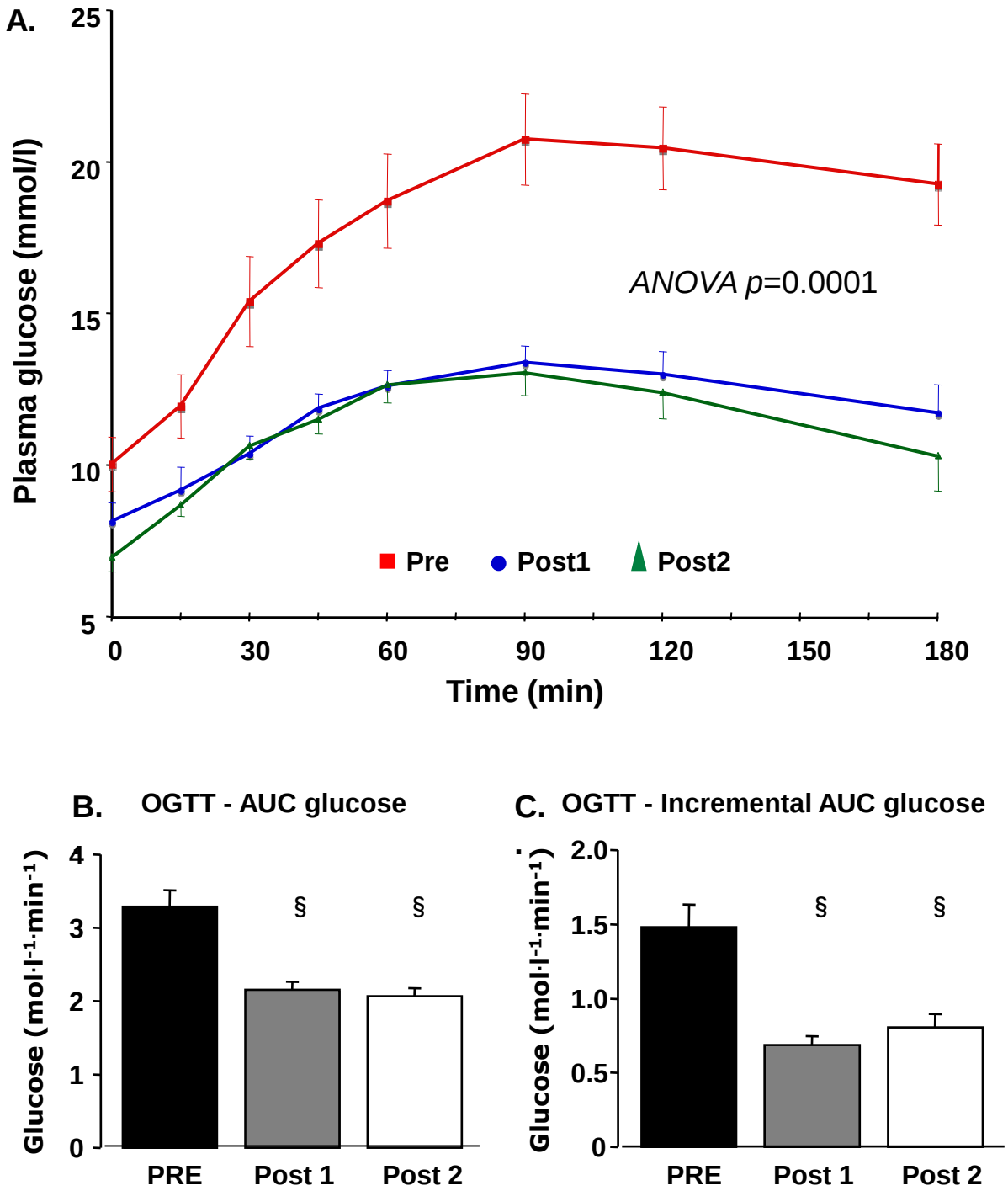

Figure 7: A. OGTT plasma glucose curves. FPG decreased after surgery. 2-hours OGTT plasma glucose also decreases about 60% in both follow-up. Time course curves did not change after surgery and hyperglycaemia continues over 180 minutes. AUC glucose and incremental AUC glucose decreases as showed at panels **B.** and **C.** Curves: *p*=ANOVA for repetitive measures or bars: Wilcoxon signed test (**p*=0.05; #*p*=0.01 and §*p*=0.001).

Fig 8. Metabolic effects of BPD on plasma glucose, insulin and C-Peptide.

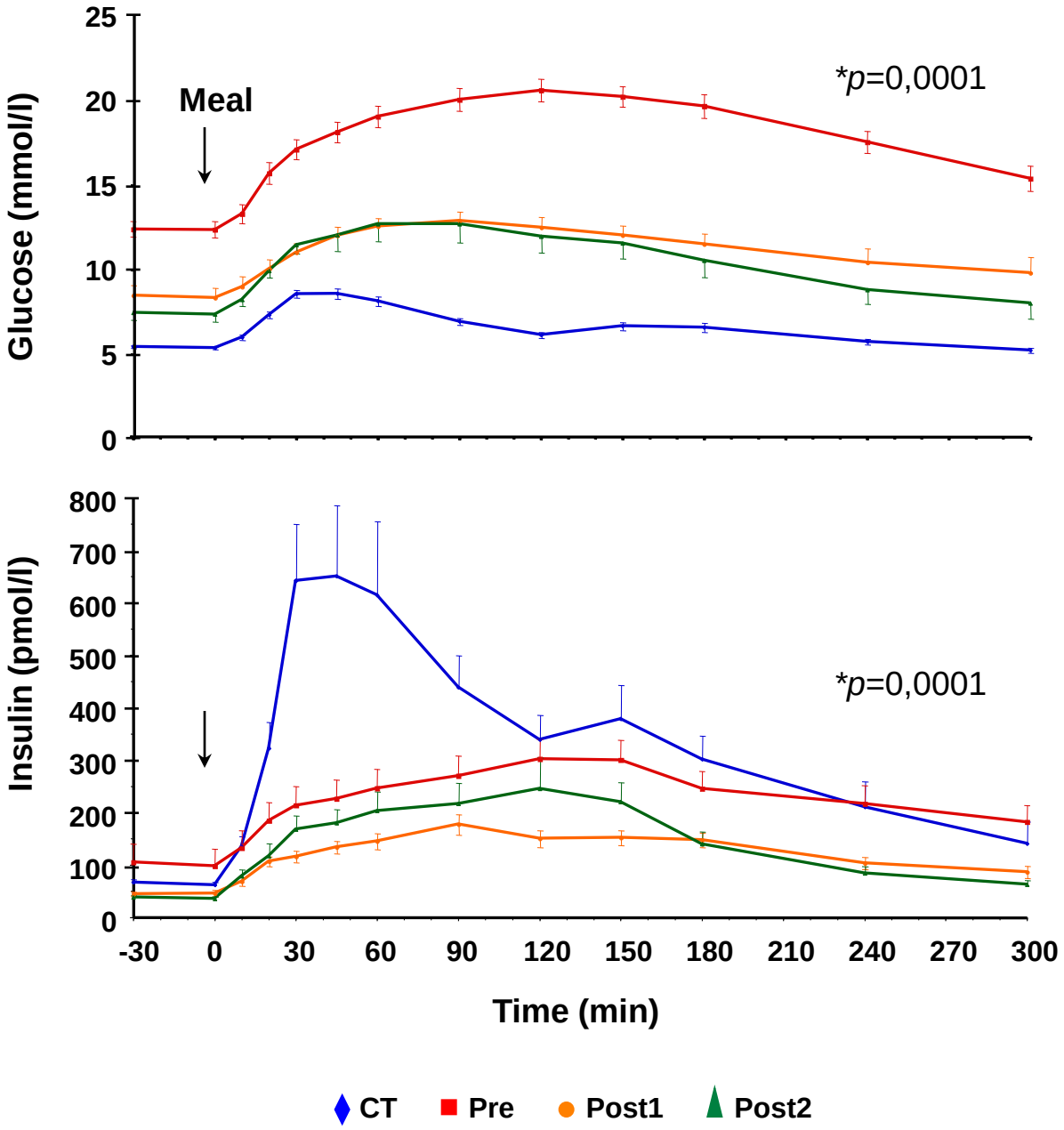

Figure 8. Data are mean ± SEM. **A.** plasma glucose curve decreases earlier after surgery and slightly on Post 2. **B.** plasma insulin curve decreases at first follow-up but showed an increment in plasma concentrations, mainly among the 120 minutes after stimulation. **p*= ANOVA repeated-measures.

Fig 9. Metabolic effects of BPD on plasma glucagon, GLP-1 and FFA.

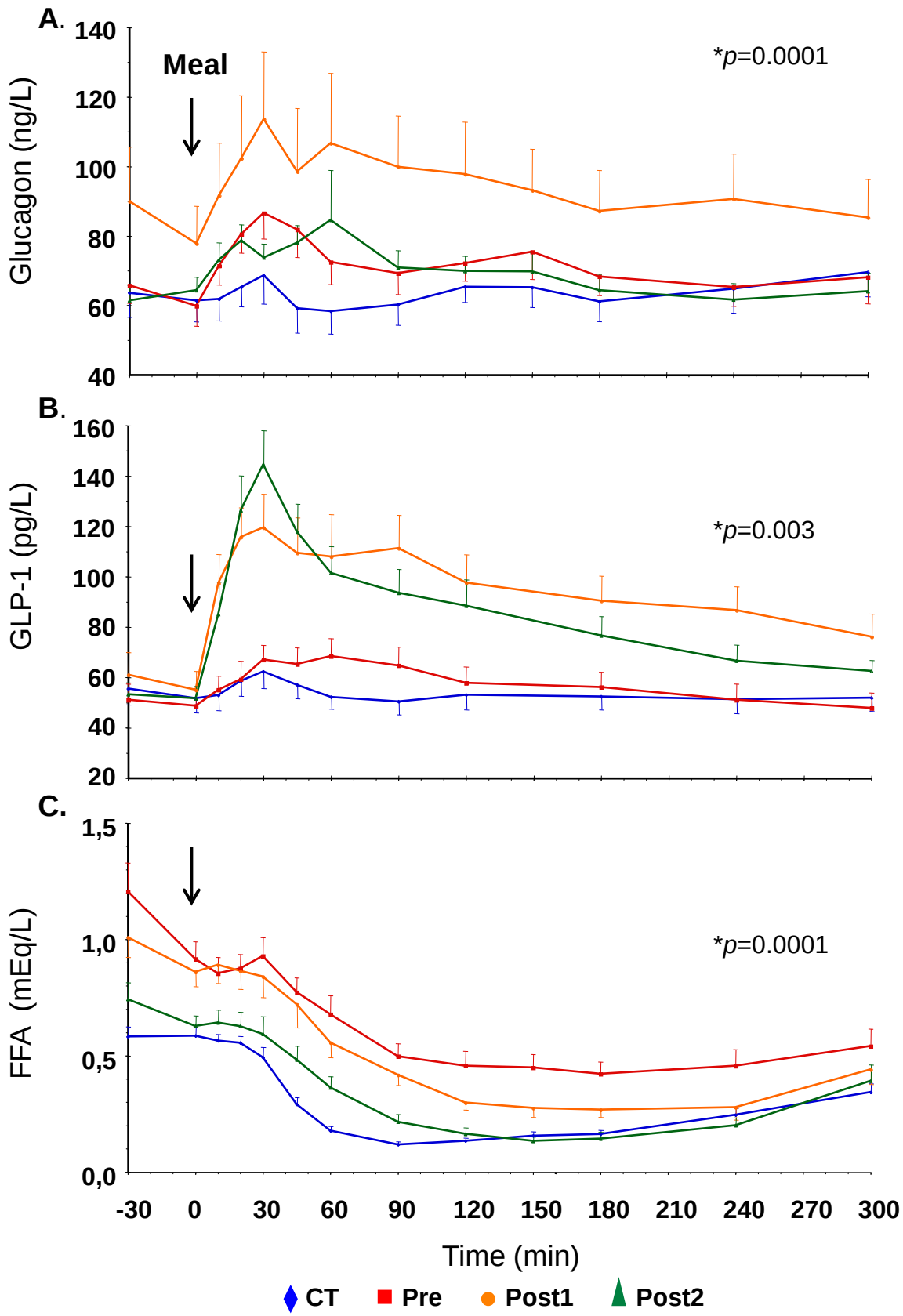

Figure 9. Data are mean ± SEM. **A.** Glucagon, **B.** GLP-1 and **C.** FFA changes after biliopancreatic diversion. FFA showed levels similar to Control group at 1 year follow-up, whereas Glucagon and GLP-1 levels higher than. *p= ANOVA for repeated-measures (Pre, Post1 and Post2).

Fig 10. Changes in insulin secretion rate after BPD.

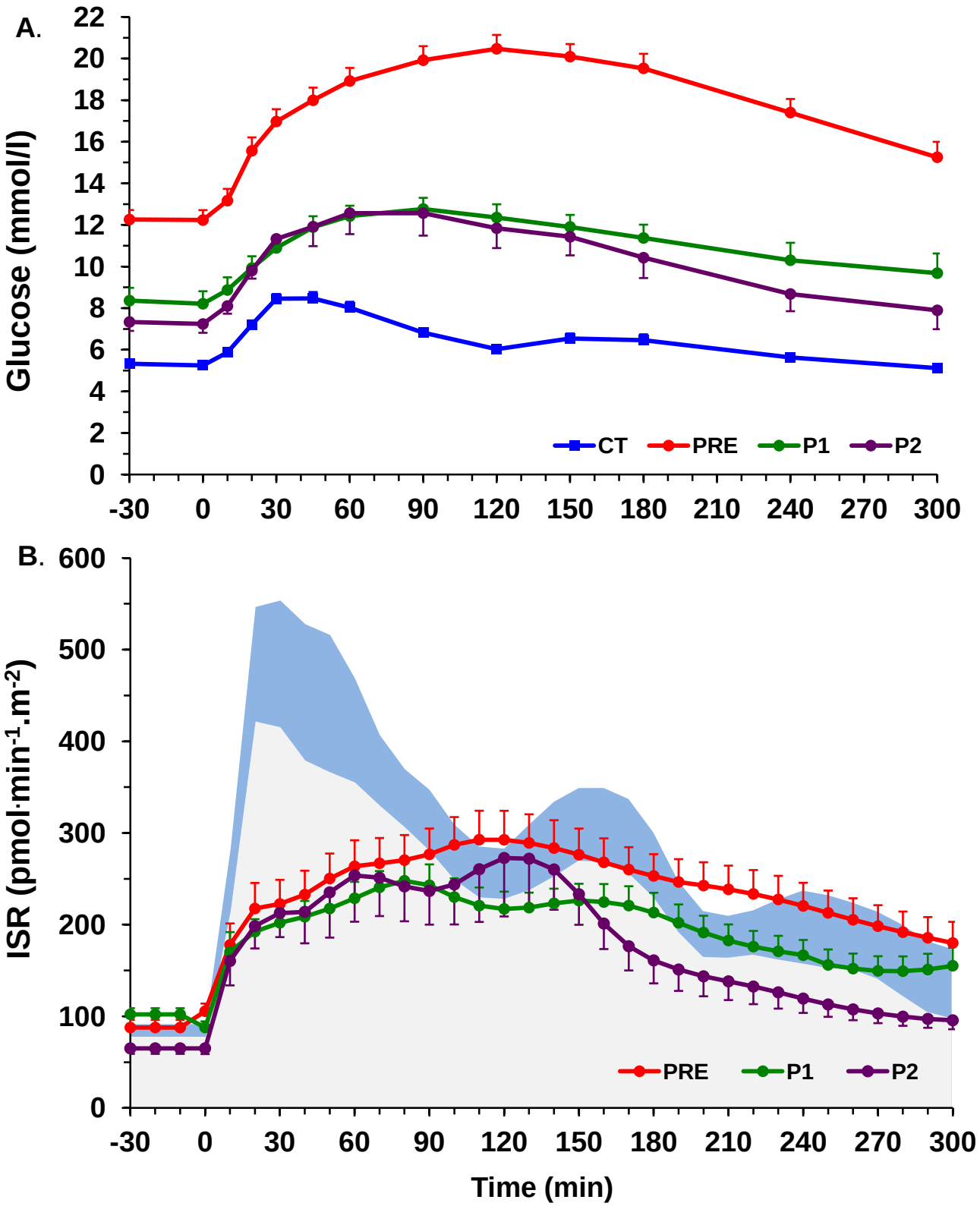

Figure 10. Data are mean ± SEM. **A.** plasma glucose concentration decreases during mixed meal without reached normal levels. **B.** insulin secretion rate (ISR) calculated using deconvolution of C-peptide. Blue shadow is Control group values ±1 SEM.

Fig .11: Effect of BPD on β-cell function.

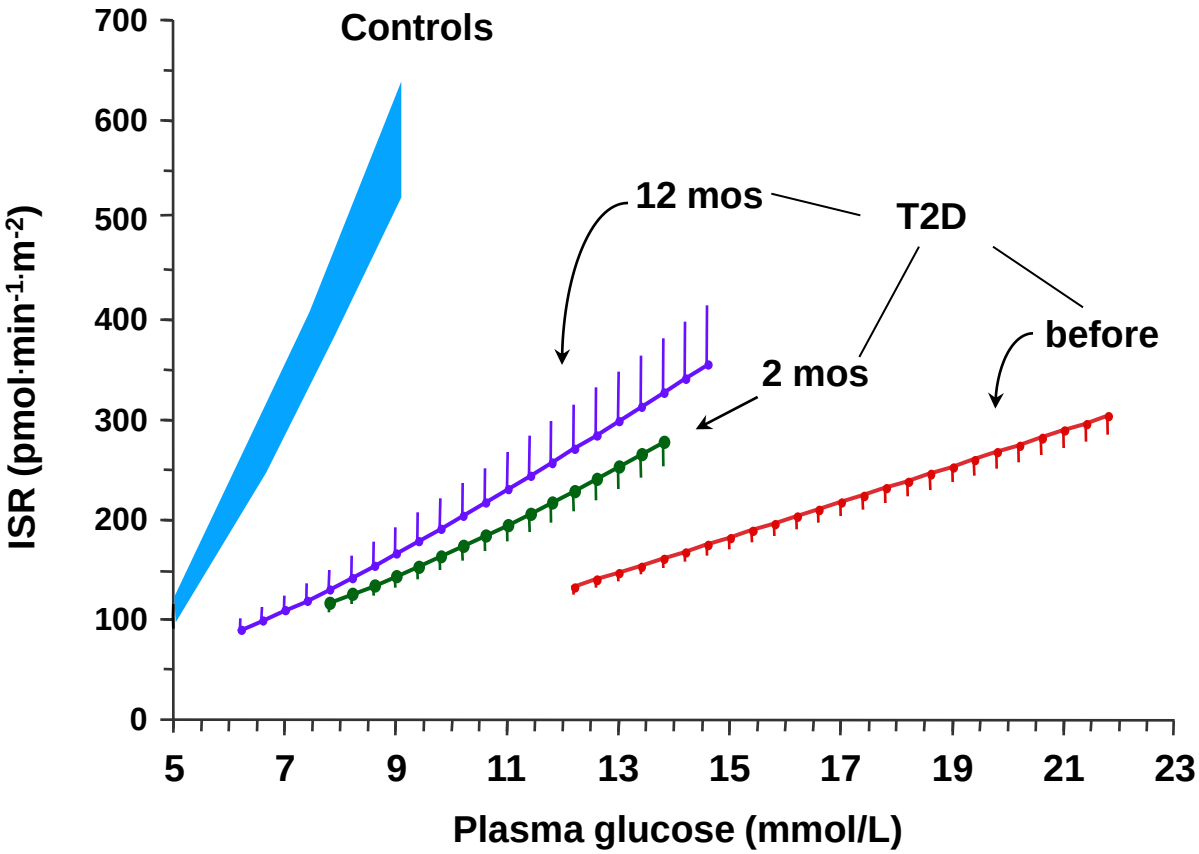

Figure 11. Data are mean ±SEM. Effect of BPD on β-cell function. Blue shadow is Control group values ±1 SEM.

Fig 12. Changes in β-cell glucose sensitivity and rate sensitivity after BPD.

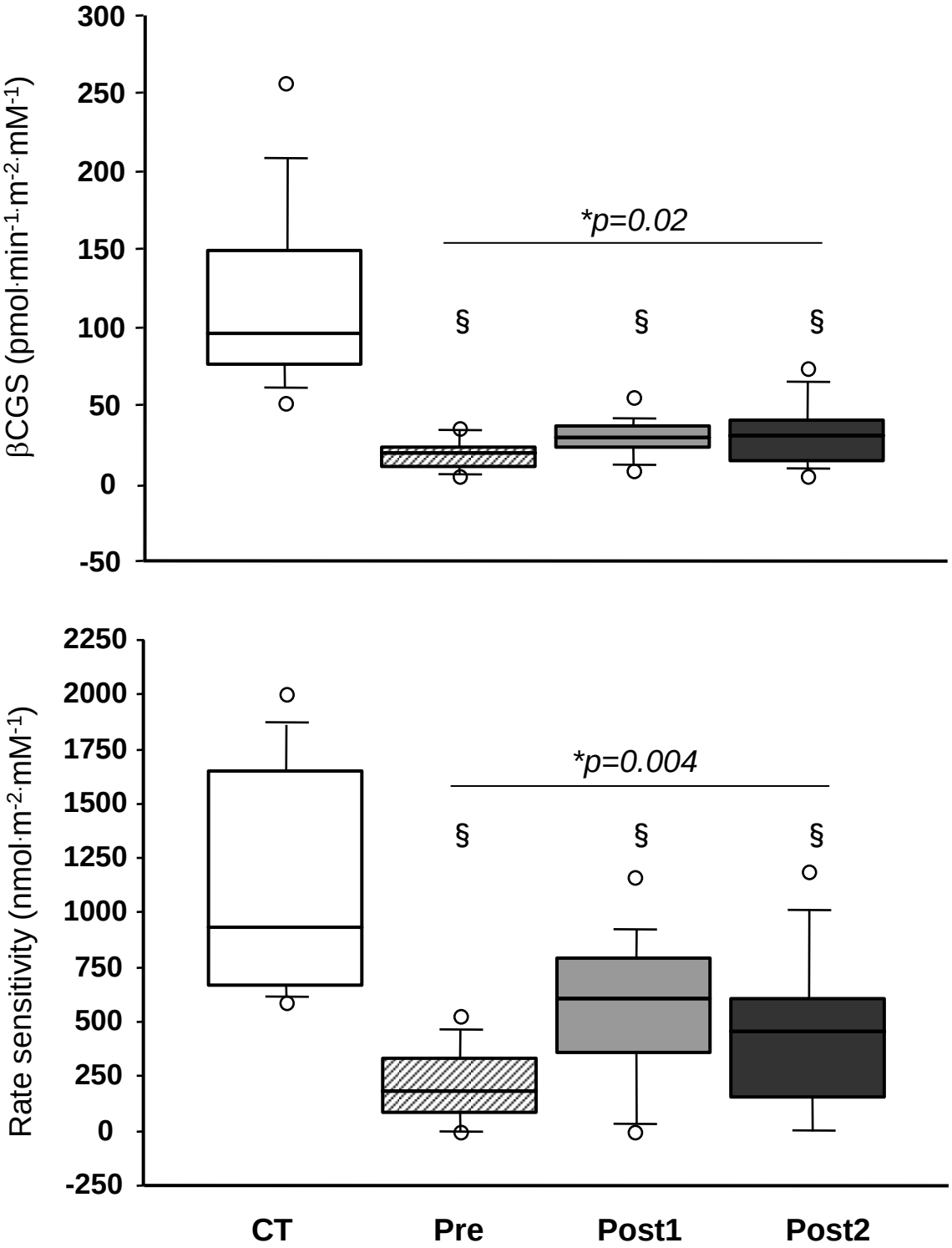

Figure 12. Data are mean ±SEM Changes in β-cell glucose sensitivity and rate sensitivity after BPD. § *p*=0.05 for the comparison *vs.* Control using Mann Whitney test. **p*= for the comparison of Pre, Post 1 and Post 2 by ANOVA repeated-measures.

Fig 13. Changes in insulin sensitivity after BPD.

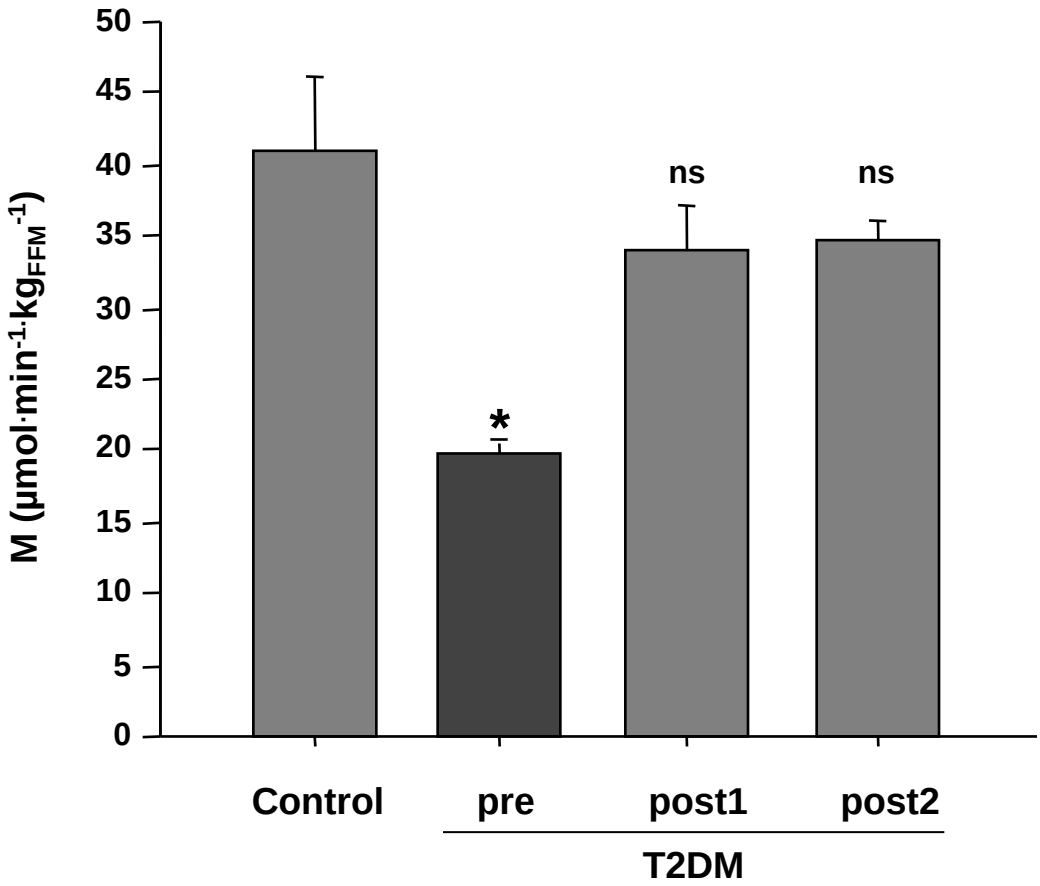

Figure 13. Data are mean ±SEM. Changes in insulin sensitivity (M value) after BPD. *$p \leq 0.05$ for the comparison *vs.* Control using Mann Whitney test.

Fig 14. Changes in insulin action after BPD.

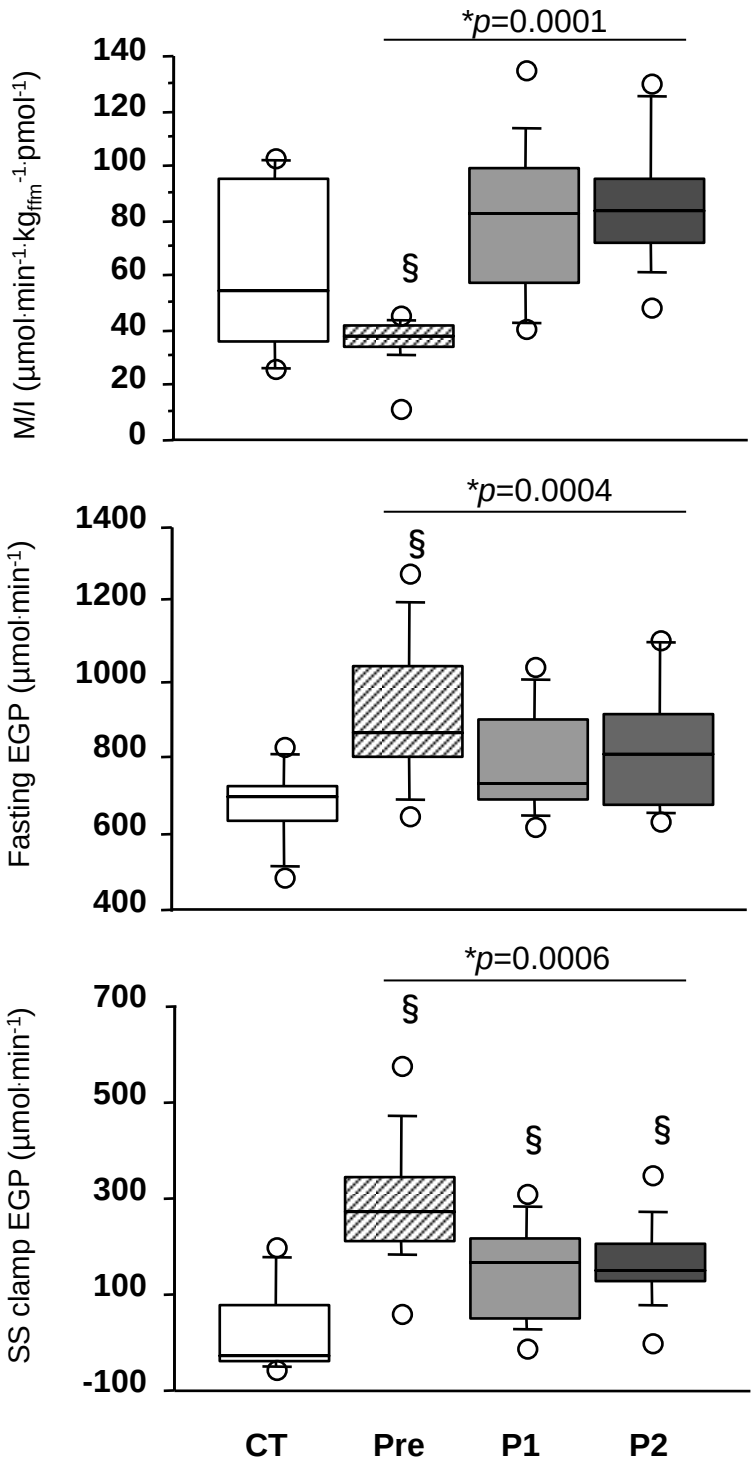

Figure 14. Effects of BPD on insulin sensitivity and on EGP during euglycaemic hyperinsulinemic clamp. § $p \leq 0.05$ for the comparison *vs.* Control using Mann Whitney test. *p= for the comparison of Pre, Post 1 and Post 2 by ANOVA repeated-measures.

Fig. 15. Changes in prehepatic insulin:glucagon ratio induced by BPD.

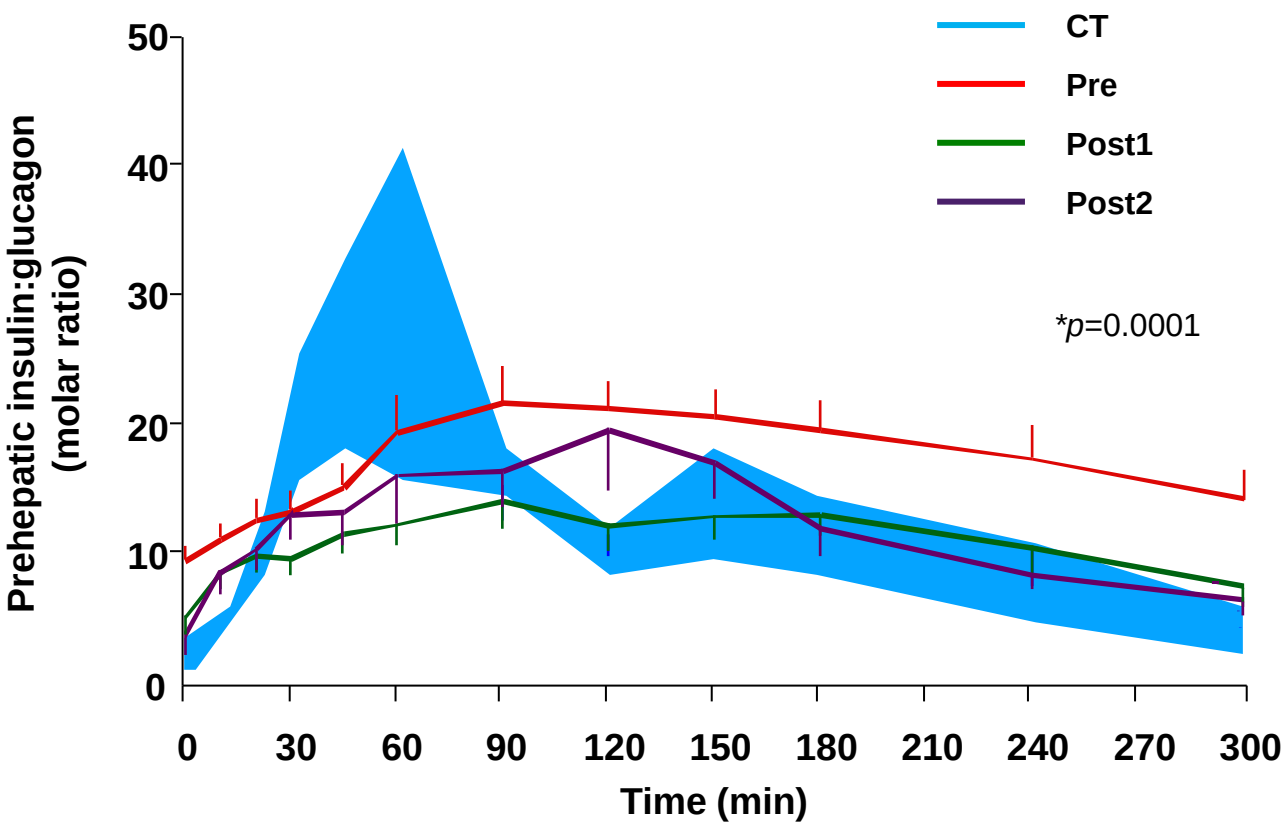

Figure 15. Data are mean ±SEM. Changes in prehepatic insulin:glucagon ratio induced by BPD. Blue shadow is Control group values ±1 SEM. *p= ANOVA repeated-measures for the comparison of Pre, Post 1 and Post 2.

Table 1. Anthropometric and biochemical characteristics[#]

	Control	Pre	Post1	Post2	*p1*	*p2*
BMI (kg/m^2)	30.3 ± 3.6	28.3 ± 2.1	24.8 ± 2.0[§]	23.3 ± 1.7[§]	0.0007	0.0007
Fasting glucose (mmol/l)	5.3 ± 0.1	12.2 ± 0.5[§]	8.3 ± 0.6[§]	7.3 ± 0.4[§]	0.0007	0.0007
Fasting Insulin (pmol/l)	63 ± 7	105 ± 35	46 ± 6[*]	36 ± 5[#]	0.003	0.0007
Fasting Fat Free Acids (µEq/l)	0.58 ± 0.4	0.86 ± 0.48[§]	0.71 ± 0.36[§]	0.67 ± 0.46	ns	0.0008
Fasting Glucagon (ng/ml)	62.5 ± 6.4	62.7 ± 5.0	83.9 ± 13.1	63.6 ± 4.2	ns	ns
Fasting GLP-1 (pM)	55.5 ± 7.5	48.3 ± 5.9	57.1 ± 7.5	53.2 ± 5.0	ns	ns
Triglycerides (mmol/l)	1.0 ± 0.1	1.9 ± 0.2[§]	1.9 ± 0.2[§]	1.9 ± 0.3[§]	ns	ns
Total Cholesterol (mmol/l)	4.8 ± 0.2	5.3 ± 0.4	3.5 ± 0.2[§]	3.5 ± 0.1[§]	0.007	0.009
HDL cholesterol (mmol/l)	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.01	ns
LDL cholesterol (mmol/l)	3.2 ± 0.2	3.3 ± 0.4	1.5 ± 0.1[§]	1.8 ± 0.1[§]	0.002	0.01

Data are mean ± SEM. *p1* and *p2* are respectively for the comparison of Pre *vs.* Post 1 and Pre *vs.* Post 2 by Wilcoxon signed rank test; * $p \leq 0.05$; [#]$p \leq 0.01$ or [§]$p \leq 0.001$ for the comparison *vs* Control using Mann Whitney test.

Table 2. Metabolic effects of MMT on glucose metabolism, insulin and glucagon secretion and incretins[#]

	Control	Pre	Post1	Post2	*p1*	*p2*
AUC Glucose ($mol \cdot l^{-1} \cdot h^{-1}$)	1.8 ± 0.4	5.5 ± 0.1[§]	3.4 ± 0.2[§]	3.1 ± 0.2[§]	0.0007	0.001
AUC Insulin ($nmol \cdot l^{-1} \cdot h^{-1}$)	94.4 ± 12.1	71 ± 9.9	39 ± 2.6[§]	45 ± 5.9[#]	0.002	0.01
AUC Glucagon ($ng \cdot ml^{-1} \cdot h^{-1}$)	19 ± 1.7	21 ± 1.6	28 ± 3.8*	21 ± 1.4	ns	ns
AUC GLP-1 ($nM \cdot l^{-1} \cdot h^{-1}$)	16 ± 1.9	17 ± 1.8	28 ± 3.1[#]	24 ± 2.1[#]	0.002	0.03
AUC Fat Free Acids ($\mu Eq^{-1} \cdot l^{-1}$)	69 ± 4.8	163 ± 16.3[§]	124 ± 16.3[§]	85 ± 11.0	0.05	0.001
Inc. Glucose AUC ($mol \cdot l^{-1} \cdot h^{-1}$)	0.3 ± 0.03	1.8 ± 0.1[§]	0.9 ± 0.1[§]	0.9 ± 0.1[§]	0.001	0.004
Inc. Insulin AUC ($nmol \cdot l^{-1} \cdot h^{-1}$)	77 ± 11.0	42 ± 5.1*	25 ± 2.7[§]	34 ± 6.1[#]	0.006	ns
Mean Pre-hep. Ins./glucag. ratio	8.8 [8.9]	17.6 [10.5]*	13.4 [9.5]	9.6 [7.2]	0.01	0.001
Inc. Glucagon AUC ($ng \cdot ml^{-1} \cdot h^{-1}$)	0.69 ± 0.9	3.3 ± 0.9	4.9 ± 1.5*	1.6 ± 1.2	ns	ns
Inc. AUC GLP-1 ($nM \cdot l^{-1} \cdot h^{-1}$)	- 0.2 ± 0.1	2.3 ± 0.5*	11.1 ± 1.5[§]	9.9 ± 2.1[#]	0.001	0.004
Inc. AUC Fat Free Acids ($\mu Eq^{-1} \cdot l^{-1}$)	-103 ± 11.6	-149 ± 22.0	-157 ± 16.0*	-118 ± 15.2	ns	ns

Data are mean ± SEM. *p1* and *p2* are respectively for the comparison of Pre *vs.* Post 1 and Pre *vs.* Post 2 by Wilcoxon signed rank test; * $p \leq 0.05$; [#]$p \leq 0.01$ or [§]$p \leq 0.001$ for the comparison *vs* Control using Mann Whitney test.

Table 3: β-cell function parameters[#]

	Control	Pre	Post 1	Post 2	*p1*	*p2*
Fasting ISR ($pmol \cdot min^{-1} \cdot m^{-2}$)	84 [53]	115 [52]*	83 [22]	61 [38]	ns	0.002
Total insulin output ($nmol \cdot m^{-2}$)	77 [46]	80 [51]	62 [19]*	52 [37]*	0.04	0.06
ß-cell glucose sensitivity ($pmol \cdot min^{-1} \cdot m^{-2} \cdot mM^{-1}$)	96 [73]	19 [12]§	30 [14]§	31 [26]§	0.01	0.01
Rate sensitivity ($nmol \cdot m^{-2} \cdot mM^{-1}$)	934 [981]	187 [247]§	605 [436]$^{\#}$	453 [453]§	0.004	0.04
Potentiation factor (fold)	1.5 [0.5]	1.24 [0.44]§	1.11 [0.43]$^{\#}$	1.49 [1.09]	ns	0.02
Insulin clearance basal ($l \cdot min^{-1} \cdot m^{-2}$)	----	2.85 ± 0.94	3.86 ± 0.78	3.22 ± 0.85	0.002	ns
Insulin clearance MTT ($l \cdot min^{-1} \cdot m^{-2}$)	----	1.65 ± 0.71	2.09 ± 0.52	1.91 ± 0.66	0.01	ns

Data are median [IQR] or mean ± SEM . *p1* and *p2* are respectively for the comparison of Pre *vs.* Post 1 and Pre *vs.* Post 2 by Wilcoxon signed rank test; * $p \leq 0.05$; $^{\#}p \leq 0.01$ or $^{\S}p \leq 0.001$ for the comparison *vs* Control using Mann Whitney test.

Table 4: Euglycaemic hyperinsulinaemic clamp data[#]

	Control	Pre	Post1	Post2	*p1*	*p2*
M (µmol/Kg_{FFM}^{-1}·min^{-1})	40 [26]	19 [3][§]	36 [19]	34 [9]	0.0007	0.0007
M/I (µmol.$min^{-1}$$kgffm^{-1}$.$pmol^{-1}$)	54 [59]	38 [8][*]	82 [41]	84 [23]	0.0007	0.0007
Fasting Plasma Insulin (pmol/l)	58 [33]	65 [48]	40 [24][*]	34 [8][#]	0.002	0.0007
SS Clamp Plasma Insulin (pmol/l)	633 [363]	531 [132]	418 [63][§]	418 [107][§]	0.0008	0.005
Rd clamp (umol·min^{-1})	1608 [1480]	1235 [357]*	1658 [558]	1701 [305]	0.01	0.001
EGP (µmol·min^{-1})	696 [91]	869 [239]*	733 [213]	806 [233]	0.0007	0.01
EGP clamp (µmol·min^{-1})	-27 [116]	271 [133]§	168 [170]*	150 [75]*	0.003	0.008

Data are median [IQR]. *p1* and *p2* are respectively for the comparison of Pre *vs.* Post 1 and Pre *vs.* Post 2 by Wilcoxon signed rank test;
* $p \leq 0.05$; $^{\#}p \leq 0.01$ or $^{\S}p \leq 0.001$ for the comparison *vs* Control using Mann Whitney test.

Table 5. Anthropometric and biochemical characteristics divided by remitters and non remitters[#]

	Pre		p^1	Post 1		p^2	Post 2		p^3
	Remitters	Non remitters		Remitters	Non remitters		Remitters	Non remitters	
age (years)	55 ± 2	57 ± 1							
BMI (kg/m^2)	28.7 ± 0.6	28.1 ± 0.9	ns	25.3 ± 0.8	24.5 ± 0.7	ns	22.8 ± 0.8	23.7 ± 0.6	ns
HbA$_{1c}$%	8.4 ± 0.5	8.6 ± 0.5	ns	6.6 ± 0.5	6.7 ± 0.3	ns	5.3 ± 0.3	6.5 ± 0.2	0.004
Fasting Insulin (pmol/l)	73.0 ± 12.9	114.3 ± 54.7	ns	42.7 ± 9.5	48.7 ± 8.4	ns	36.6 ± 8.2	34.7 ± 6.0	ns
Fasting C-peptide (nmol/l)	0.98 ± 0.64	0.71 ± 0.95	0.04	0.66 ± 0.61	0.68 ± 0.90	ns	0.55 ± 0.86	0.41 ± 0.52	ns
Fasting glucose (mmol/l)	11.3 ± 0.6	12.9 ± 0.6	ns	6.4 ± 0.4	9.5 ± 0.7	0.007	5.7 ± 0.5	8.3 ± 0.4	0.005
Fasting Fat Free Acids (mEq/l)	0.8 ± 0.8	1.0 ± 0.1	ns	0.9 ± 0.1	0.8 ± 0.1	ns	0.6 ± 0.1	0.7 ± 0.1	ns
Fasting Glucagon (pg/ml)	56.8 ± 6.6	66.8 ± 7.1	ns	65.7 ± 11.4	96.0 ± 20.0	ns	62.9 ± 5.6	64.2 ± 6.3	ns
Fasting GLP-1 (pg/ml)	43.4 ± 9.9	55.2 ± 8.1	ns	44.1 ± 9.8	73.8 ± 13.4	ns	48.1 ± 7.6	56.8 ± 6.3	ns

Data are mean ± SEM. *p1* and *p2* are respectively for the comparison of Pre *vs.* Post 1 and Pre *vs.* Post 2 by Wilcoxon signed rank test; * $p \leq 0.05$; $^{\#}p \leq 0.01$ or $^{\S}p \leq 0.001$ for the comparison *vs* Control using Mann Whitney test.

Table 6. Lipid profile divided by remitters and non remitters[#]

	Pre		p^1	Post 1		p^2	Post 2		p^3
	Remitters	Non remitters		Remitters	Non remitters		Remitters	Non remitters	
Triglycerides (mmol/l)	1.8 ± 0.2	2.1 ± 0.3	ns	2.1 ± 0.3	1.8 ± 0.2	ns	1.6 ± 0.2	2.1 ± 0.4	ns
Total Cholesterol (mmol/l)	4.6 ± 0.7	5.7 ± 0.5	ns	3.5 ± 0.3	3.4 ± 0.2	ns	3.5 ± 0.2	3.5 ± 0.2	ns
HDL cholesterol (mmol/l)	1.0 ± 0.1	1.3 ± 0.7	ns	1.1 ± 0.1	0.9 ± 0.1	ns	1.1 ± 0.1	1.1 ± 0.1	ns
LDL cholesterol (mmol/l)	2.7 ± 0.6	3.6 ± 0.5	ns	1.5 ± 0.2	1.5 ± 0.1	ns	1.9 ± 0.2	1.8 ± 0.1	ns

Data are mean ± SEM. *p1* and *p2* are respectively for the comparison of Pre *vs.* Post 1 and Pre *vs.* Post 2 by Wilcoxon signed rank test; * $p \leq 0.05$; $^{\#}p \leq 0.01$ or $^{\S}p \leq 0.001$ for the comparison *vs* Control using Mann Whitney test.

Table 7. Effects of BPD on glucose metabolism, insulin and glucagon secretion and incretins divided by T2DM remission[#]

	Pre		p^1	Post 1		p^2	Post 2		p^3
	Remitters	Non remitters		Remitters	Non remitters		Remitters	Non remitters	
AUC Glucose ($mol \cdot l^{-1} \cdot h^{-1}$)	5.4 ± 0.3	5.5 ± 0.2	ns	2.8 ± 0.2	3.7 ± 0.2	0.01	2.2 ± 0.2	3.6 ± 0.1	0.004
Inc. AUC Glucose ($mol \cdot l^{-1} \cdot h^{-1}$)	2.0 ± 0.1	1.6 ± 0.2	ns	1.0 ± 0.1	0.9 ± 0.1	ns	0.5 ± 0.1	1.2 ± 0.1	0.01
AUC Insulin ($nmol \cdot l^{-1} \cdot h^{-1}$)	79 ± 8.8	66 ± 15.6	ns	45 ± 2.3	34 ± 3.5	0.03	57 ± 11.2	38 ± 6.2	ns
Inc. AUC Insulin ($nmol \cdot l^{-1} \cdot h^{-1}$)	57 ± 5.3	31 ± 5.7	0.02	32 ± 1.3	20 ± 3.5	0.01	45 ± 12.3	28 ± 6.3	ns
AUC C-peptide ($nmol \cdot l^{-1} \cdot h^{-1}$)	664 ± 39	424 ± 64	ns	494 ± 19	384 ± 36	0.05	540 ± 100	330 ± 47	0.05
Inc. AUC C-peptide ($nmol \cdot l^{-1} \cdot h^{-1}$)	369 ± 26	212 ± 42	0.01	297 ± 22	181 ± 29	0.02	357 ± 94	209 ± 37	ns
AUC GLP-1 ($nM \cdot l^{-1} \cdot h^{-1}$)	15 ± 2.6	19 ± 2.4	0.05	26 ± 5.0	30 ± 4.2	ns	21 ± 3.2	27 ± 2.7	ns
Inc. AUC GLP-1 ($nM \cdot l^{-1} \cdot h^{-1}$)	17 ± 0.6	2.8 ± 0.7	ns	13 ± 2.0	9.5 ± 2.2	ns	11 ± 3.3	9.5 ± 2.9	ns
AUC Glucagon ($ng \cdot ml^{-1} \cdot h^{-1}$)	19 ± 2.2	23 ± 2.1	ns	25 ± 6.0	31 ± 5.2	ns	20 ± 2.6	21 ± 1.7	ns
AUC FFA ($\mu Eq^{-1} \cdot l^{-1}$)	131 ± 22	183 ± 21	ns	134 ± 21	117 ± 12	ns	69 ± 23	93 ± 12	ns
Inc. AUC FFA ($\mu Eq^{-1} \cdot l^{-1}$)	-139 ± 18	-156 ± 37	ns	-144 ± 24	-166 ± 22	ns	-102 ± 36	-126 ± 14	ns

Data are mean ± SEM. *p1* and *p2* are respectively for the comparison of Pre *vs.* Post 1 and Pre *vs.* Post 2 by Wilcoxon signed rank test; * $p \leq 0.05$; $^{\#}p \leq 0.01$ or $^{\S}p \leq 0.001$ for the comparison *vs* Control using Mann Whitney test.

Table 8: β-cell function parameters divided by remitters and non remitters.

	Pre		p^1	Post 1		p^2	Post 2		p^3
	Remitters	Non remitters		Remitters	Non remitters		Remitters	Non remitters	
Fasting ISR (pmol·min^{-1}·m^{-2})	132[17]	34[51]	0.02	81[20]	86[28]	ns	85[35]	57[24]	0.07
Total insulin output (nmol·m^{-2})	90[8]	44[37]	0.05	64[11]	52[25]	ns	53[37]	43[29]	ns
ß-cell glucose sensitivity (pmol·min^{-1}·m^{-2}·mM^{-1})	20[4]	11[17]	ns	32[12]	24[22]	0.07	46[33]	16[18]	0.006
Rate sensitivity (nmol·m^{-2}·mM^{-1})	247[127]	166[293]	ns	508[342]	692[390]	ns	610[697]	212[455]	ns
Potentiation factor (fold)	1.34[0.5]	1.19[0.4]	ns	1.17[0.5]	1.11[0.4]	ns	1.8[1.1]	1.3[0.6]	ns
Insulin clearance basal (l·min^{-1}·m^{-2})	3.1±0.3	2.7±1.4	ns	3.7±0.3	3.9±0.3	ns	3.3±0.5	3.2±0.3	ns
Insulin clearance MTT (l·min^{-1}·m^{-2})	1.8±0.3	1.5±0.2	ns	2.3±0.3	2.0±0.1	ns	2.2±0.3	1.8±0.2	ns

Data are median [interquartile range] or are mean ± SEM . p^1, p^2 *and* p^3 for the comparison remitters *vs* non-remitters at baseline, 2 months and 1 year respectively by Man-Whitney test.

Table 9: Euglycaemic hyperinsulinaemic clamp data by remitters and non remitters[#]

	Pre		p^1	Post 1		p^2	Post 2		p^3
	Remitters	**Non remitters**		**Remitters**	**Non remitters**		**Remitters**	**Non remitters**	
M (µmol/Kg_{FFM}^{-1}·min^{-1})	20 [5]	19 [2]	ns	39 [6]	24 [16]	0.07	38 [10]	31 [9]	0.05
M/I (µmol·min^{-1}· kg_{ffm}^{-1}·$pmol^{-1}$)	38 [7]	36 [9]	ns	94 [17]	68 [44]	ns	90 [41]	81 [19]	ns
SS Clamp Plasma Insulin (nmol/l)	532 [95]	513 [147]	ns	423 [67]	408 [67]	ns	385 [136]	431 [63]	ns
Rd clamp (umol·min^{-1})	1294 [388]	1235 [330]	ns	1863 [348]	1526 [662]	ns	1734 [681]	1701 [212]	ns
EGP (µmol·min^{-1})	827 [264]	958 [231]	ns	717 [139]	802 [248]	ns	775 [196]	807 [231]	ns
EGP clamp (µmol·min^{-1})	266 [121]	291 [185]	ns	38 [143]	203 [100]	0.06	145 [48]	176 [115]	ns

Data are median [interquartile range]. p^1, p^2 *and* p^3 for the comparison remitters *vs* non-remitters at baseline, 2 months and 1 year respectively by Man-Whitney test.

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