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Postgraduate Doctoral Studies in Biomedical Fields

Mechanisms of insulin resistance and β-cell failure in type 2 diabetes. Effective diet against ominous octet.

Doctoral Thesis

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Identification

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Acknowledgments

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I. SUMMARY

Background and Aims: Dietary intervention is one of the key components in type 2 diabetes (T2D) management. Vegetarian diet is a promising alternative in the nutritional treatment of T2D. The aims of our study were:

1. To compare the effects of vegetarian and conventional diabetic diet with the same caloric restriction on insulin resistance, volume of visceral fat and plasma concentrations of oxidative stress markers after a 12-weeks-diet-intervention and subsequent 12-weeks of diet plus aerobic exercise training in subjects with T2D.

2. To explore the effect of 12 weeks of diet intervention and subsequent 12 weeks of diet combined with aerobic exercise training on -cell function and to evaluate the role of gastrointestinal peptides in subjects with T2D.

3. To study quality of life, Beck depression score and changes in eating behaviour in response to a vegetarian and a conventional diabetic diet.

4. To explore the role of changes in fatty acid composition of serum phospholipids in diet-induced changes in insulin sensitivity in subjects with T2D.

5. To follow-up our patients 1 year from the end of the intervention.

Methods: Subjects with T2D (n=74) were randomly assigned to experimental group (EG, n=37) following vegetarian diet or control group (CG, n=37) following conventional diabetic diet with the same caloric restriction. Participants were examined at baseline, 12 weeks of diet intervention and 24 weeks (second 12 weeks of diet were combined with aerobic exercise). Insulin sensitivity was measured by hyperinsulinemic isoglycemic clamp. Visceral and subcutaneous fat were measured by magnetic resonance imaging. -cell function was assessed during standard meal tests and quantified with a mathematical model. Quality of life was assessed using 2 questionnaires: Weight-Loss Quality-of-Life (OWLQOL) and Weight-Related Symptoms (WRSM). We used the Three-Factor Eating Questionnaire to monitor changes in eating behaviour and the Beck Depression Inventory to screen for depressive symptoms. The fatty acid composition of serum phospholipids was measured by gas liquid chromatography.

Results: 43% of EG and 5% of CG participants reduced diabetes medication. Body weight decreased by 6.2±5.8 kg in EG and by 3.2±4.5 kg in CG (interaction group x time p=0.001). Insulin sensitivity increased more in EG (by 30% vs. 20% in CG, interaction group x time p=0.04). Visceral and subcutaneous fat decreased more in EG (interaction group x time p=0.007 and p=0.02, respectively). Plasma adiponectin increased in EG (p=0.02); leptin decreased in EG (p=0.02). Vitamin C, superoxide dismutase and reduced glutathione increased in EG (p=0.002, p<0.001 and p=0.02, respectively). Difference between groups enlarged after exercise. Changes in insulin sensitivity and enzymatic oxidative stress markers correlated with changes in visceral fat.

Both insulin secretion at the reference level and glucose sensitivity increased in weeks 0-12 (by 33±54% and by 26±53%, respectively, p<0.001) and remained unchanged in weeks 12-24. Plasma concentrations of pancreatic polypeptide (PP) decreased in weeks 0-12 (p<0.05) and did not change significantly in weeks 12-24. Changes in parameters of -cell function correlated negatively with plasma concentrations of PP. Both diets elicited a positive effect on the quality of life, mood and eating behaviour, however the positive effects of a vegetarian diet were greater.

Linoleic acid (18:2n6) increased in EG (p=0.04) while it decreased in CG (p=0.04) in response to dietary interventions. In EG, changes in 18:2n6 correlated positively with changes in MCR (r=+0.22; p=0.04) and negatively with changes in visceral fat (r=-0.43; p=0.01).

Neither weight nor waist circumference changed significantly in either group. HbA1c increased (p 0.05) similarly in both groups.

Conclusions: Insulin sensitivity increased more with vegetarian diet. Vegetarian diet led to a greater reduction in visceral fat and greater improvement in plasma concentrations of adipokines and oxidative stress markers. Differences between groups enlarged after addition of exercise. After diet-induced weight loss, -cell function improved in T2D subjects and remained unchanged after the addition of exercise. We demonstrate for the first time that these changes are associa ted with a decrease in PP secretion. Both diets elicited a positive effect on the quality of life , mood and eating behaviour, however the p ositive effects of a vegetarian diet were greater. We demonstrated that the insulin -sensitizing effect of vegetarian diet might be related to the increased proportion of 18:2n6 in serum phospholipids. 1 year after the end of the intervention, the positive effects of a vegetarian diet compared to a conventional diet were partially maintained.

SOUHRN

Úvod a cíle: Dietní intervence je pilí em lé by diabetu 2. typu (T2D). Vegetariánská strava je zajímavou alternativou.

Cíle naší studie byly následující:

1. Porovnat ú inky vegetariánské a konven ní diabetické diety se srovnatelnou kalorickou restrikcí na inzulínovou rezistenci, objem viscerálního tuku a plazmatické koncentrace marker oxida ního stresu po 12 -týdenní dietní intervenci a dalších 12 týdnech diety plus fyzické aktivity u pacient s T2D.

2. Zkoumat ú inek 12-týdenní dietní intervence a dalších 12 týdn diety plus fyzické aktivity na funkci -bun k a vyhodnotit potenciální roli gastrointestinálních peptid u pacient s T2D.

3. Porovnat ú inek vegetariánské a konven ní diabetické diety na kvalitu života, Beckovo skóre deprese a jídelní chování u pacient s T2D.

4. Zkoumat roli zm n složení mastných kyselin v sérových fosfolipidech ve zm nách v inzulínové senzitivit u pacient s T2D.

5. Sledovat naše pacienty 1 rok od ukon ení studie .

Metody: Pacienti s T2D (n=74) byli náhodn rozd leni do exper imentální skupiny (ES, n=37), která konzumovala vegetariánskou stravu, a kontrolní skupiny (KS, n=37), která dodržovala konven ní diabetickou dietu se stejnou kalorickou restrikcí. Ú astníky studie jsme vyšet ili na za átku , po 12 týdnech dietní intervence a po 24 týdnech (druhých 12 týdn dietní intervence bylo kombinováno s aerobním cvi ením). Inzulínovou senzitivitu jsme m ili pomocí hyperinzulínového isoglykemického clampu. Objem viscerálního a podkožního tuku byl stanoven pomocí zobrazování magnetickou rezonancí. Funkce -bun k byla stanovena b hem standardního meal testu a kvantifikována pomocí matematického modelu. Kvalita života byla stanovena pomocí 2 dotazník : Weight-Loss Quality-of-Life (OWLQOL) a Weight-Related Symptoms (WRSM). Použili jsme T ífaktorový dotazník ke zjišt ní zm n jídelního chování a Beck v dotazník ke zjišt ní depresivních p íznak . Složení mastných kyselin v sérových fosfolipidech jsme m ili pomocí plynové kapalinové chromatografie.

Výsledky: U 43% ú astník v ES a 5% v KS jsme snížili antidiabetika. T lesná hmotnost se snížila o 6,2±5,8 kg v ES a o 3,2±4,5 kg v KS (interakce skupina x as p=0,001). Inzulínová senzitivita se zvýšila více v ES (o 30% vs. 20% v KS, interakce skupina x as p=0,04). Objem viscerálního a podkožního tuku se snížil více v ES (interakce skupina x as p=0,007 a p=0,02). Plazmatické koncentrace adiponektinu se zvýšily v ES (p=0,02); leptin klesl v ES (p=0,02). Vitamín C, superoxiddismut áza a redukovaný glutathion stouply v ES (p=0,002, p<0,001 a p=0,02). Rozdíly mezi skupinami se zv tšily b hem cvi ebního programu . Zm ny v inzulínové senzitivit a enzymatických markerech oxida ního stresu korelovaly se zm nami v objemu viscerálního tuku.

Sekrece inzulínu p i referen ní glykémii a glukózová senzitivita se zvýšily v týdnech 0-12 (o 33±54% a o 26±53%, p<0,001), beze zm ny v týdnech 12-24. Plazmatické koncentrace pankreatického polypeptidu (PP) klesly v týdnech 0-12 (p<0,05), beze zm ny v týdnech 12-24. Zm ny v parametrech funkce -bun k korelovaly negativn se zm nami v plazmatických koncentracích PP.

Ob diety m ly pozitivní ú inek na kvalitu života, náladu a jídelní chování , nicmén pozitivní ú inky byly v tší u vegetariánské stravy.

Kyselina linolová (18:2n6) se zvýšila v ES (p=0,04) a klesla v KS (p=0,04). V ES korelovaly zm ny v 18:2n6 pozitivn se zm nami v MCR (r=+0,22; p=0,04) a negativn se zm nami ve viscerálním tuku (r=-0,43; p=0,01).

Hmotnost i obvod pasu se 1 rok od ukon ení intervence ani v jedné skupin významn nezm nily. HbA1c stoupl (p 0,05) srovnateln v obou skupinách.

Záv ry: Inzulínová senzitivita se zvýšila více p i vegetariánské strav . Vegetariánská strava vedla k v tšímu poklesu viscerálního tuku a v tšímu zlepšení plazmatických koncentrací adipokin a marker oxida ního stresu. Rozdíly mezi skupinami se zv tšily b hem cvi ebního programu . B hem váhového úbytku v d sledku dietní intervence došlo ke zlepšení funkce -bun k. Poprvé jsme ukázali, že tyto zm ny jsou spojeny s poklesem sekrece PP. Ob diety m ly pozitivní ú inek na kvalitu života, náladu a jídelní chování, nicmén pozitivní ú inky byly v tší u vegetariánské stravy. Ukázali jsme, že pozitivní ú inky vegetariánské stravy na inzulínovou senzitivitu jsou spojeny se zvýšeným obsahem 18:2n6 v sérových fosfolipidech. 1 rok od konce intervence p etrvaly áste n pozitivní ú inky vegetariánské stravy ve srovnání s konven ní diabetickou dietou .

I. PATHOPHYSIOLOGICAL MECHANISMS OF INSULIN RESISTANCE AND -CELL FAILURE IN TYPE 2 DIABETES

1. Introduction

Insulin resistance and -cell failure represent the core pathophysiologic al defects in type 2 diabetes. It has been recognized that the -cell failure occurs much earlier and is more severe than previously thought. Subjects in the upper tertile of impaired glucose tolerance (IGT) are maxima lly insulin resistant and have lost over 80% of their -cell function. In addition to the muscle, liver, and -cell, the fat cell (accelerated lipolysis), gastrointestinal tract (incretin deficiency or resistance), -cell (hyperglucagonemia), kidney (increased glucose reabsorption), and brain (insulin resistance) all play important roles in the development of glucose intolerance in subjects with type 2 diabetes (1). Collectively, these eight players comprise the ominous octet (2) (Fig. 1) and indicate that therapy must be started early to prevent or slow the progressive -cell failure that already is well established in IGT subjects. A treatment paradigm shift is recommended in which combination therapy is initiated with lifestyle intervention (diet and exercise), metformin (which improves insulin sensitivity and has anti atherogenic effects), a thiazolidinedione (which improves insulin sensitivity, preserves -cell function, and exerts antiatherogenic effects), and an incretin analogue (which preserves -cell function and promotes weight loss). Sulfonylureas are not recommended because, after an initial improvement in glycemic control, they are associated with a progressive rise in HbA1C and progressive loss of -cell function.
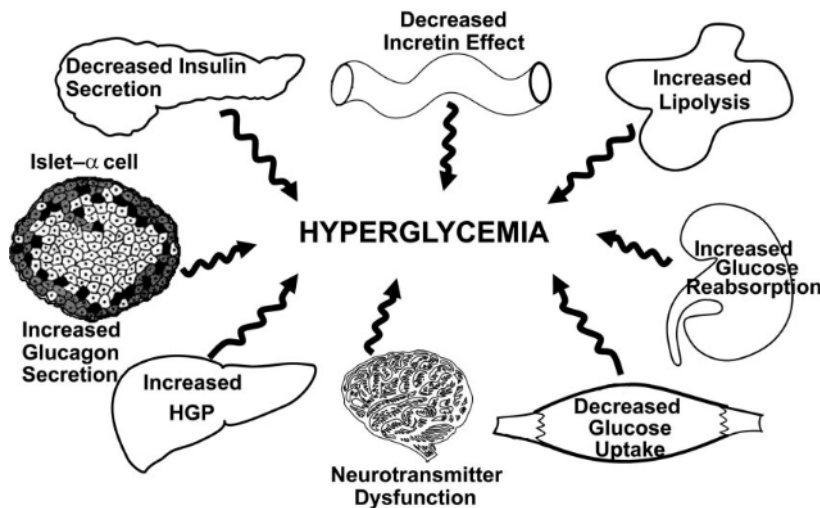
FIG. 1. The ominous octet. See text for a more detailed explanation (2).

1.1. Natural history of type 2 diabetes

The natural history of type 2 diabetes has been well described in multiple populations (3). Individuals prone to develop type 2 diabetes inherit a set of genes from their parents that make their tissues resistant to insulin (4). In liver, the insulin resistance is manifested by an overproduction of glucose during the basal state despite the presence of fasting hyperinsulinemia (5) and an impaired suppression of hepatic glucose production (HGP) in response to insulin (6), as occurs following a meal (7). In muscle, the insulin resistance is manifested by impaired glucose uptake following ingestion of a carbohydrate meal and results in postprandial hyperglycemia (7,8). Although the origins of the insulin resistance can be traced to their genetic background (4), the epidemic of diabetes that has developed in western countries is related to the epidemic of obesity and physical inactivity (9). Both obesity (10) and decreased physical activity (11) are insulin-resistant states and, when added to the genetic burden of the insulin resistance, place a major stress on the pancreatic -cells to augment their secretion of insulin to offset the defect in insulin action. As long as the -cells are able to augment their secretion of insulin sufficiently to offset the insulin resistance, glucose tolerance remains normal (12). However, with time the -cells begin to fail and initially the postprandial plasma glucose levels and subsequently the fasting plasma glucose concentration begin to rise, leading to the onset of overt diabetes (13). Collectively, the insulin resistance in muscle and liver and -cell failure, have been referred to as the triumvirate (14). The resultant hyperglycemia and poor metabolic control may cause a further decline in insulin sensitivity, but it is the onset and pace of progressive -cell failure that determines the rate of diabetes progression.

2. Insulin resistance

Both the liver and muscle are severely resistant to insulin in individuals with type 2 diabetes (3). However, when discussing insulin resistance, it is important to distinguish what is responsible for the insulin resistance in the basal or fasting state and what is responsible for the insulin resistance in the insulin-stimulated state.

2.1. Insulin signal transduction

For insulin's action, it must first bind to and then activate the insulin receptor by phosphorylating key tyrosine residues on the   chain (15,16). This results in the translocation of insulin receptor substrate (IRS)-1 to the plasma membrane, where it interacts with the insulin receptor and also undergoes tyrosine phosphorylation. This leads to the activation of PI 3-kinase and Akt, resulting in glucose transport into the cell, activation of nitric oxide synthase with arterial vasodilation (17,18), and stimulation of multiple intracellular metabolic processes.

It has been demonstrated that the ability of insulin to tyrosine phosphorylate IRS-1 is severely impaired in lean type 2 diabetic individuals (16,19), in obese normal glucose tolerant individuals (19), and in the insulin-resistant, normal glucose tolerant offspring of two type 2 diabetic parents (20) (Fig. 2). This defect in insulin signaling leads to decreased glucose transport, impaired release of nitric oxide with endothelial dysfunction, and multiple defects in intramyocellular glucose metabolism. In contrast to the severe defect in IRS-1 activation, it has been shown that the mitogen-activated protein (MAP) kinase pathway, which can be activated by Shc, is normally responsive to insulin (19) (Fig. 2). The MAP kinase pathway, when stimulated, leads to the activation of a number of intracellular pathways involved in inflammation, cellular proliferation, and atherosclerosis (21,22).

Thus, the block at the level of IRS-1 impairs glucose transport into the cell and the resultant hyperglycemia stimulates insulin secretion. Because the MAP kinase pathway retains its sensitivity to insulin (19,23), this causes excessive stimulation of this pathway and activation of multiple intracellular pathways involved in inflammation and atherogenesis. This, in part, explains the strong association between insulin resistance and atherosclerotic cardiovascular disease in nondiabetic, as well as in type 2 diabetic, subjects (24–26).
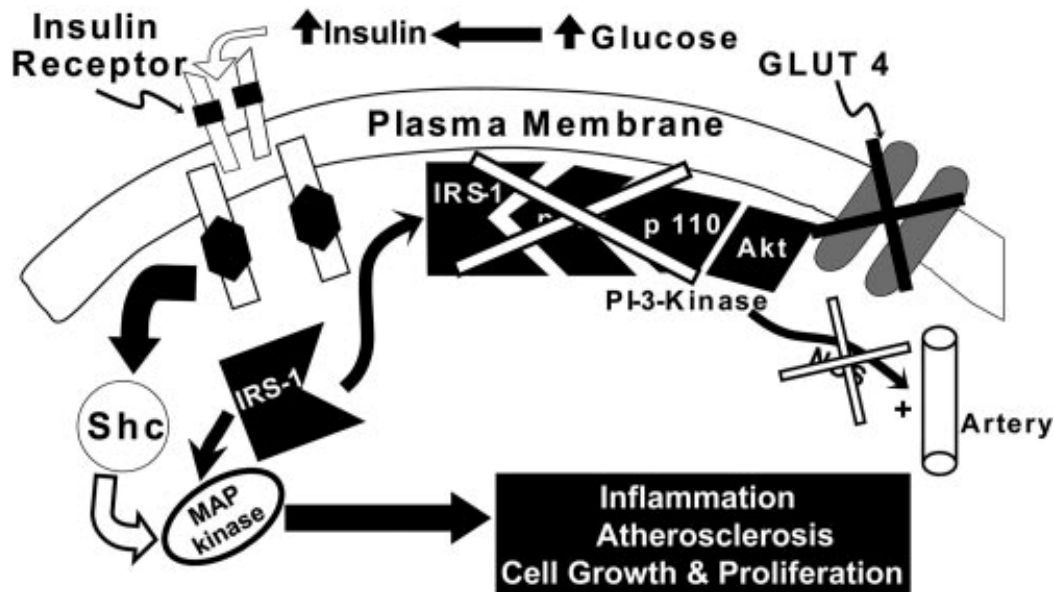
FIG. 2. Relationship between impaired insulin signal transduction and accelerated atherogenesis in insulin - resistant subjects, i.e., type 2 diabetes and obesity (16),(19).

2.2. Liver

The brain has an obligate need for glucose and is responsible for 50% of glucose utilization under basal or fasting conditions. This glucose demand is met primarily by glucose production by the liver and to a smaller extent the kidneys. The fasting hepatic glucose production (HGP) in increased in patients with type 2 diabetes compared to healthy subjects (5). As the rate of basal HGP rises, so also does the fasting plasma glucose concentration, and these two variables are strongly correlated with an *R* value of 0.847 (*P*<0.001). This overproduction of glucose by the liver occurs in the presence of fasting plasma insulin levels that are increased 2.5- to 3-fold, indicating severe resistance to the suppressive effect of insulin on HGP. The increase in basal HGP is explained entirely by an increase in hepatic gluconeogenesis (27).

In addition to hepatic insulin resistance, multiple other factors contribute to accelerated rate of HGP including: *1*) increased circulating glucagon levels and enhanced hepatic sensitivity to glucagon (28); *2*) lipotoxicity leading to increased expression and activity of phosphoenolpyruvate carboxykinase and pyruvate carboxylase (29), the rate-limiting enzymes for gluconeogenesis; and *3*) glucotoxicity,

leading to increased expression and activity of glucose-6-phosphatase, the rate-limiting enzyme for glucose escape from the liver (30).

2.3. Muscle

Using the euglycemic insulin clamp technique in combination with tritiated glucose to measure total body glucose disposal, it has been demonstrated that lean type 2 diabetic individuals are severely resistant (3). In patients with type 2 diabetes, the presence of multiple intramyocellular defects in insulin action has been documented (31), including impaired glucose transport and phosphorylation (8), reduced glycogen synthesis (32), and decreased glucose oxidation (33). However, more proximal defects in the insulin signal transduction system play a paramount role in the muscle insulin resistance (19).

2.4. -cell function

Although the plasma concentrations of insulin are typically increased in response to the development of insulin resistance during the natural history of type 2 diabetes, this does not mean that the -cell is functioning normally. To the contrary, recent studies have demonstrated that the onset of -cell failure occurs much earlier and is more severe than previously appreciated. By the time when the diagnosis of diabetes is made, the patient has lost over 80% of his/her -cell function, and it is essential that the physician intervene aggressively with therapies known to correct known pathophysiological disturbances in -cell function.

There are no cut points that distinguish normal glucose tolerance (NGT) from IGT and from type 2 diabetes. Rather, glucose intolerance is a continuum; therefore, the current diagnostic criteria (34) for IGT and type 2 diabetes are quite arbitrary and, like plasma cholesterol, glucose tolerance should be viewed as a continuum of risk. The higher the 2-h plasma glucose concentration, even within the range of IGT, the greater is the risk for microvascular complications.

In a postmortem analysis, Butler et al. (35) quantified relative -cell volume and related it to the fasting plasma glucose concentration. As individuals progressed from NGT to impaired fasting glucose (IFG), there was a 50% decline in -cell volume,

suggesting a significant loss of -cell mass long before the onset of type 2 diabetes. With the progression to overt diabetes, there was a further and significant loss of -cell volume. Although -cell volume should not be viewed to be synonymous with -cell mass, these results suggest that significant loss of -cell mass occurs long before the onset of type 2 diabetes, according to current diagnostic criteria (34).

2.5. Adipocytes

Considerable evidence implicate s impaired adipocyte metabolism and altered fat topography in the pathogenesis of glucose intolerance in type 2 diabetes (36,37). Fat cells are resistant to insulin's anti -lipolytic effect, leading to day-long elevation in the plasma FFA concentrations (36,37). Chronically increased plasma FFA levels stimulate gluconeogenesis (38), induce insulin resistance (39), and impair insulin secretion (39). These FFA-induced disturbances are referred to as lipotoxicity. Dysfunctional fat cells produce excessive amounts of insulin resistance–inducing, inflammatory, and atherosclerotic provoking adipocytokines and fail to secrete normal amounts of insulin-sensitizing adipocytokines such as adiponectin (36). Enlarged fat cells are insulin resistant and have diminished capacity to store fat (40). When adipocyte storage capacity is exceeded, lipid "overflows" into muscle, liver, and -cells, causing muscle/hepatic insulin resistance and impaired insulin secretion (36). Lipid can also overflow into arterial vascular smooth cells, leading to the acceleration of atherosclerosis.

It has been demonstrated that a physiological elevation in the plasma FFA concentration stimulates HGP (41) and impairs insulin-stimulated glucose uptake in liver (42) and muscle (43,44). It has also been shown that elevated plasma FFA levels inhibit insulin secretion.

2.6. Gastrointestinal tract

Glucose ingestion elicits a much greater insulin response than an intravenous glucose infusion that mimics the plasma glucose concentration profile observed with oral glucose (45). Most of this incretin effect can be explained by two hormones: GLP -1 and GIP. GLP-1 secretion by the L-cells of the distal small intestine is deficient, while GIP secretion by the K-cells of the more proximal small intestine is increased, but

there is resistance to the stimulatory effect of GIP on insulin secretion (46). GLP-1 also is a potent inhibitor of glucagon secretion (45), and the deficient GLP-1 response contributes to the paradoxical rise in plasma glucagon secretion and impaired suppression of HGP that occurs after ingestion of a mixed meal (47).

The gut microbiota, composed of hundreds of billions of bacteria, play an important role in maintaining key physiologic functions for the human body (48) Experimental data explored how the gut microbiota were able to control the energy metabolism, and thereby the development of adiposity (49). Some nutrients with prebiotic properties, which escape the digestion in the upper part of the gut, modify the composition of the gut microbiota in favor of bacteria that could play a beneficial role on glucose homeostasis, namely by modulating the endocrine function of the gut, and by reinforcing the gut barrier (48). Clearly, the gut is a major endocrine organ and contributes to the pathogenesis of type 2 diabetes.

2.7. Pancreatic -cells

It has been demonstrated that the basal plasma glucagon concentration is elevated in patients with type 2 diabetes (28). The important contribution of elevated fasting plasma glucagon levels to the increased basal rate of HGP in patients with type 2 diabetes was provided by Baron et al. (50). Compared with control subjects, patients with type 2 diabetes had a markedly elevated rate of basal HGP, which correlated closely with the increase in fasting plasma glucagon concentration. Following somatostatin infusion, plasma glucagon concentrations declined by 44%, while basal HGP decreased by 58%. These results conclusively demonstrate the important role of hyperglucagonemia in the pathogenesis of fasting hyperglycemia in type 2 diabetes. There also is evidence that the liver may be hypersensitive to the stimulatory effect of glucagon in hepatic gluconeogenesis (28).

2.8. Kidney

The kidney filters about 162 g of glucose every day. 90% of the filtered glucose is reabsorbed by the high capacity SGLT2 transporter in the convoluted segment of the proximal tubule, and the remaining 10% of the filtered glucose is reabsorbed by the SGLT1 transporter in the straight segment of the descending proximal tubule (51).

The result is that no glucose appears in the urine. In animal models of both type 1 and type 2 diabetes, the maximal renal tubular reabsorptive capacity (Tm), for glucose is increased (52). In humans with type 1 diabetes, the Tm for glucose has been shown to be increased. In human type 2 diabetes, the Tm for glucose has not been systematically examined. No studies in either type 1 or type 2 diabetic individuals have examined the splay in the glucose titration curve in h umans. However, cultured human proximal renal tubular cells from type 2 diabetic patients demonstrate markedly increased levels of SGLT2 mRNA and protein and a fourfold increase in the uptake of   -methyl-D-glucopyranoside (AMG), a nonmetabolizeable glucose analog (53).

The adaptive response of the kidney to conserve glucose, which is essential to meet the energy demands of the body, especially the brain and other neural tissues, which have an obligate need for glucose, becomes maladaptive in the diabetic patient. Instead of dumping glucose in the urine to correct the hyperglycemia, the kidney chooses to hold on to the glucose. Even worse, the ability of the diabetic kidney to reabsorb glucose appears to be augmented by an absolute increase in the renal reabsorptive capacity for glucose.

2.9. Brain

It is well established that the current epidemic of diabetes is being driven by the epidemic of obesity (54). It has been demonstrated that, in rodents, insulin is a powerful appetite suppressant (55). Obese individuals, both diabetic and nondiabetic, are characterized by insulin resistance and compensatory hyperinsulinemia. Nonetheless, food intake is increased in obese subjects despite the presence of hyperinsulinemia, and one could postulate that the insulin resistance in peripheral tissues also extends to the brain.

Impaired appetite regulation by insulin in obese subjects has been studied using functional magnetic resonance imaging (MRI) to examine the cerebral response to an ingested glucose load (56). After glucose ingestion, two hypothalamic areas with consistent inhibition were noted: the lower posterior hypothalamus, which contains the ventromedial nuclei, and the upper posterior hypothalamus , which contains the

paraventricular nuclei. In both of these hypothalamic areas, which are key centers for appetite regulation, the magnitude of the inhibitory response following glucose ingestion was reduced in obese, insulin-resistant, normal glucose tolerant subjects, and there was a delay in the time taken to reach the maximum inhibitory response, even though the plasma insulin response was markedly increased in the obese group. Whether the impaired functional MRI response in obese subjects contributes to or is a consequence of the insulin resistance and weight gain remains to be determined. Nonetheless, these results suggest that the brain, like other organs (liver, muscle, and fat) in the body, may be resistant to insulin. Studies by Obici et al. (57,58) in rodents have also provided evidence for cerebral insulin resistance leading to increased HGP and reduced muscle glucose uptake.

3. Pathogenesis of -cell failure

3.1. Age

Advancing age plays an important role in the progressive -cell failure that characterizes type 2 diabetes. A progressive age-related decline in -cell function has been demonstrated (59). This is consistent with the well-established observation that the incidence of diabetes increases progressively with advancing age.

3.2. Genes

-cell failure also clusters in families, and studies in first-degree relatives of type 2 diabetic parents and in twins have provided strong evidence for the genetic basis of the -cell dysfunction (60,61). Impaired insulin secretion has been shown to be an inherited trait in Finnish families with type 2 diabetes with evidence for a susceptibility locus on chromosome 12 (62). Recently, a number of genes associated with -cell dysfunction in subjects with type 2 diabetes have been described, the transcription factor TCF7L2 being the best established (63,64).

Unfortunately, at present there are no known therapeutic interventions that can reverse either the age-related decline or genetic-related factors responsible for impaired insulin secretion. However, there are a number of causes of -cell failure

that can be reversed or ameliorated.

3.3. Insulin resistance

Insulin resistance, by placing an increased demand on the β-cell to hypersecrete insulin, also plays an important role in the progressive β-cell failure of type 2 diabetes. Therefore, interventions aimed at enhancing insulin sensitivity are of paramount importance. The precise mechanisms, via which insulin resistance leads to β-cell failure, remain unknown. It commonly is stated that the β-cell, by being forced to continuously hypersecrete insulin, eventually wears out. Although simplistic in nature, this explanation lacks a mechanistic cause. An alternate hypothesis, for which considerable evidence exists, is that the cause of the insulin resistance is also directly responsible for the β-cell failure. Thus, just as excess deposition of fat (LC-fatty acyl CoAs, diacylglycerol, and ceramide) in liver and muscle has been shown to cause insulin resistance in these organs, deposition of fat in the β-cell leads to impaired insulin secretion and β-cell failure. Similarly, hypersecretion of islet amyloid polypeptide (IAPP), which is co-secreted in a one-to-one ratio with insulin, can lead to progressive β-cell failure.

3.4. Lipotoxicity

Elevated plasma free fatty acid (FFA) concentrations impair insulin secretion, and this has been referred to as lipotoxicity (65). Interventions, such as weight loss and thiazolidinediones, that mobilize fat out of the β-cell would be expected to reverse lipotoxicity and preserve β-cell function.

3.5. Glucotoxicity

Chronically elevated plasma glucose levels also impair β-cell function, and this has been referred to as glucotoxicity (66). Strict glycemic control is essential not only to prevent the microvascular complications of diabetes but also to reverse the glucotoxic effect of chronic hyperglycemia on the β-cells (67,68), as well as on hepatic and muscle insulin resistance.

3.6. Incretins

Abnormalities in the incretin axis have been shown to play an important role in the progressive -cell failure of type 2 diabetes. GLP-1 and glucose-dependent insulinotrophic polypeptide (also called gastric inhibitory polypeptide [GIP]) account for 90% of the incretin effect (45). In type 2 diabetes, there is a deficiency of GLP-1 (45) and resistance to the action of GIP (46). The deficiency of GLP-1 can be observed in individuals with IGT and worsens progressively with progression to type 2 diabetes (69). In addition to deficiency of GLP-1, there is resistance to the stimulatory effect of GLP-1 on insulin secretion (70). In contrast to GLP-1, plasma levels of GIP are elevated in type 2 diabetes, yet circulating plasma insulin levels are reduced (71). This suggests that there is -cell resistance to the stimulatory effect of GIP on insulin secretion, and this, in fact, has been demonstrated (46). Recent studies have shown that tight glycemic control can restore the -cells' insulin secretory response to GIP (72). Thus, -cell resistance to GIP is another manifestation of glucotoxicity.

4. Effective diet against ominous octet

4.1. Caloric restriction

Caloric restriction is the number one strategy to lose weight and to improve glycemic control. Weight loss is typically accompanied by improvements in glycemic control and insulin sensitivity. A reduction in caloric intake can have profound effects on glucose control, insulin secretion and insulin resistance before any changes in obesity occur (73,74). To further study this, Kelley et al. (75) measured insulin sensitivity by a hyperinsulinemic euglycemic clamp in obese patients with type 2 diabetes after: 1) 1 week of bodyweight maintenance; 2) 1 week of caloric restriction (800 kcal/day); 3) 2 months of further restriction (400 kcal/day), followed by 1 month of refeeding (intake was increased by 200 kcal/week during this month), and 1 week of a diet aimed at bodyweight maintenance; and 4) 1 week of energy restriction (800 kcal/day). Under these highly controlled conditions, the reduction in hepatic glucose production and improvement in insulin secretion and insulin sensitivity were substantial after the initial energy restriction and only insulin sensitivity showed a further improvement after the bodyweight loss (12.7 ±2.0 kg). The final week of energy restriction did not

affect these parameters.

In addition to bodyweight, alterations in dietary composition may also be associated with changes in insulin resistance. There is currently debate regarding the best diet for patients with type 2 diabetes with respect to the percentage of fat and carbohydrate in the diet. It has been recommended that fat intake is less than 35% of total energy, protein 10-20%, and carbohydrates 45-60% of total energy intake. Both the American Diabetes Association and the European Association for the Study of Diabetes have opted to individualize the diet to meet the patient's needs with the goal of minimizing diabetic complications (76,77).

4.2. Reduced intake of saturated fatty acids

A few studies have reported that dietary saturated fat can adversely affect insulin sensitivity (78,79). In a study of 162 healthy men and women, insulin sensitivity was significantly impaired (-10%, $P = 0.03$) after administration of a diet high in saturated fatty acids (17% of energy) for 3 months (78). Likewise, Xiao et al. (79) reported a decrease in insulin sensitivity following oral ingestion of emulsions containing predominantly saturated fatty acids (45% of energy) over 24 h in overweight men and women. Reductions in saturated fat intake have been reported to increase insulin sensitivity, an effect that is independent of changes in body weight (2,80).

4.3. Polyunsaturated fatty acids

Previous studies have suggested that the consumption of specific dietary fats, particularly low omega-6 polyunsaturated fatty acids and high trans unsaturated fatty acids, increases the risk of type 2 diabetes, but the role of omega-3 fats remains unclear (81).

Omega-3 fatty acids, particularly long-chain omega-3 fatty acids from seafood sources, alter the expression of peroxisome proliferator –activator receptor genes, which are involved in signaling nutrition status (82), and of the production of inflammatory cytokines, which are associated with type 2 diabetes (83). These findings suggest that omega-3 fatty acids could lower the risk of type 2 diabetes (84). In epidemiologic studies, intake of long-chain omega-3 fatty acids was associated

with better glucose tolerance in some studies (85,86), but not in others (87,88). Some intervention studies have found that omega-3 intake resulted in an increase in glycated hemoglobin (89,90), and in fasting blood glucose (87,90).

Epidemiologic studies of the relation of long-chain fatty acid intake with T2DM have reported conflicting results (91–96). Furthermore, because studies have suggested that environmental contaminants such as dioxins (97), found in fish, might raise the risk of type 2 diabetes, the risks and benefits of fish intake remain controversial (98). Recently, the association was examined between dietary long-chain fatty acids and incidence of type 2 diabetes in 3 prospective cohorts of women and men. 195,204 US adults (152,700 women and 42,504 men) without preexisting chronic disease at baseline were followed for 14 to 18 years. No evidence was found that higher consumption of long-chain fatty acids and fish reduced the risk of type 2 diabetes. Instead, higher intakes may modestly increase the incidence of this disease (99).

4.4. Reduced glycemic index

In a recent meta-analysis of prospective cohort studies, there was a 40% increase in risk of type 2 diabetes in participants whose diets were in the highest quintile of glycemic index versus the lowest (99). A meta-analysis by Brand-Miller et al. (100) of 14 randomized clinical trials reported that low glycemic index diets reduced HbA1c by 0.43 percentage points (95% CI 0.13–0.73) more than high-glycemic index diets in individuals with diabetes.

4.5. Increased intake of dietary fiber

In a randomized, crossover study in patients with type 2 diabetes, consuming a diet containing 50 g/day of dietary fiber for 6 weeks decreased 24-h glucose and insulin concentrations by 10% and 12%, respectively, compared to a diet containing a more moderate amount of fiber (24 g/day) (101). In observational studies, dietary fiber intake is inversely associated with diabetes incidence (102) and insulin resistance (103). Dietary fiber, in particular viscous fibers (104), may improve glycemic control by 1) delaying gastric emptying, which reduces the rate of glucose absorption, 2) decreasing the rate of glucose uptake by increasing the thickness of the unstirred water layer, 3) being fermented into propionate in the colon, which inhibits glucose

production in hepatocytes, and 4) increasing satiety, which promotes weight loss and improved insulin sensitivity (104,105).

4.6. Vitamins and micronutrients

People with diabetes should be encouraged to consume adequate amounts of vitamins and minerals from natural food sources, particularly fruits, nuts, and vegetables (76,106). Foods rich in antioxidants (tocopherols, carotenoids, vitamin C, and flavonoids) and other water and fat-soluble vitamins are especially encouraged. Consumption of foods rich in folate (e.g. citrus fruits and legumes) will ensure adequate folate status, and possibly reduce risk of coronary heart disease, while diets that include oily fish (e.g. salmon, tuna) and whole grain breads or cereals provide fat- and water-soluble vitamins (76,77).

Supplementation with a multivitamin preparation is recommended for selected patients with diabetes (e.g. the elderly, pregnant or lactating women, and individuals on strict calorie-restricted diets) who may be at particular risk of micronutrient deficiency (76). There are some claims that chromium and vanadium supplementation may improve glycemic control, but there is still in sufficient evidence to support such claims, and megadose supplementation may actually be unsafe (76,77).

4.6.1. Vitamin D

Vitamin D deficiency has been shown to alter insulin synthesis and secretion in both humans and animal models (107,108). It has been reported that vitamin D deficiency may predispose to glucose intolerance, altered insulin secretion and type 2 diabetes mellitus. Vitamin D replenishment improves glycaemia and insulin secre tion in patients with type 2 diabetes with established hypovitaminosis D, thereby suggesting a role for vitamin D in the pathogenesis of type 2 diabetes mellitus. The presence of vitamin D receptors (VDR) and vitamin D-binding proteins (DBP) in pancreatic tissue and the relationship between certain allelic variations in the VDR and DBP genes with glucose tolerance and insulin secretion have further supported this hypothesis (109,110). The mechanism of action of vitamin D in type 2 diabetes is thought to be mediated not only through regulation of plasma calcium levels, which regulate insulin synthesis and secretion, but also through a direct action on pancreatic beta-cell

function (111).

4.6.2. Reduction in heme-iron intake

Serum ferritin, the storage form of iron, was positively correlated with insulin resistance (112,113), and predicted the development of hyperglycemia (114) and type 2 diabetes (115) in observational studies. Hua et al. (116) reported greater insulin sensitivity and lower serum ferritin levels in lacto-ovo vegetarians compared with omnivores matched for age and body mass index. In this study, serum ferritin and insulin resistance were strongly and positively correlated (r = 0.80, *P* = 0.0001). Lowering body iron by phlebotomy in six male omnivores to levels similar to those seen in vegetarians resulted in a 40% enhancement of insulin-mediated glucose disposal (116). Heme-iron intake has been positively related to diabetes incidence, whereas non-heme iron, found in plants, was negatively correlated (117).

4.7. Vegetarian diet and type 2 diabetes

Observational and clinical trials indicate a benefit of vegetarian and vegan diets for diabetes management. The consistency of observed beneficial outcomes from studies employing vegetarian and vegan diets warrant additional research and future expansion of dietary guidelines to endorse vegan and vegetarian diets as a viable alternative to conventional dietary interventions (118,119).

Several possible mechanisms may explain the beneficial effects of vegetarian diet for diabetes management (120): higher intake of fiber (101), lower intake of saturated fat (and a higher P/S ratio) (2), higher intake of non-heme iron and reduction in iron stores (121), higher intake of vegetable protein in place of animal protein (122), higher intake of antioxidants (123) and plant sterols (124).

4.7.1. Observational studies

Several studies have reported that diabetes prevalence is lower among vegetarians compared with omnivores (125–127). Seventh-day Adventists are a population of interest because nearly all avoid tobacco, alcohol, and caffeine, while roughly half are omnivores and half are vegetarians. Overall, Adventists have only 45% of the

diabetes prevalence of the general population (126). In three large Adventist cohort studies, the prevalence of diagnosed diabetes was 1.6 to 2 times higher among non-vegetarians compared with vegetarians or vegans (125–127). Further adjustment for body weight reduced this difference only slightly. Regular consumption of even small amounts of meat was associated with an increased risk of diabetes in this population (127). In a 17-year study of 8401 Seventh-day Adventists, those who ate meat at least once per week were 29% more likely to develop diabetes compared with those eating no meat. Those who consumed any processed meats (specifically salted fish and frankfurters) were 38% more likely to develop diabetes. Long-term adherence (over 17 years) to a vegetarian diet was associated with a 74% reduced risk of developing diabetes relative to long-term adherence to a diet that included at least weekly meat intake. There was no association between an index of animal product consumption (meats, dairy, and eggs), with diabetes incidence. Other large cohort studies have also reported that meat consumption is associated with an increased risk of type 2 diabetes (128,129).

4.7.2. Interventional trials

Because vegetarian and vegan diets are associated with a lower body weight (130), increased insulin sensitivity (131,132) and reduced risk of diabetes, intervention trials have tested their effectiveness for diabetes management. Early studies reported a dramatic decrease in medication use when following a plant-based diet. Subsequent studies demonstrated a greater improvement in insulin sensitivity and glycemic control with a vegetarian diet compared to the conventional diabetic diet.

Anderson and Ward (133) tested the effect of a low-fat, high-carbohydrate (9% of energy from fat, 70% from carbohydrate) near-vegetarian diet containing 65 g of fiber and 65 g of cholesterol per day in 20 normal-weight men with type 2 diabetes treated by insulin in a 16-day trial. Energy intake was individualized to prevent changes in body weight. By the end of the study period, insulin use was discontinued in nine participants and, in the remainder was reduced from a mean of 26 to 11 units per day ($P < 0.001$).

The effect of a low-fat vegan diet on type 2 diabetes was first tested in a small 12-

week pilot study in 1999, which included 11 patients with T2D (134). Fasting plasma glucose decreased 28%, compared to 12% for a more conventional portion-controlled, energy-restricted diabetes diet, and weight loss was also significantly greater in the vegan group (7.2 kg, compared to 3.8 kg). Of six participants in the vegan group on oral hypoglycemic agents, medication use was discontinued in one and reduced in three. Insulin was reduced in both vegan-group participants using insulin. In contrast, none of the control-group participants on oral hypoglycemic agents reduced medication use.

A similar dietary intervention was subsequently tested in 64 healthy (non-diabetic), postmenopausal, overweight women with no energy intake limit. After 14 weeks, weight decreased by 5.8 kg in the low-fat vegan group, compared to a 3.8 kg weight reduction in a control group asked to follow the diet guidelines of the National Cholesterol Education Program ($P = 0.012$) (135). The index of insulin sensitivity increased by 24% in the intervention group, but remained unchanged in the control group. After an additional 2 years of observation, net weight reduction continued to be greater for participants in the low-fat vegan group compared with the control group (-3.1 kg versus -0.8 kg, $P = 0.02$) (136).

In a 22-week randomized trial, 99 individuals with type 2 diabetes were randomly assigned to either a low-fat, low-glycemic-index, vegan diet with no limits on energy or carbohydrate intake and no restrictions on portion sizes, or to a control group, in which each member received individualized diet instruction according to 2003 American DiabetesAssociation (ADA) guidelines (137). Overall, HbA1c decreased by 0.96 percentage points in the vegan group and 0.56 points in the control group ($P = 0.09$). Excluding those who changed medications during the study period,HbA1c decreased 1.2 points in the vegan group, compared to 0.4 points in the ADA group ($P = 0.01$); body weight decreased 6.5 kg in the vegan group and 3.1 kg in the control group ($P < 0.001$).

Following the same patients for an additional year showed that clinical improvements were partially preserved (138). HbA1c changes from baseline to last available value or last value before medication adjustment were -0.40 in the vegan group and +0.01 in the ADA group ($P = 0.03$). Body weight changes, compared to baseline values, were

largely maintained in both the vegan group (-4.4 kg) and the ADA group (-3.0 kg), without a significant between-group difference ($P = 0.25$).

II. OUR RESEARCH

The thesis comprises of 5 related papers that investigate the potential of a vegetarian diet compared to a conventional hypocaloric diet in the treatment of type 2 diabetes. Paper 1 describes the effect of both diets on insulin resistance, visceral fat and oxidative stress markers. Paper 2 explores the potential of both hypocaloric diets to improve -cell function and the connectedness with the secretion of gastrointestinal peptides. Paper 3 compares the effect of both diets on quality of life, mood and eating behaviour. Paper 4 explores the possible mechanisms of the insulin-sensitizing properties of a vegetarian diet, i.e. the effect on the fatty acid profile in membrane phospholipids. Paper 5 presents the results of a post-trial monitoring 1 year after the end of the diet intervention.

5.1. Aims and hypotheses

Vegetarian Diet Improves Insulin Resistance and Oxidative Stress Markers More Than Conventional Diabetic Diet in Subjects with Type 2 Diabetes (Appendix 1)

Aim: The aim of our study was to compare the effects of vegetarian and conventional diabetic diet with the same caloric restriction on insulin resistance, volume of visceral fat and plasma concentrations of oxidative stress markers after a 3-months-diet-intervention and to test whether the positive changes will be sustainable or even augmented after adding aerobic exercise training for other 3 months.

Hypothesis: Our hypothesis was that vegetarian diet would be more effective in reducing insulin resistance and volume of visceral fat and improving oxidative stress markers than conventional diabetic diet and that the difference between groups would enlarge after addition of exercise.

Improvement in -cell function after Diet-induced Weight Loss is Associated with Decrease in Pancreatic Polypeptide in Subjects with Type 2 Diabetes (Appendix 2)

Aim: The aim of our study was to explore the effect of a lifestyle intervention program (12 weeks of diet intervention and subsequent 12 weeks of diet combined with aerobic exercise training) on -cell function and to evaluate the role of GIP and anorectic gut hormones PYY, PP and oxyntomodulin in subjects with T2D.

Hypothesis: Our hypothesis was that a lifestyle intervention program would improve -cell function and this improvement might be related to changes in gastrointestinal peptides.

Vegetarian diet improves quality of life and mood more th an conventional diet in patients with type 2 diabetes (Appendix 3)

Aim: The aim was to study quality of life, Beck depression score and changes in eating behaviour in response to a vegetarian and a conventional diabetic diet.

Hypothesis: Our hypothesis was that both hypocaloric diets would affect the studied parameters similarly.

Beneficial effect of a vegetarian diet on the fatty acid profile in membrane phospholipids in subjects with type 2 diabetes (Appendix 4)

Aim: The aim was to explore the role of changes in fatty acid composition of serum phospholipids in diet-induced changes in MCR in subjects with type 2 diabetes (T2D).

Hypothesis: Our hypothesis was that increased insulin sensitivity induced by a vegetarian diet would be related to changes in th e serum phospholipids fatty acid pattern.

Vegetarian vs. conventional diabetic diet – a 1-year-follow-up (Appendix 5)

Aim: The aim of the post-trial monitoring was to follow-up our patients at 6 months and 1 year from the end of the intervention.

Hypothesis: Our hypothesis was that the vegetarian participants in our study should be able to maintain their reduced weight and improved glycemic control more than the participants consuming a conventional diet.

5.2. Methods

This chapter briefly describes the investigational and analytical methods used in the interventional study (paper 1-4) and in the post-trial monitoring (paper 5).

A 24-week, randomized, open, parallel design was used. Seventy-four patients with Type 2 diabetes were randomly assigned to either the experimental group (n = 37), which received a vegetarian diet, or the control group (n = 37), which received a conventional diabetic diet. Both diets were isocaloric, calorie restricted (-500 kcal∕day). All meals during the study were provided. The second 12 weeks of the diet were combined with aerobic exercise. Participants were examined at baseline, 12 weeks and 24 weeks.

5.2.1. Power analysis for planning of the study:

The power analysis of the repeated measures model was performed using statistical software PASS 2005 (Number Cruncher Statistical Systems, Kaysville, UT, USA; 24-28). The factors included in the model are the between-subject factor (control group vs. experimental group), within-subject factor (individual stages of the trial) and between-factor interaction. The last term expresses the measure of divergence between the courses of the time profiles in control and experimental groups.

Insulin resistance: Assumptions: The insulin resistance in the control group should decrease by 5% within the first 3 months and by 10% within the additional 3-month period, while the declines in the treated group should be 20% within each period. We

assume about 5% standard deviation for repeated sampling and the autocorrelation within the subject reaching the value about 0.7. Result: The probability that 5 subjects per group are sufficient for finding significance at the 95% level of statistical significance for both factors and the between factor interaction is more than 95%.

Weight loss: Assumptions: The weight in the control group should decr ease from 95 kg by 1 kg within each period, while the declines in the treated group should be 3 kg within each period. We assume about 1 % standard deviation for repeated sampling and the autocorrelation wi thin the subject reaching the value about 0.7. Result: The probability that 7 subjects per group are sufficient for finding significance at the 95% level of statistical significance for both factors and the between factor interaction is more than 95%.

Body composition: Assumptions: The ratio of subcutaneous to visceral fat in the control group should be constant within the first 3 months and should decrease by 5% within the additional 3 -month period, while the declines in the experimental group should be 10% and 20% within the first and the second period, respectively. We assume about 10% standard deviation for repeated sampling and the autocorrelation within the subject reaching the value about 0.7. Result: The probability that 19 subjects per group are s ufficient for finding significance at the 95% level of statistical significance for both factors and the between factor interaction is more than 95%.

5.2.2. Subjects

Subjects with Type 2 diabetes treated by oral hypoglycaemic agents were recruited from February to May 2008. Inclusion criteria were: Type 2 diabetes, age 30 –70 years, HbA1c between 6 and 11% (42 –97 mmol ⁄ mol), BMI between 25 and 53 kg ⁄ m2, and willingness to change dietary habits and follow a prescribed exercise program. Exclusion criteria w ere HbA1c < 6% (< 42 mmol ⁄ mol) or > 11% (> 97 mmol ⁄ mol), use of insulin, abuse of alcohol or drugs, pregnancy, lactation, or current use of a vegetarian diet. Out of 161 patients pre -chosen by their endocrinologists, 74 met the inclusion criteria and g ave written informed consent (Fig. 1). Baseline characteristics of the study population is given in Table 1. For detailed characteristics please refer to Appendix 1.

February – May 2008 → 161 persons with T2DM were screened

→ 87 were excluded
32 did not meet inclusion criteria
55 refused to participate

June 2008 → 74 underwent randomization

37 were assigned to vegetarian diet | 37 were assigned to conventional diabetic diet

2 withdrew owing to lack of motivation | 4 withdrew
2 lacked motivation
2 had personal reasons

November - December 2008 → 35 completed 3 months of diet intervention | 35 completed 3 months of diet intervention

4 withdrew owing to lack of motivation | 2 withdrew owing to lack of motivation

February - March 2009 → 31 completed another 3 months of diet and exercise | 31 completed another 3 months of diet and exercise

37 were included in the analysis | 37 were included in the analysis

Figure 1. Enrollment of the Participants and Completion of the Study.

Characteristic	Experimental group (n=37)	Control group (n=37)
Age - years	54.6±7.8	57.7±4.9
Sex - no. (%)		
Male	17 (46)	16 (48)
Female	20 (54)	17 (52)
Smokers - no. (%)	9 (24)	5 (15)
Weight - kg	101.1±17.1	100.8±17.8
BMI - $kg.m^{-2}$	35.1±6.1	35.0±4.6
Waist circumference - cm	113.7±11.2	113.8±13.1
Blood biomarkers		
Cholesterol total - $mmol.l^{-1}$	4.4±0.8	4.2±0.9
LDL-cholesterol - $mmol.l^{-1}$	2.54±0.6	2.57±0.8
HDL-cholesterol - $mmol.l^{-1}$	1.07±0.3	1.09±0.2
Triglycerides - $mmol.l^{-1}$	2.1±0.9	2.1±0.9
HbA1c - %	6.3±1.4	6.2±1.2
Medications		
Oral hypoglycemic agents - no. (%)		
Metformin	29 (78)	28 (85)
Sulfonylurea	20 (54)	13 (39)
Thiazolidinedione	7 (19)	5 (15)
Other	8 (22)	3 (9)
Lipid-lowering therapy - no. (%)	22 (59)	16 (48)
Antihypertensive therapy - no. (%)	25 (68)	22 (67)

Table 1. Baseline characteristics of the study population

5.2.3. Study design

A 24-week, randomized, open, parallel, metabolically controlled des ign was used. The subjects were randomly assigned to either the experimental group (n = 37), which received a vegetarian diet, or the control group (n = 37), which received a conventional diabetic diet. Both diets were designed to be isocaloric and calorie restricted (-500 kcal ⁄ day), with caloric intakes based on the measurement of resting energy expenditure of each subject by indirect calorimetry (metabolic monitor VMAX; Sensor Medics, Anaheim, CA, USA) (139). The second 12 weeks of the diet were combined with aerobic exercise. All participants started with a 1 -week tutorial, where they learned in detail how to compose and prepare their diet. Participants attended weekly 1-h meetings with lectures and cooking classes. All meals duri ng the study were provided. Participants were examined at baseline, 12 weeks and 24 weeks. The study protocol was approved by the Institutional Ethics Committee.

5.2.4. Diet

The vegetarian diet (~60% of energy from carbohydrates, 15% protein and 25% fat) consisted of vegetables, grains, legumes, fruits and nuts. Animal products were limited to maximum of one portion of low-fat yogurt a day. The conventional diabetic diet was administered according to the dietary guidelines of the Diabetes and Nutrition Study Group (DNSG) of the European Association for the Study of Diabetes (EASD). It contained 50% of total energy from carbohydrates, 20% protein, less than 30% fat (7% saturated fat, < 200 mg/day of cholesterol/day).

Vegetarian meals were provided in two vegetarian restaurants and the conventional diabetic diet meals were provided at the Institute for Clinical and Experimental Medicine, Prague. To meet the vitamin B12 needs of the experimental group, while maintaining the same level of intervention in the two groups, vitamin B12 was supplemented in both the experimental group and the control group (50 µg/day). Alcoholic beverages were limited to one per day for women and two per day for men.

5.2.5. Exercise program

Participants were asked not to alter their exercise habits during the first 12 weeks. During weeks 13–24 they were prescribed an individualized exercise program based on their history of physical activity and an initial spiroergometric examination. Participants exercised at 60% of their maximal heart rate twice a week for 1 h under professional supervision, plus once a week at home or at the sports centre with the same intensity; they were given a sport-tester Polar FT4 (Polar, Kempele, Finnland) and a pedometer (Omron HJ-113, Omron, Kyoto, Japan) for individual physical activities and were repeatedly instructed on how to use them.

5.2.6. Compliance

Records of all visits to pick up meals were kept. At weeks 0, 12 and 24, a 3-day dietary record was completed by each participant (two weekdays and one weekend day). A registered dietician analysed all 3-day dietary records using a country-specific food-nutrient database (140). At weeks 3, 8, 14 and 19, a registered dietician made unannounced telephone calls and each participant recalled his or her 24-h diet. This data set was not statistically analysed, but allowed the investigators to check the

adherence and to provide additional counseling. Participants were divided according to their adherence to the prescribed diet into the high, medium or low adherence group. High adherence was defined as the average daily energy intake being no more than 100 kcal in excess of the intake prescribed; medium adherence was less than 200 kcal in excess. If criteria for neither high nor medium adherenc e were met, the participants were included in the low adherence group. An additional criterion for high adherence to the vegetarian diet was the average daily cholesterol intake being £ 50 mg and, for medium adherence, being less than 100 mg. In the contro l group, the average daily cholesterol li mit was less than 200 mg for high adherence and less than 300 mg for medium adherence.

5.2.7. *Physical activity*

Physical activity was assessed by pedometer Omron HJ -113 (Omron, Kyoto, Japan; each participant comple ted a 3-day record, 2 weekdays and 1 weekend day) and with two questionnaires: the International Physical Activity Questionnaire (IPAQ) (141) and the Baecke questionnaire (142) at weeks 0, 12, and 24. Records of each participant's visits at the sports centre were kept. Adherence to the exercise program was defined as more than 75% of prescribed visits at the centre (18/24).

5.2.8. *Medication*

Participants were asked to continue their preexisting medication regimens, except when hypoglycemia occurred repeatedly (fasting plasma glucose determined at the laboratory <4.4 mmol.l^{-1} or capillary glucose reading <3.4 mmol.l^{-1} accompanied by hypoglycemic symptoms). In such cases, medications were reduced by a study physician following the medication protocol. All participants were given an Accu - Chek Go glucometer (Roche, Basel, Switzerland) and were instructed on how to use it.

5.2.9. *Procedures*

All measurements were performed at 0, 12, and 24 weeks on an outpatient basis, after 10-12h overnight fasting with only tap water allowed ad libitum. Height and weight were measured using a periodically calibrated scale accurate to 0.1 kg. Waist circumference was measured with a tape measure placed at the midpoint between the

lowest rib and the upper part of the iliac bone. Blood pressure and heart rate were measured after participants had rested in a seated position for 5 min using a digital M6 Comfort monitor (Omron, Kyoto, Japan). Three measurements were taken at 2 - min intervals. The first measurement was disregarded, and a mean value was calculated for the remaining two measurements.

5.2.9.1. Hyperinsulinemic isoglycemic clamp. The hyperinsulinemic ($1 \text{ mU.kg}^{-1}.\text{min}^{-1}$) isoglycemic clamp, lasting 3 hours, was conducted as previously described (143). Insulin sensitivity was estimated as the metabolic clearance rate of glucose (MCR) calculated during the last 20 min. of the clamp after correction for changes in glucose pool size (143).

5.2.9.2. Meal tests. Insulin secretion was tested after stimulation with standard breakfast (453 kcal, 45% carbohydrates, 17% proteins, 38% lipids). Plasma concentrations of glucose, immunoreactive insulin and C -peptide were measured at 0, 30, 60, 120, and 180 min.

5.2.9.3. Magnetic resonance imaging. 27 water-suppressed magnetic resonance images centered to the intervertebral disc of L2/L3 with TR/TE=450/10 ms and thickness of 10 mm were acquired in breath -hold. The post-processing of MRI with the calculation of subcutaneous and visceral abdominal fat volume was done in MATLAB (The Math Works, Natick, Massachusetts, USA); the inner border of subcutaneous region was detected semi -automatically (144) while the abdominal fat voxels were selected by thresholding.

5.2.10. Analytical methods

Serum glucose was analyzed using the Beckman Analyzer glucose -oxidase method (Beckman Instruments Inc., Fullerton, CA, USA). Plasma immunoreactive i nsulin and C-peptide concentrations were determined using Insulin and C -peptide IRMA kits (Immunotech, Prague, Czech Republic). HbA1c was measured by HPLC (Tosoh, Tokyo, Japan). Plasma lipids concentrations were measured by enzymatic methods (Roche, Basel, Switzerland). HDL -cholesterol was measured after double

precipitation with dextran and MgCl$_2$. LDL-cholesterol was estimated using the Friedewald equation, if triglyceride concentration was within normal limits.

5.2.10.1. Oxidative stress markers. The amount of lipid peroxidation was determined as thiobarbituric acid reactive substances (TBARS) (145) using home-made kits. The activity of superoxide dismutase, catalase, seleno-dependent glutathione peroxidase, and the level of ascorbic acid were analyzed by standard methods (146),(147) using following kits: Superoxide dismutase assay kit (Sigma-Aldrich Corp., St. Louis, MO, USA), Catalase and Glutathione peroxidase assay kits (Cayman Chemical, Ann Arbor, MI, USA), and home-made kits for ascorbic acid. Glutathione reductase activity was measured by the decrease of NADPH (Sigma-Aldrich Corp., St. Louis, MO, USA). The whole blood level of reduced glutathione was determined by HPLC kit Glutathione in whole blood (Chromsystems, Munich, Germany).

5.2.10.2. Adipokines. Plasma concentrations of total adiponectin and resistin were measured using ELISA kits (Raybiotech, Norcross, GA, USA), HMW-adiponectin using ELISA kits (Millipore, Billerica, MA, USA) and leptin using Milliplex (Millipore, Billerica, MA, USA).

5.2.10.3. Gastrointestinal peptides. Plasma concentrations of chosen gastrointestinal peptides were measured in fasting state and after clamp-induced hyperinsulinemia (at 180 min. of hyperinsulinemic isoglycemic clamp). Concentrations of GIP, PP and PYY were measured using Milliplex kits (Millipore, Billerica, MA, USA). Oxyntomodulin was measured using ELISA kits (Millipore, Billerica, MA, USA).

5.2.10.4. Fatty acid composition in serum phospholipids. Serum lipids were extracted according to Folch (148). Lipid classes were separated by thin layer chromatography using hexane-diethylether-acetic acid (80:20:3, v/v) as a solvent system. Fatty acid in serum phospholipids was converted to methyl esters using 1% solution of Na in methanol and the fatty acid methyl esters were eluted with hexane. Gas chromatography of the fatty acid methyl esters was performed on a GS 5890A (Hewlett Packard, USA) instrument equipped with a flame-ionization detector. A carbowax-fused silica capillary column (25m x 0.25 mm i.d.) was used. The column temperature was 150, 225°C (2°C/min), hydrogen was used as the carrier gas.

Individual peaks of fatty acid methyl esters were identified by comparing retention times with those of authentic standards (Sigma, Czech Republic). The composition of serum fatty acid (spectrum of 17 main fatty acid) was analyzed. The product/precursor ratios of the serum fatty acid were used to calculate indices reflecting the activities of enzymes involved in hepatic fatty acid metabolism: elongase (18:0/16:0), D6 desaturase (18:3n8/18:2n6), D5 desaturase (20:4n6/20:3n6, and D9 desaturase (16:1n7/16:0) (149,150).

5.2.11. Modeling analysis of -cell function

Insulin secretory rates (ISRs) were calculated from plasma C -peptide levels by deconvolution (151) and expressed per square meter of estimated body surface area. The dependence of ISR on glucose le vels was modeled separately for each patient and each study day. The -cell model used in the present study, describing the relationship between insulin secretion and glucose concentration, has been described previously in detail (152,153).

Insulin secretion consists of two components. The first component represents the dependence of insulin secretion on absolute glucose con centration (G) at any time point and is characterized by a dose -response function, f(G), relating the two variables. Characteristic parameters of the dose response are insulin secretion at a fixed glucose concentration of 9 mmol/litre (approximately the fa sting glucose level in diabetic subjects) and the mean slope in the observed glucose range. The dose response is modulated by a potentiation factor, P(t), which accounts for several potentiating agents (prolonged exposure to hyperglycemia, nonglucose subst rates, gastrointestinal hormones, and neurotransmitters). The first secretion component is thus the product, P(t)f(G).

The potentiation factor is set to be a positive function of time and to average one during the experiment. It thus expresses a relative potentiation of the secretory response to glucose. The excursion of the potentiation factor was quantified using ratios between mean values at times 160 –180 and 0–20 min.

The second insulin secretion component represents a dynamic dependence of insulin

secretion on the rate of change of glucose concentration. This component is termed the derivative component and is determined

by a single parameter. Rate sensitivity is related to early insulin release (152,153).

The model parameters [the parameters of the dose response, f(G), and the potentiation factor, P(t)] were estimated from glucose and C-peptide concentration by regularized least squares, as described previously (154,155). Regularization involves the choice of smoothing factors that were selected to obtain glucose and C-peptide model residuals with SDs close to the expected measurement error (1% for glucose and 5% for C-peptide). Estimation of the individual model parameters was performed blinded to the randomization of patients for treatment.

5.2.12. Assessment of quality of life, mood and eating behaviour

Quality of life was assessed using 2 questionnaires: Weight-Loss Quality-of-Life (OWLQOL) and Weight-Related Symptoms (WRSM) (156). We used the Three-Factor Eating Questionnaire (157) to monitor changes in eating behaviour and the Beck Depression Inventory to screen for depressive symptoms (158).

5.12.13. Follow-up

62 patients (31 from V and 31 from C) who completed the study were invited for a follow-up 6 at months and 1 year after the end of the intervention. 47 patients (76%; 23 from V and 24 from C) attended the 6-month-follow-up, 44 (71%; 21 from V and 23 from C) attended the 1-year-follow-up. During one year after the end of the intervention, the patients had discontinued the original diet intervention and they consumed comparable diets. We measured their weight, waist circumference, HbA1c and blood lipids. The patients completed a 3-day dietary record and their physical activity was assessed by a Omron HJ-113 pedometer (Omron, Kyoto, Japan).

5.2.14. Statistical analyses

The intention-to-treat analysis included all participants. For evaluation of the relationships between continuous variables and factors we used a repeated measures

ANOVA model with between-subject and within-subject factors and interactions. Factors group, subject and time were included in the model. Interaction between group and time (group x time) was calculated for each variable. Within each group, paired comparison t tests were calculated to test whether the changes from baseline to three months, from baseline to six months and from three to six months were statistically significant. Pearson correlations were calculated for the relationship between dependent and independent variables.

5.3. Results

<u>Vegetarian Diet Improves Insulin Resistance and Oxidative Stress Markers More Than Conventional Diabetic Diet in Subjects with Type 2 Diabetes (Appendix 1)</u>

92% of the participants completed the first three months (95% in EG, and 89% in CG); 84% of participants in each group completed all six months. Adherence to the prescribed diet at six months was high among 55% participants in EG and 32% in CG, medium among 22.5% in EG and 39% in CG, and low among 22.5% in EG and 29% in CG. Pedometer readings and self-reported energy expenditure showed no significant between-group differences. Adherence to the prescribed exercise program was 85.5% (90.3% in EG and 80.6% in CG).

5.3.1. Dietary intake

Both groups reduced energy intake ($p<0.001$ for each group). Percentage of consumed carbohydrates (out of daily caloric intake) increase d in EG ($p=0.002$). Percentage of consumed fats decreased in both groups ($p=0.03$). Percentage of consumed proteins decreased in EG ($p<0.001$). Cholesterol intake decreased in EG ($p<0.001$). For details of baseline dietary intake and its changes please refer to Appendix 1.

5.3.2. Glycemic control, insulin sensitivity

Diabetes medication was reduced in cases of repeated hypoglycemia in 43% of EG participants and in 5% of CG participants. HbA1c fell in both groups during the first 12 weeks ($p<0.001$). It remained reduced after exercise. Decrease from baseline to six

months was significant only in EG (-0.65±1%; p=0.002 vs. -0.21±1.1%; ns. in CG), however the difference between groups was not statistically significant. Metabolic clearance rate of glucose (MCR) increa sed in both groups during the first 12 weeks (p<0.001 for each group). After exercise there were insignificant trends toward increase in EG and toward decrease in CG. MCR increased more in EG from baseline to six months (by 30% vs. 20% in CG; group x time p=0.04; Fig. 2F).

5.3.3. Body weight and abdominal fat

Body weight decreased in both groups after diet intervention (p<0.001) and it was maintained after exercise. Weight loss was greater in EG (-6.2±5.8 kg vs. -3.2±4.5 kg in CG; group x time p=0.001; Fig. 2A). Waist circumference decreased in both groups after diet intervention (p<0.001), more in EG (-6.4±3.5 cm in EG vs. -5.3±3.5 cm in CG; group x time p=0.001). After exercise it further decreased in EG (-1.9±3 cm; p<0.01) whereas it remained unchanged in CG (+0.72±3.8 cm; ns). Volume of subcutaneous fat decreased in both groups after diet intervention (p<0.001). After exercise it further decreased in EG by 2% (p<0.05) whereas it insignificantly increased in CG by 2% (p=0.06; group x time p=0.02; Fig. 2D). Volume of visceral fat decreased in both groups after diet intervention (p<0.001). After exercise it further decreased in EG by 4%, whereas it remained unchanged in CG (group x time p=0.007; Fig. 2E).

5.3.4. Oxidative stress markers

Plasma concentrations of vitamin C increased by 16% in EG after diet intervention (p=0.002) and remained elevated after exercise whereas changes were not significant in CG (group x time p=0.002; Fig. 3A). Superoxide dismutase increased in EG in successive steps by 49% (p<0.001) whereas in CG it gradually decreased by 30% (p<0.001; group x time p<0.001; Fig. 3B). Catalase increased in both groups (p<0.01). TBARS decreased in both groups (p<0.001). Reduced glutathione increased in EG gradually by 27% (p=0.02; Fig. 3C) whereas it decreased in CG by 11% (p=0.05; group x time p<0.001). Glutathione reductase decreased in EG gradually by 42% (p<0.001; Fig. 3D) while glutathione peroxidase increased in CG by 20%

(p<0.001; Fig. 3E) and glutathione transferase increased in both groups, mo re in CG (by 59% vs. 14% in EG; group x time p=0.003; Fig. 3F).
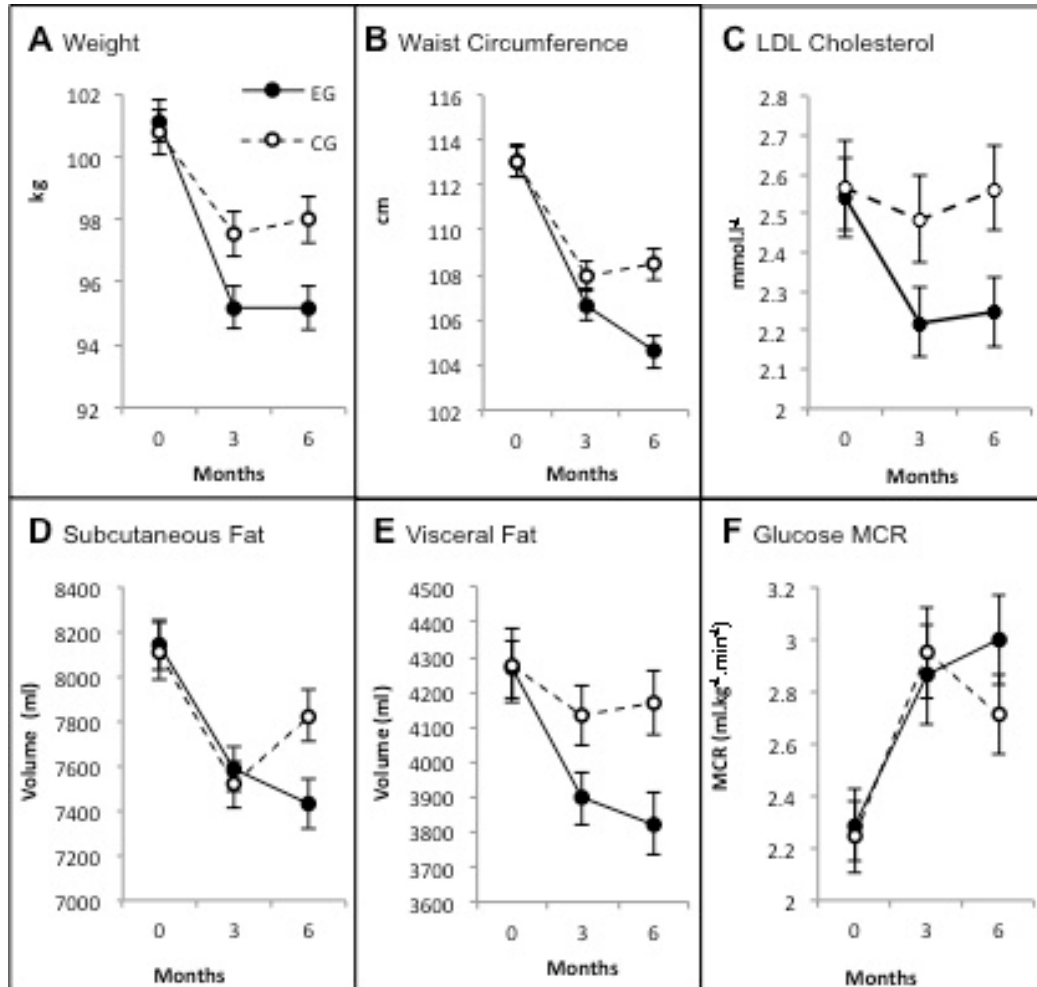
Figure 2. Anthropometric Parameters, LDL -cholesterol and Insulin Sensitivity during the Study. Error bars represent standard error of the mean. P -values for interaction between group a nd time assessed by repeated measures ANOVA are p<0.001 for Weight (A), p<0.001 for Waist Circumference (B), p=0.05 for LDL-cholesterol (C), p=0.02 for Subcutaneous Fat (D), p=0.007 for Visceral Fat (E), p=0.04 for Metabolic Clearance Rate of Glucose (F).

5.3.5. Adipokines

Plasma concentrations of both total and HMW adiponectin increased in EG by 19% and 15%, respectively from baseline to six months while it did not change significantly in CG (group x time p=0.02; Fig. 3G and group x time p=0.05, respectively). Resistin decreased after diet intervention in EG by 19% and remained reduced after exercise whereas it did not change in CG first (week 12) and after exercise it increased by 24% (p=0.01; group x time p=0.005; Fig. 3H). Leptin decreased similarly in both groups after diet intervention. It increased back in CG after exercise. Decrease from baseline to six months was significant only in EG (by 35%; p=0.02; group x time p=0.05; Fig. 3I).

5.3.6. Risk factors of atherosclerosis

LDL-cholesterol decreased by 8% in EG after diet intervention (p=0.05) and remained reduced after exercise while it did not change in CG (group x time p=0.05; Fig. 2C). HDL-cholesterol increased by 5% in CG from baseline to six months (p<0.01). It increased by 6% in EG after exerci se training (p=0.02; group x time p=0.07). Fibrinogen decreased in both groups after exercise training (p=0.02 for EG and p=0.04 for CG).

5.3.7. Regression analysis, correlations

Regression analysis showed that changes in volume of visceral fat volume wer e strongly associated with changes in MCR and plasma concentrations of enzymatic oxidative stress markers; each kilogram of lost visceral fat was associated with an increase in MCR by 1.2 ml.kg^{-1}.min^{-1}, with an increase in superoxide dismutase by 1.7 U.ml^{-1} and with an increase in reduced glutathione by 0.9 mmol.l^{-1}. The Pearson's correlation of MCR change with change in volume of visceral fat was r= -0.63; p<0.001. Correlation of changes in superoxide dismutase and reduced glutathione with changes in volume of visceral fat was r=-0.55; p<0.001and r=-0.45; p=0.02, respectively.
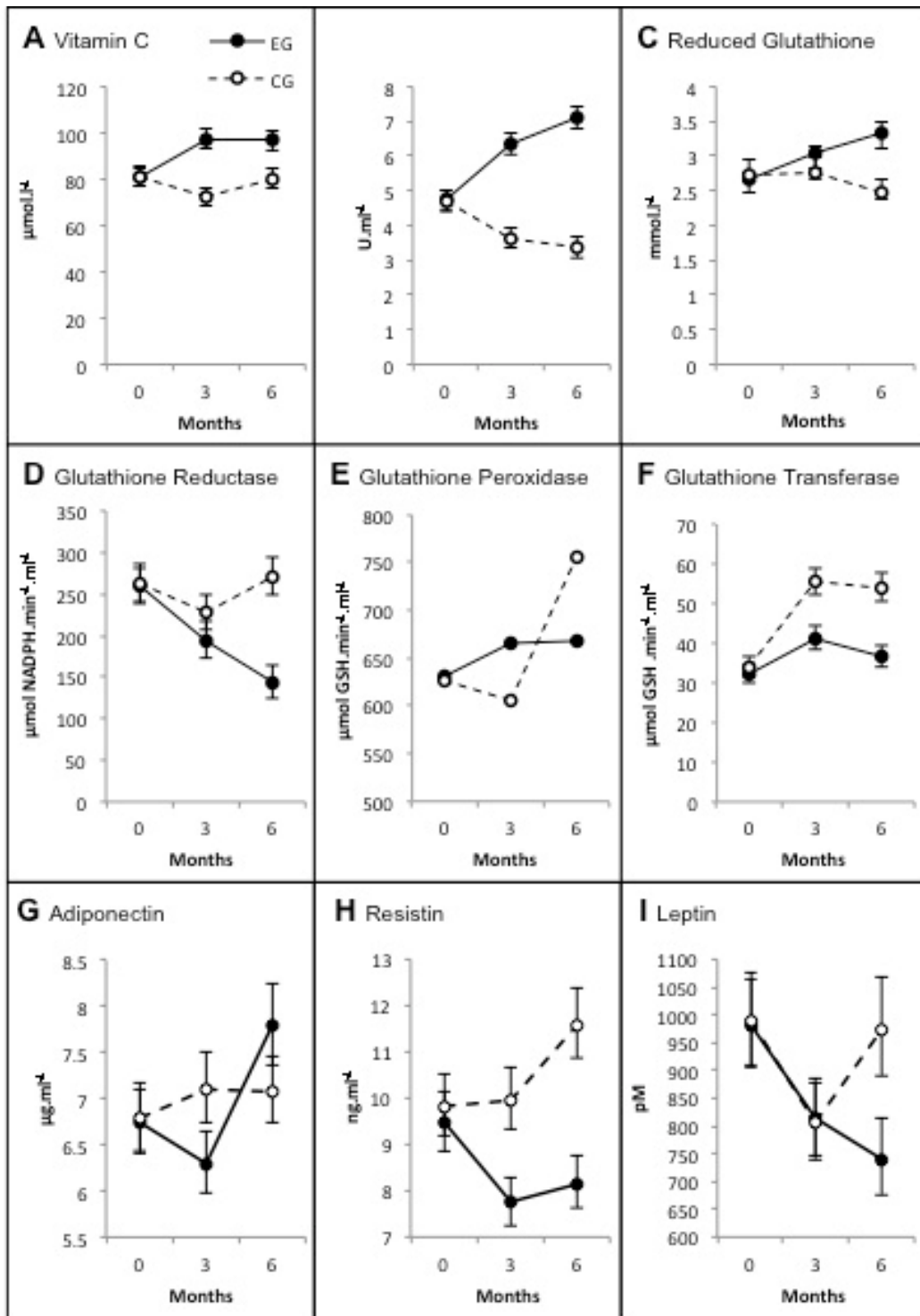
Figure 3. Plasma Levels of Oxidative Stress Markers and Adipokines During the Study. Error bars represent standard error of the mean. P -values for interaction between group and ti me assessed by repeated measures ANOVA are P=0.002 for Vitamin C (A), p<0.001 for Superoxide dismutase (B),

p<0.001 for Reduced Glutathione (C), p<0.001 for Glutathione Reductase (D), P=0.004 for Glutathione Peroxidase (E), P=0.003 for Glutathione Transfe rase (F), P=0.02 for Adiponectin (G), P=0.005 for Resistin (H) and P=0.05 for Leptin (I).

Improvement in -cell function after Diet-induced Weight Loss is Associated with Decrease in Pancreatic Polypeptide in Subjects with Type 2 Diabetes (Appendix 2)

5.3.8. Glucose, immunoreactive insulin, C -peptide

Changes in plasma concentrations of glucose , immunoreactive insulin and C-peptide after standard breakfast are shown in Fig. 4. Both fasting and stimulated plasma concentrations of glucose decreased in the first 12 weeks (p<0.001). Further decrease of glucose after the addition of exercise was sign ificant only at 120 minutes of the meal test (p=0.04), but the decrease of stimulated plasma concentrations of glucose as a whole was not significant (p=0.08). Both fasting and stimulated plasma concentrations of immunoreactive insulin decreased in the fir st 12 weeks (p<0.001). After the addition of exercise stimulated plasma concentrations of immunoreactive insulin further decreased (p=0.02) due to decrease in concentrations at 120 and 180 minutes. Both fasting and stimulated plasma concentrations of C -peptide decreased in the first 12 weeks (p<0.001). After the addition of exercise, stimulated plasma concentrations of C-peptide further decreased (p=0.01) due to decrease in concentrations at 120 and 180 minutes.
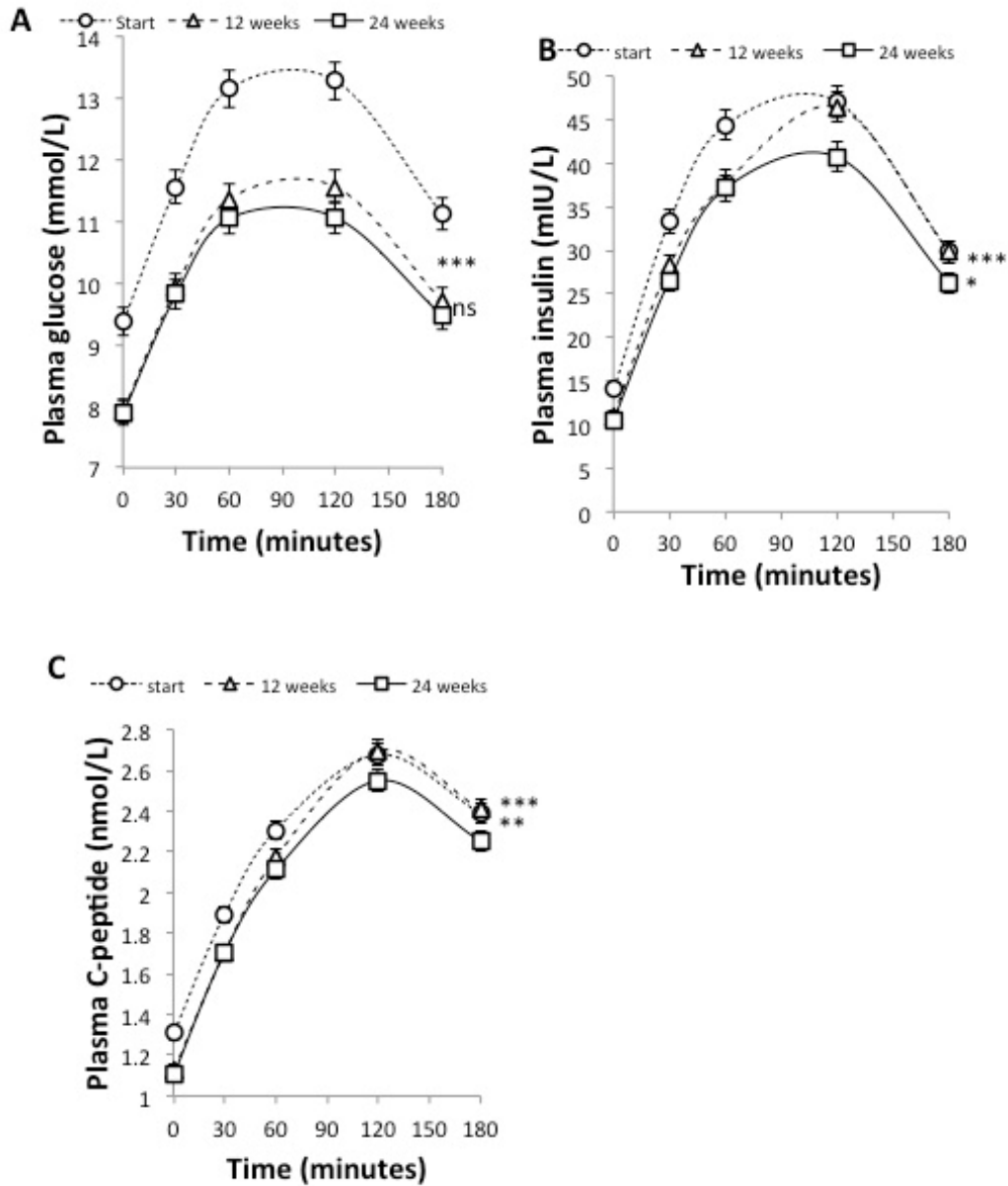
Figure 4. Plasma concentrations after standard breakfast at start (circles, dotted line), 12 weeks (triangles, dashed line), and 24 weeks (sqauares, full line). Error bars represent 95% CIs. P-values: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. A: Glucose. $F(64;2) = 217.6$; $p<0.001$. B: Immunoreactive ins ulin. $F(64;2) = 350.6$; $p<0.001$. C: C-peptide. $F(64;2) = 547.7$; $p<0.001$.

5.3.9. -cell function

Changes in parameters of -cell function during the study are shown in Fig. 5. Insulin secretion at glucose level 9 mmol/l increased by 33% in the first 12 weeks (p<0.001; from 184 pmol.min^{-1}.m^{-2} [95% CI, 169 to 201] to 244 pmol.min^{-1}.m^{-2} [95% CI, 226 to 264]) and remained unchanged after addition of exercise (244 pmol.min^{-1}.m^{-2} [95% CI, 224 to 264]). Glucose sensitivity increased by 26% in the first 12 weeks (p<0.001; from 42 pmol.min^{-1}.m^{-2}.mM^{-1} [95% CI, 39 to 46] to 53 pmol.min^{-1}.m^{-2}.mM^{-1} [95% CI, 49 to 58]) and remained unchanged after the addition of exercise (56 pmol.min^{-1}.m^{-2}.mM^{-1} [95% CI, 51 to 61]). Neither rate sensitivity nor excursion of potentiation factor changed significantly during the study.

5.3.10. Gastrointestinal peptides

The concentrations of gastrointestinal peptides at baseline, week 12 and 24 are shown in Table 2 in Appendix 2. Fasting plasma concentration of PP decreased by 17% from start to 12 weeks (p=0.03) and it did not change significantly from week 12 to week 24. Fasting plasma concentration of GIP did not change significantly from start to 12 weeks; however, it decreased by 19% from start to 24 weeks (p=0.002). Fasting plasma concentrations of oxyntomodulin and PYY did not change significantly during the study.

After clamp-induced hyperinsulinemia (evaluating all the clamps), plasma concentrations of GIP decreased by 14% (p=0.02), PYY decreased by 12% (p=0.02), and PP increased by 20% (p=0.005). Plasma concentrations of oxyntomodulin did not change after hyperinsulinemia.

Plasma concentration of PP after clamp -induced hyperinsulinemia decreased from start to 12 weeks (p=0.01). It did not change significantly from week 12 to week 24. Changes in plasma concentrations of no other chosen gastointestinal peptid e after clamp-induced hyperinsulinemia were significant.
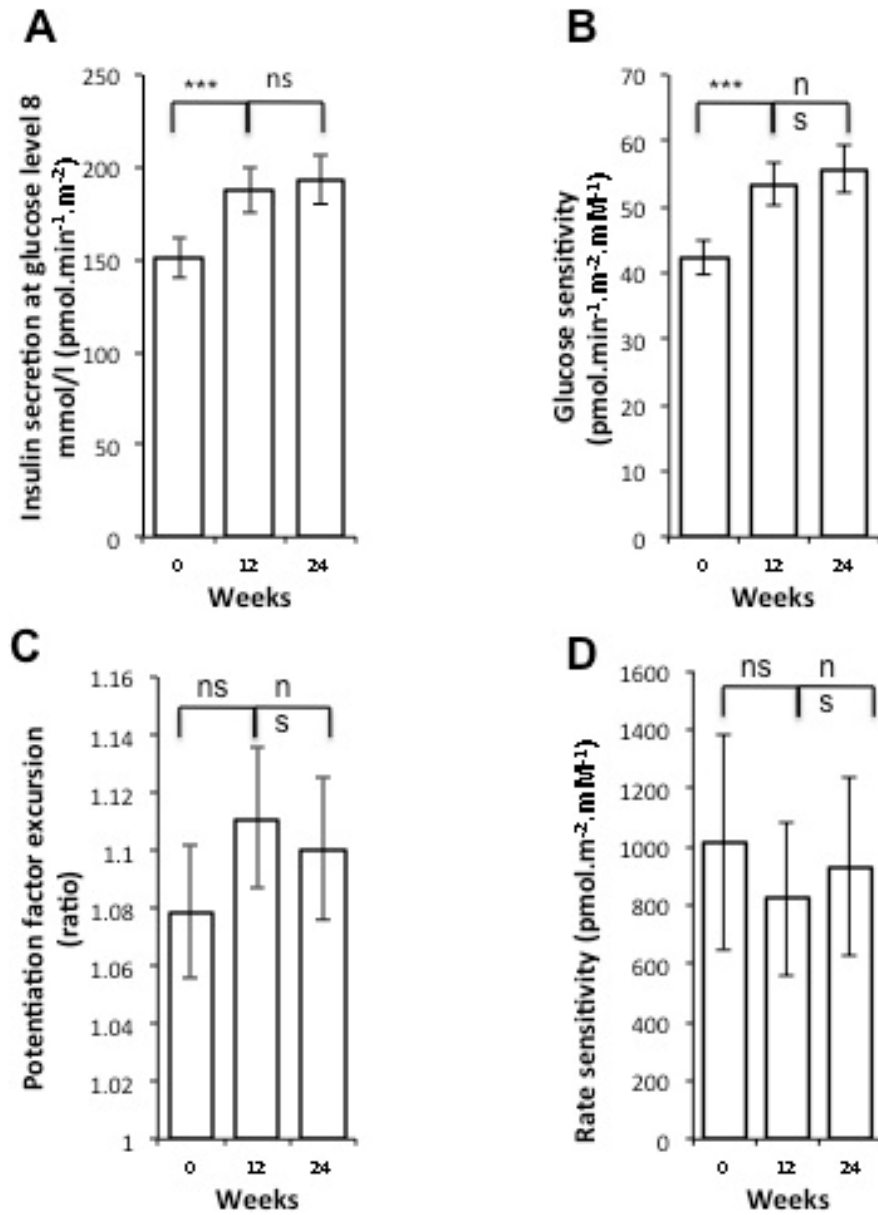
Figure 5. Parameters of β-cell function. Error bars represent 95% CIs. P-values: *** p<0.001. A: Insulin secretion at glucose level 9 mmol/l. F(64;2) = 7.5; p<0.001. B: Glucose sensitivity. F (64;2) = 10.8; p<0.001. C: Metabolic clearance rate of glucose (MCR). F(64;2) = 6.5; p<0.01.

5.3.11. Correlations between changes in parameters of -cell function and changes in gastrointestinal peptides

Changes in insulin secretion at the reference le vel correlated negatively with changes in plasma concentrations of PP during hyperinsulinemia (r= -0.36; p<0.001; after adjustment for changes in BMI: r= -0.31; p=0.001; Fig. 6A) and with changes in volume of visceral fat (r=-0.22; p=0.04). Glucose sensitivi ty correlated negatively with plasma concentrations of PP - both fasting and during hyperinsulinemia (r= -0.21; p=0.01 and r=-0.22; p=0.01, respecively; after adjustment for changes in BMI: r= -0.23; p=0.007 and r=-0.2; p=0.04, respectively; Fig. 6 B and C). No correlation was found between duration of diabetes and improvement in either parameter of -cell function.

Changes in volume of subcutaneous fat correlated positively with changes in fasting plasma concentrations of PP (r=+0.41; p<0.001) and with chang es in fasting plasma concentrations of PYY (r=+0.39; p=0.001). Changes in volume of visceral fat did not correlate with changes in plasma concentrations of any measured gastrointestinal peptide.
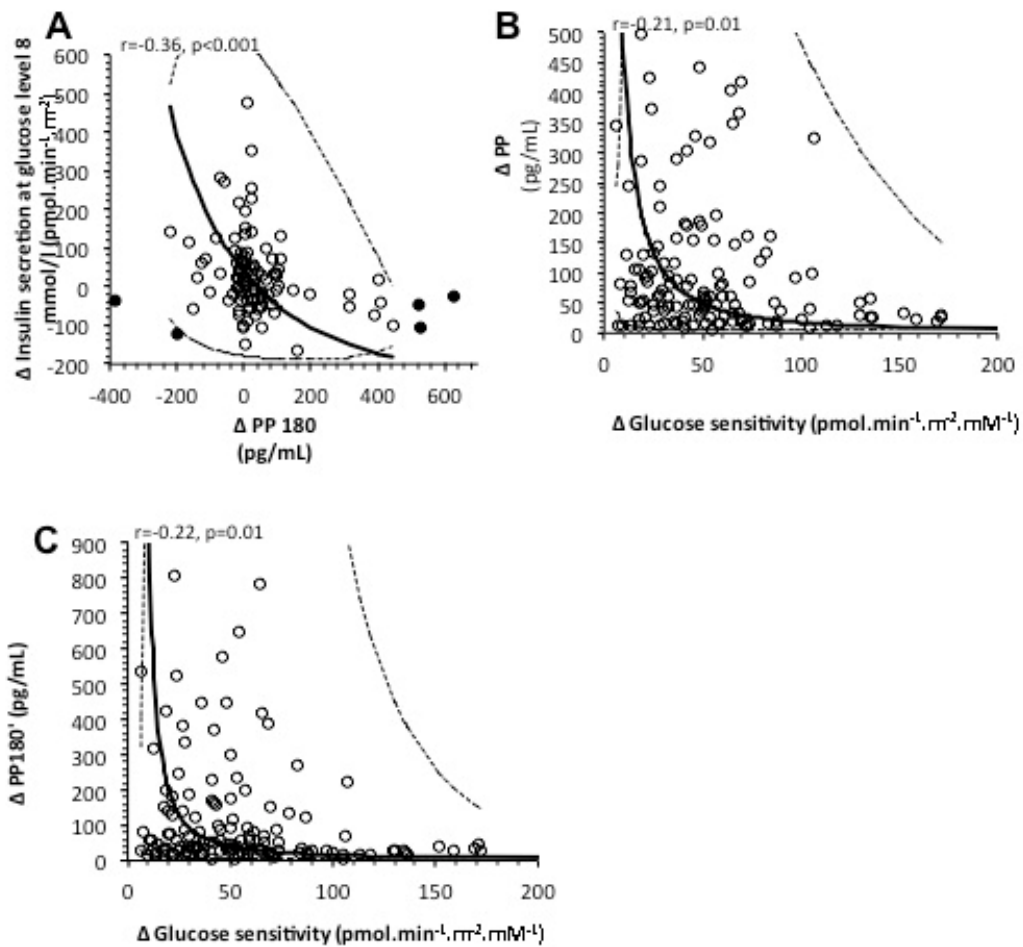
Figure 6. Correlations between changes in parameters of -cell function and changes in gastroitestinal peptides. Full line… the main correlation axis, dotted line…the 95% confidence ellipsoid. A: Correlation between changes in insulin secretion at 8 mmol/l and changes in plasma concentrations of pancreatic polypeptide (PP) during hyperinsulinemia; r= -0.36, p< 0.001; B: Correlation between changes in glucose sensitivity and changes in fasting plasma concentrations of PP; r=-0.21, p= 0.01; C: Correlation between changes in glucose sensitivity and changes in plasma concentrations of PP during hyperinsulinemia; r= -0.22, p= 0.01.

<u>Vegetarian diet improves quality of life and mood more than conventional diet in patients with type 2 diabetes (Appendix 3)</u>

5.3.12. Quality of life and Beck Depression Inventory

The parameters of quality of life, Beck Depression Inventory and Three -Factor Eating Questionnaire are shown in Fig. 7 . Quality of life (the OWLQoL score) increased in both groups comparably in weeks 12 -0. It further increased in VG in weeks 24 -12 while it did not change significantly in CG. Quality of life increased more in VG from baseline to 24 weeks (group x time p=0.01 ; Fig. 7A). The negative weight-related symptoms (WRSM) decreased in both groups in weeks 12 -0 and remained reduced in weeks 24-12 (Fig. 7B). The Beck depression score decreased in both groups in weeks 12-0, but the decrease was significant only in VG from baseline to 24 weeks (p<0.001; group x time p=0.03 ; Fig. 7C).

5.3.13. Eating behaviour

Dietary restraint increased in both groups in weeks 12-0, more in CG, and did not change significantly in weeks 24 -12 in either group (group x time p=0.04 ; Fig. 7D). Disinhibition decreased in both groups in weeks 12 -0, but the decrease was significant only in VG from baseline to 24 weeks (p<0.001; group x time p=0.01 ; Fig. 7E). Feelings of hunger decreased in both groups in weeks 12 -0 with a trend toward a greater decrease in VG and did not change significantly in weeks 24 -12 in either group (Fig. 7F).
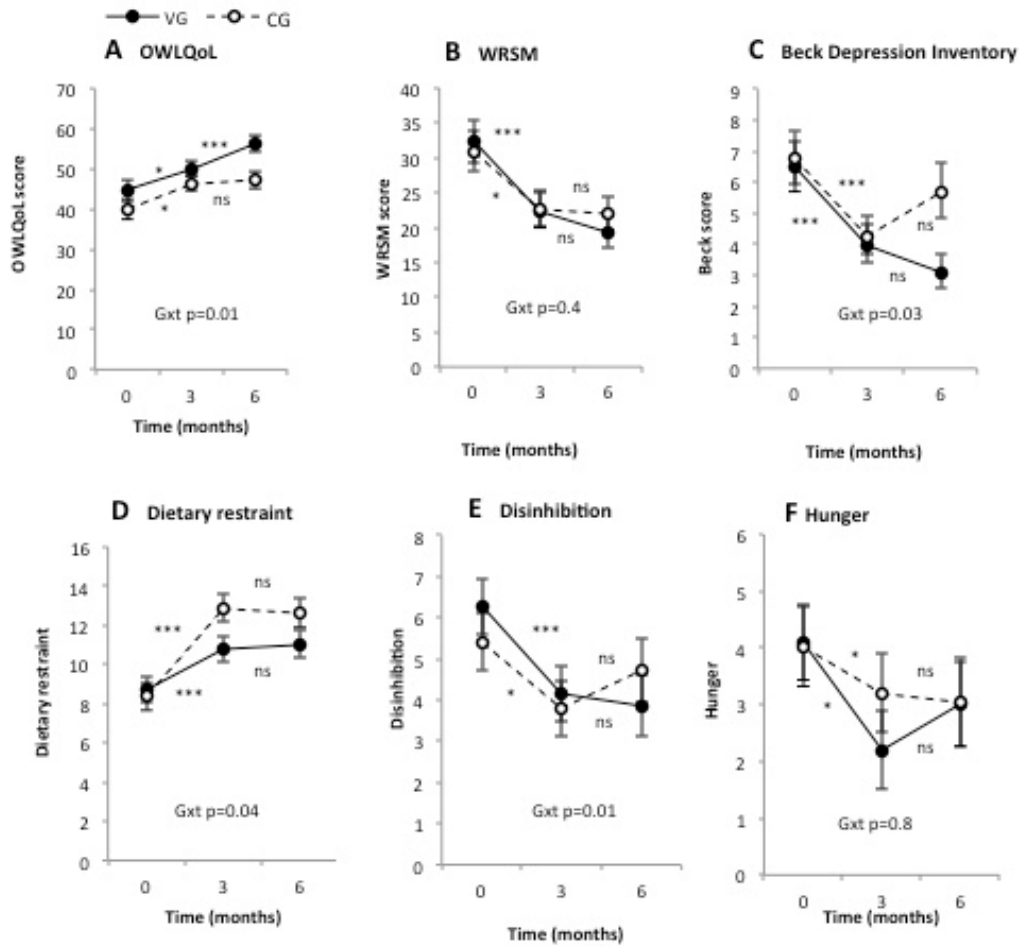
Fig.7. Quality of life, Beck Depression Inventory and Three-Factor Eating Questionnaire. Data are means ± 95% Confidence intervals. Significant changes from baseline to 12 weeks and from 12 to 24 weeks for within-group changes assessed by paired comparison t-tests are indicated by * for p<0.05, ** for p<0.01, and *** for p<0.001. Gxt … p-value for the interaction between group (vegetarian and control group) and time (0,12 and 24 weeks) assessed by repeated measures ANOVA.

<u>Beneficial effect of a vegetarian diet on the fatty acid profile in me mbrane</u>
<u>phospholipids in subjects with type 2 diabetes (Appendix 4)</u>

5.3.13. Fatty acid composition in serum phospholipids
Relative contents of all measured fatty acids in serum phospholipids at weeks 0, 12
and 24 in both groups are shown in Table 2 in Appendix 4.

N6 polyunsaturated fatty acids *(the sum of 18:2n6, 18:3n6, 20:2n6, 20:3n6, 20:4n6
and 22:4n6)* did not change in either group in response to dietary interventions. They
decreased after the addition of exercise training in VG (p<0.001) while the tr end
toward decrease in CG was insignificant. There were no significant differences in the
total n-6 polyunsaturated fatty acids between the groups. Patients in the VG exhibited
increased content of linoleic acid (18:2n6) by 10% compared to the CG (group x time
p<0.001; Figure 8A).

N3 polyunsaturated fatty acids *(the sum of 18:3n3, 20:5n3, 22:5n3 and 22:6n3)* did
not change in VG in response to dietary intervention while there was a insignificant
trend toward an increase in CG. After the addition of exercise , they decreased in VG
(p<0.001) while the t rend toward a decrease in CG was insignificant.

Monounsaturated fatty acids *(the sum of 16:1n7, 18:1n9, 18:1n7, 20:1n9)* did not
change in either group in either period.

Saturated fatty acids *(the sum of 14:00, 16:00, 18:00 and 20:00)* did not change in
either group in response to dietary interventions. After the addition of exercise both
groups exhibited significantly increased content of saturated fatty acids, mainly the
palmitic acid (16:00). However, stearic a cid (18:00) was decreased in the VG after the
addition of exercise.

The ratio of saturated to unsaturated fatty acids did not change in either group in
response to dietary interventions. After the addition of exercise it increased in VG
(p<0.001) while the increase in CG was not significant.

The enzymes:

There were no significant changes in fatty acid enzyme activity in response to dietary intervention in either group. However, after the addition of exercise, decreased activity of elongase (p<0.01) and increased activity of 9 desaturase (p=0.003) were observed in both groups.

Correlations

In VG, changes in the linoleic acid (18:2 n6) correlated positively with changes in metabolic clearance rate of glucose (r=+0.22; p=0.04; Fig. 8B) and negatively with changes in volume of visceral fat (r=-0.43; p=0.01; Fig. 1C). After adjustment for changes in BMI, the association between linoleic acid and metabolic clearance rate of glucose was no longer significant. Furthermore, changes in the linoleic acid (18:2 n6) correlated positively with changes in HDL -cholesterol (r=+0.36; p=0.01). The correlation between changes in the 18:2 n6 and neither triglycerides, total cholesterol nor LDL-cholesterol was significant (p=0.07; p=0.08 and p=0.27, respectively).

In CG, changes in the docosapentaenoic acid (20:5 n3) correlated positively with changes in metabolic clearance rate of glucose (r=+0.2; p=0.05) and negatively with changes in volume of visceral fat (r=-0.36; p=0.03).
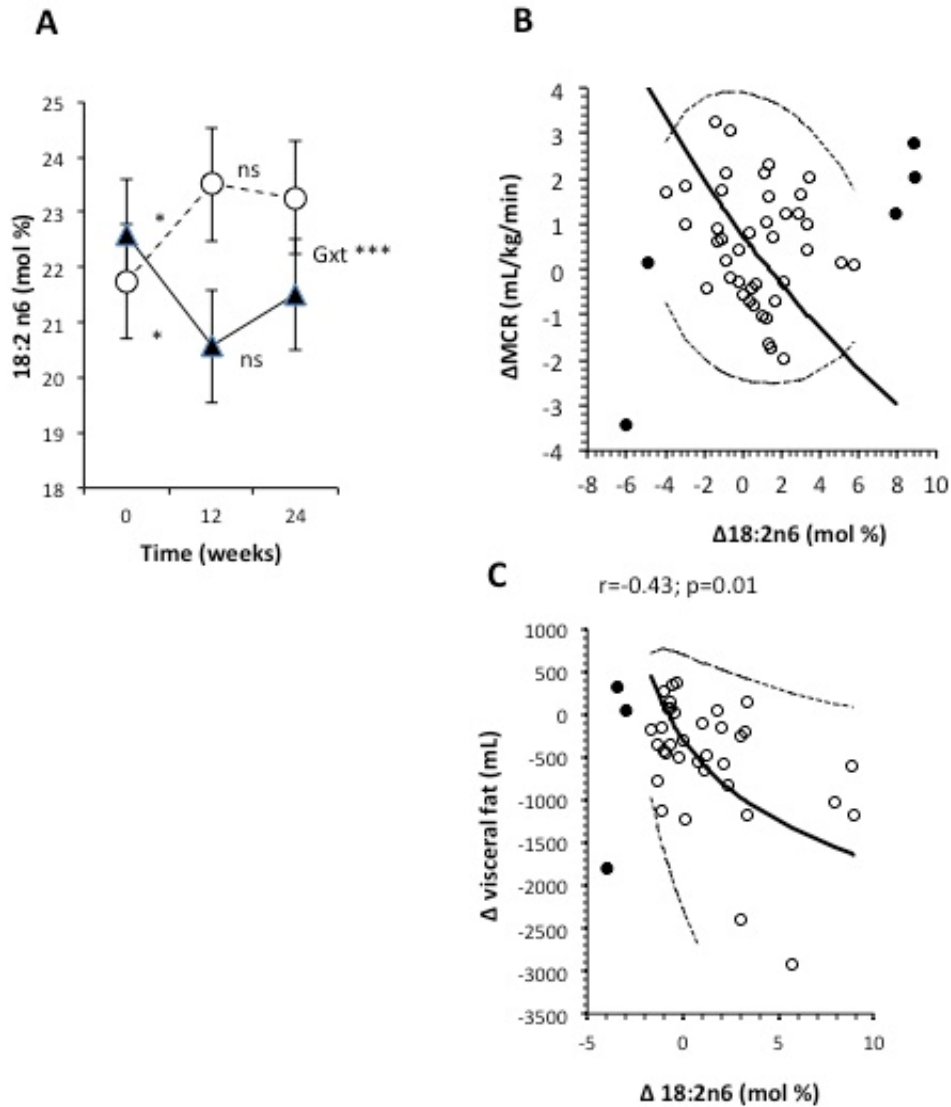
Figure 8. A: The content of linoleic acid (18:2 n6) in serum phospholipids at start, 12 weeks and 24 weeks. The control group (CG): triangles, full line; the vegetarian group (VG): circles, dashed line. Error bars represent 95% CIs. P -values: * $p < 0.05$, *** $p < 0.001$. Gxt... p value for interaction group x time. B: Correlation between changes in linoleic acid (18:2 n6) and changes in MCR (Metabolic clearance rate of glucose) in VG. Full line… the main correlation axis, dotted line… the 95% confidence ellipsoid. C: Correlation between changes in linoleic a cid (18:2 n6) and changes in volume of visceral fat in VG. Full line… the main correlation axis, dotted line… the 95% confidence ellipsoid.

Vegetarian vs. conventional diabetic diet – a 1-year-follow-up (Appendix 5)

5.3.15. Diet and physical activity

Parameters of dietary intake and pedometer readings are given in Table 1 in Appendix 5. We did not observe any differences between the groups in either parameter.

5.3.16. Oral hypoglycemic agents, which were reduced in 43% of the participants in V vs. 5% in C during the intervention, had to be increased in 14% (3/21) in V and in 26% (6/23) in C during 1 year after the end of the intervention. Insulin therapy was started in 5% (1/21) in V and in 13% (3/23) in C (Fig. 9 A).

5.3.17. Body weight, which was reduced more in V during the intervention (-6.2±5.8 kg vs. -3.2±4.5 kg in C; group x time p=0.001), increased (p 0.05) slightly 6 months after the intervention in both groups (+1.7±3.1 kg in V and +1.5±3.1 kg in C; group x time p 0.05). One year after the intervention, the trend toward weight gain was not statistically significant in either group (Fig. 9 B).

5.3.18. Waist circumference, which was reduced more in V during the intervention (-8.7±4.7 cm vs. -4.7±4.7 cm in C; group x time p=0.001), increased 6 months after the end of the intervention in V (+1.9±2.7 cm; p<0.01), while the trend toward increase was not statistically significant in C (group x time p 0.01). One year after the intervention, the trend toward increase was not statistically sig nificant in either group (Fig. 9C).

5.3.19. HbA1c, which was reduced more in V during the intervention (-0.7±1% vs. -0.2±1% in C; group x time p=0.08), increased 6 months after the end of the intervention in V (+0.7±0.9 %; p<0.01), while the trend towards an increase was not statistically significant in C. One year after the int ervention, the increase in HbA1c was comparable in both groups (+0.49±1.04%; p =0.05 in V vs. +0.42±0.8%; p<0.05 in C; group x time p=0.31; Fig. 9 D).

5.3.20. Blood lipids: Hypolipidemic agents were discontinued after the end of the intervention in 29% pat ients (6/21) in V and in 0% (0/23) in C. LDL cholesterol,

which decreased during the study only in V (-0.2±0.6 mmol/l; p=0.05), did not change significantly in either group either 6 months or 1 year after the end of the intervention, although a trend toward an increase was evident in both groups (Fig. 9E). Total cholesterol, HDL-cholesterol nor triglycerides changed significantly in either group either at 6 months or 1 year after the end of the intervention .
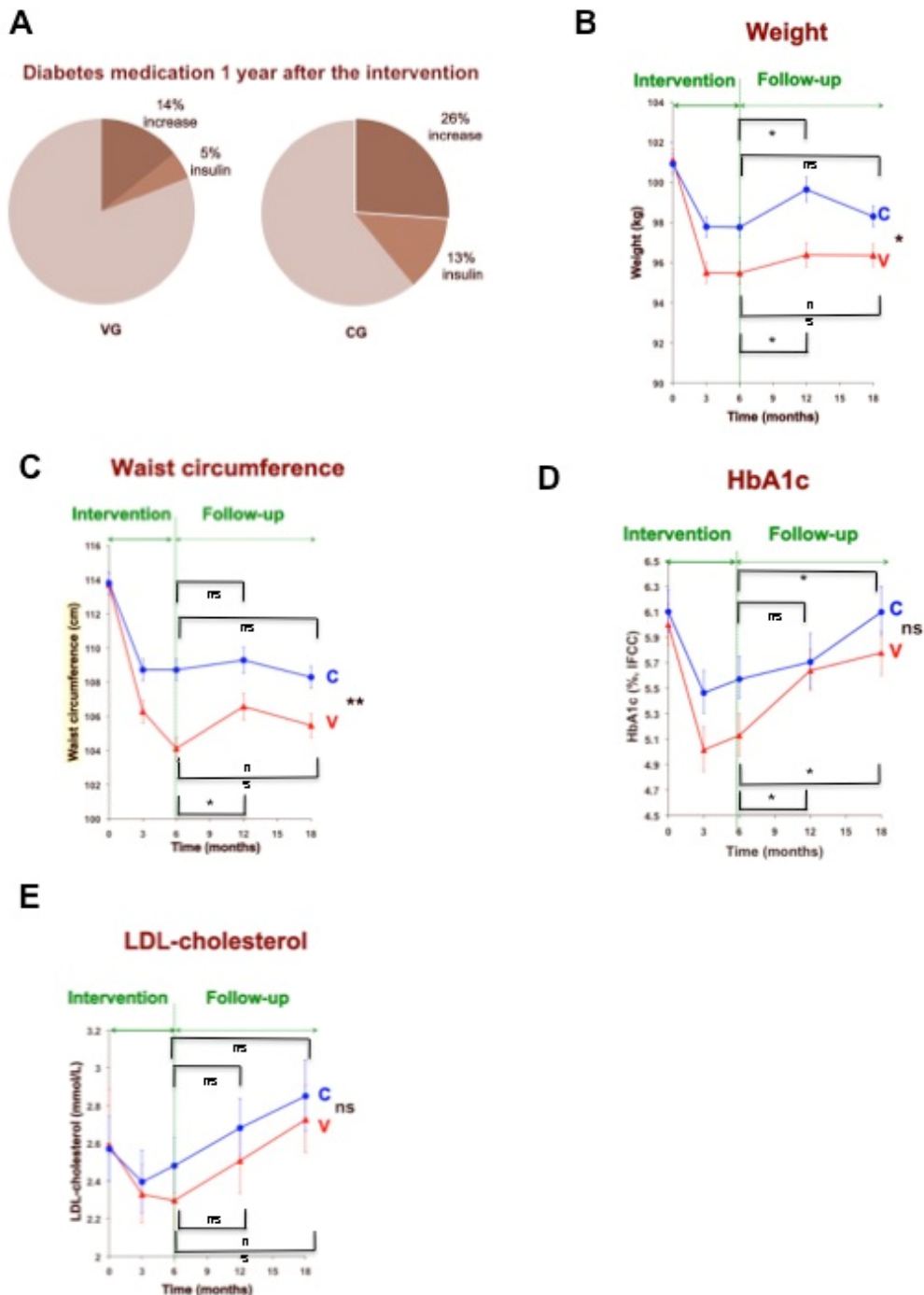
Figure 9. Diabetes medication, anthropometr ic and laboratory parameters during and 1 year after the intervention. Error bars represent 95% CIs. P -values for interaction between group and time: * p< 0.05, ** p< 0.01, *** p< 0.001. A: Diabetes medication 1 year after the intervention, B: Weight, C: Waist circumference, D: HbA1c, E: LDL -cholesterol.

5.4. Discussion

<u>Vegetarian Diet Improves Insulin Resistance and Oxidative Stress Markers More Than Conventional Diabetic Diet in Subjects with Type 2 Diabetes (Appendix 1)</u>

We found that vegetarian diet increased insulin sensitivity, reduced volume of visceral fat and improved plasma concentrations of adipokines and oxidative stress markers more than conventional diabetic diet. Difference between groups enlarged after addition of exercise. To our best kno wledge this is the first study that uncovered the effect of vegetarian diet on these parameters after diet intervention and after addition of exercise. The advantageous effects of vegetarian diet may be partly explained by weight loss, especially loss of v isceral fat and consequent increase in insulin sensitivity.

Several possible mechanisms may explain the beneficial effects of vegetarian diet (120): higher intake of fiber (101), lower intake of saturated fat (and a highe r P/S ratio) (159), higher intake of non-heme iron and reduction in iron stores (121), higher intake of vegetable protein in place of animal protein (122), higher intake of antioxidants (123) and plant sterols (124). Vegetarian diet was reported to reduce intramyocellular lipid concentrations (160) and this together with the effect on visceral fat which we observed might be responsible for a substantial portion of the effect of vegetarian diet on insulin sensitivity and enzymatic oxidative stress markers.

Our data suggest that vegetarian diet leads to a complex improvement of enzymatic and nonenzymatic oxidative stress markers. Both enzymatic and nonenzymatic antioxidant defense mechanisms work in synergy against different types of free radicals (161), which play a major role in the development and progression of diabetes and its complications (162). The changes we observed in plasma concentrations of adipokines reflect loss of adipose tissue.

Reduction of LDL-cholesterol we observed with vegetarian diet is i n concordance with previous studies where vegetarian diet has been shown to reduce LDL - cholesterol (137), postprandial lipids (163), and to reverse atherosclerosis progression (164). Of interest is different dynamics of changes in HDL -cholesterol (although the difference between groups was not significant): whereas it increased in CG from baseline to six months with no significant increase in either period, it increased in EG only after addition of exercise. Previous studies showed no increase or even decrease in HDL-cholesterol with vegetarian diets, however this decrease is lower than reduction in LDL-cholesterol. Isolated increase in HDL -cholesterol observed in other diets does not by far confer the same benefits (165).

The strengths of the study include the parallel design, in which all participants started simultaneously, allowing the investigators to use weekly meetings in both groups to encourage further compliance. Providing all meals for the participants and exercising under professional supervision ensured the best possible compliance. The study duration was reasonably long allowing sufficient time for adaptation to the diet. The study investigated several metabolic parameters, the results giving well -matched pieces of a puzzle, applicable outside the research setting.

We are aware of several limitations of our study. T he number of subjects did not allow confirming the superior effect of vegetarian diet on HbA1c observed by Barnard et al. (137). Lower adherence to the prescribed diet in the control group during the exercise is pointing out to the potenti al weakness of the conventional diabetic diet: portion size limits may increase feelings of hunger during exercise, leading inevitably to exceeding of prescribed energy intake limits.

The limited adherence to conventional diabetic diet has been a common problem in dietary intervention studies for a long time (166),(137). Especially during exercise it became evident that it is easier to follow vegetarian diet than conventional diabetic diet. This may be partly responsible for greater reduction in volume of visceral fat and insulin resistance with vegetarian diet after aerobic exercise.

In conclusion, our results indicate that vegetarian diet is more effective in increasing

insulin sensitivity, reducing volume of visceral fat and improving plasma concentrations of adipokines and oxidative stress markers than conventional diabetic diet. Vegetarian diet could be a more convenient alternative in nutritional therapy of T2D, especially in combination with aerobic exercise.

Improvement in -cell function after Diet-induced Weight Loss is Associated with Decrease in Pancreatic Polypeptide in Subjects with Type 2 Diabetes (Appendix 2)

12-weeks of diet-induced weight loss resulted in improvement of -cell function (implicated by increase in both insulin secretion at the reference level and glucose sensitivity). After the addition of exercise for subsequent 12 weeks the parameters of -cell function did not change (although a trend toward increase was observed). This finding is in accordance with previous work showing that lifestyle intervention with weight loss has the potential to restore -cell function in subjects with T2D (167). Predominant role of reduced -cell glucose sensitivity and glucose-stimulated insulin response over insulin resistance in peripheral tissues has been documented in progression from normal glucose tolerance to diabetes (168). Improvement in -cell function, namely the increase in insulin secretion at the reference level and glucose sensitivity after diet-induced weight loss observed in our study, may be, in light of our study, a physiological way to possibly reverse diabetes.

The improvement in -cell function may be related to other potential factors involved in response to diet-induced weight loss: reduced lipotoxicity, glucotoxicity, oxidative stress, inflammatory cytokines, adopokines and mediators produced in the liver, endothelium, central nervous system and gastrointestinal tract.

We observed decrease in both fasting and hyperinsulinemic plasma concentrations of PP in response to dietary intervention. This is in accordance with the finding that normalisation of fasting plasma glucose by short-term treatment with diet plus insulin is associated with decreases in basal and stimulated secretory activity of the pancreatic polypeptide cells in type 2 diabetes (169). Thus, the decrease of PP in our study may be caused by a decrease in fasting plasma glucose. We suggest that elevated plasma concentrations of PP may be viewed as a negative marker, in accordance with the finding that Pima Indians, who have a high risk of type 2

diabetes, exhibit marked hyperinsuli nemia and elevated plasma levels of PP (170). In this light, the decrease of PP concentrations in response to dietary intervention in our study may be perceived positively. In our study, changes in both parameters of -cell function correlated negatively with plasma concentrations of PP. The correlations remained significant after adjustment for chang es in BMI. To our best knowledge this is a new finding.

In our study, we observed a decrease in fasting plasma concentrations of GIP. GIP, secreted strongly in response to fat ingestion, plays a role in the translation of excessive amounts of dietary fat into adipocyte tissue stores (171). Patients with T2D are resistant to the biological effects of GIP (172). Specific GIP receptor antagonists improve glucose tolerance and -cell function by amelioration of insulin resistance in *ob/ob* mice (173). These effects are similar to improvements of metabolism after bariatric surgery in humans (174). The blockade of GIP action offers promise as a new and potentially important app roach to treat obesity-related diabetes (175). Thus, it may be that the diet i ntervention induces cellular changes in the -cell and the K cell, which in turn helps to restore normal incretin function. The identification of the factors that mediate this effect and its related mechanism could not be accomplished in the current study but represents an area of emerging interest. Collectively, these observations lead us to suggest that weight loss is an important determinant of improved -cell function, improved K cell function, and possibly the cross -talk between these cells.

We used physiological stimulation by standard mixed meal where insulin secr etory responses are related to the incretin axis, which allows clinical scientists to study -cell function during a physiological postprandial pertubation. A potential weakness of our study is that due to lack of appropriate inhibitor we did not measure the concentrations of GLP-1. We realize that alterations in GLP-1 levels may contribute to the changes we observed in -cell function. Our analysis did not find any relationship between -cell function and GIP as Solomon did (167), but we measured the fasting plasma concentrations of the gastrointestinal hormones and their concentrations after clamp-induced hyperinsulinemia, not the concentrations during the meal test. Diet-induced weight loss involved reduction in volume of both visceral and subcutaneous fat. Reduction in volume of visceral fat was greater and gradual,

with further significant reduction during the exercise period whereas volume of subcutaneous fat decreased only during the f irst 12 weeks and did not change significantly after the addition of exercise training. Changes in insulin secretion at the reference level correlated negatively with changes in volume of visceral fat. This finding corresponds well with the recent focus o n ectopic fat accumulation and lipotoxicity as a possible mechanism of compromising the   -cell function (173). We may assume that while volume of visce ral fat was reduced as well as ectopic fat in muscle, liver, cardiomyocytes and   -cells diminished.

Changes in volume of visceral fat did not correlate with changes in plasma concentrations of any measured gastrointestinal peptide. Changes in volume of subcutaneous fat correlated positively with changes in fasting plasma concentrations of PP and PYY. The correlation between changes in the volume of subcutaneous fat and those in fasting plasma concentrations of PP is a new finding and it is in contrast to the study by Tong et al. demonstrating an association between PP levels and volume of visceral fat, but not subcutaneous fat, as measured by CT scan, in healthy individuals (176). Regarding PYY, Lien et al. showed decrease in plasma concentrations of PYY after weight loss but it is not clear whether the decrease in PYY concentrations was related to loss of visceral or subcutaneous fat (177). On the other hand, Kim et al. did not prove any correlation between fasting and stimulated PYY levels and body mass index (178). Further studies are needed to elucidate the possible relationship between subcutaneous fat and plasma concentrations of PP and PYY suggested by the results of our study.

In our study, hyperinsulinemic -isoglycemic-clamp-induced hyperinsulinemia resulted in increased plasma concentrations of PP. This finding, together with the observed decrease in plasma concentrations of PP after diet -induced weight loss and consequent decrease in both fasting and stimulated insulinemia, and togeth er with the negative correlation of plasma concentrations of PP and insulin secretion at the reference level and glucose sensitivity of the   -cells, suggests a possible feedback of insulin secretion and secretion of PP (probably via paracrine signalling): Insulin secretion might increase secretion of PP and PP would in turn decrease insulin secretion. The dearth of experimental data at present in this respect does not allow us to draw any conclusions and leaves this possible physiological regulation

hypothetical. Also, possible PP resistance and its relationship to insulin resistance have so far not been described.

In conclusion, after 12 weeks of diet-induced weight loss, -cell function improved in T2DM and remained unchanged after addition of exercise for subsequent 12 weeks. We demonstrate for the first time that these changes are associated with decrease in PP secretion. Data from this study provide evidence that calori c restriction can improve -cell function, insulin resistance, and the role of gastrointestinal hormones, all of which are imperative in the treatment of T2D. We suggest that PP may play an important role in mediating the improvement of -cell function and that combined diet and exercise interventions provide an effective means to up -regulate gut peptide function in subjects with T2D.

Vegetarian diet improves quality of life and mood more than conventional diet in patients with type 2 diabetes (Appendix 3)

Both hypocaloric diets elicited a positive effect on the quality of life and Beck Depression Inventory (although the average Beck Depression Inventory score of our patients did not reach the threshold of depressive symptoms). The positive effect was greater with a vegetarian diet. The positive effect of a vegetarian diet on health -related quality of life has been shown previously (179). It has been demonstrated that restriction in meat intake may improve mood (180) and that vegetarian diet is associated with less negative emotions compared to omnivores (181).
The mechanism by which a vegetarian diet improves mood may partly be explained by the differences in the rate of neurotransmitter synthesis and receptor dynamics which has been reported in some studies (182).

The Three-Factor Eating Questionnaire revealed that the dietary restraint increased in both groups, suggesting an increase in the voluntary control over food intake with the aim to influence the body weight. The increase was greater in CG , suggesting that the participants in CG felt more constrained by their prescribed diet than did the participants in VG. This in accordance with the Barnard's study showing a less pronounced dietary restraint with a calorie -restricted vegan diet compared to a conventional diabetes diet (183). Disinhibition decreased in both groups in response

to the dietary interventions (weeks 12-0), but the decrease was significant only in VG from baseline to 24 weeks, suggesting that the participants in VG were less likely to overeat e.g. in stressful situations. Feelings of hunger decreased in both groups in response to the dietary interventions (weeks 12-0) with a trend toward a greater decrease in VG. This would suggest an easier adherence with a vegetarian diet in the long-term.

Generally, the changes in the studied parameters were more marked in response to the dietary interventions (in weeks 12-0) and did not change dramatically after the addition of exercise (in weeks 24-12). Differences between study groups were significant despite large inter-individual variations in the studied parameters.

In conclusion, a vegetarian diet led to a greater improvement in the quality of life and mood. Patients consuming a vegetarian diet felt less constrained than those consuming the conventional diet. Disinhibition decreased with a vegetarian diet. Feelings of hunger decreased in both groups in response to the dietary interventions with a trend toward a greater decrease in vegetarian group. All these results suggest that vegetarian diet is sustainable in the long-term and may exhibit desired improvements not only in physical, but also in mental health, of patients with type 2 diabetes.

<u>Beneficial effect of a vegetarian diet on the fatty acid profile in membrane phospholipids in subjects with type 2 diabetes (Appendix 4)</u>

We demonstrated that vegetarian diet increases the content of linoleic acid (18:2 n6) in serum phospholipids. Increased content of linoleic acid (18:2 n6) was associated with increased insulin sensitivity in VG. This result is in accordance with previous research showing the beneficial effects of increased content of linoleic acid in serum phospholipids on insulin action (150,184). It suggests that increased content of linoleic acid (18:2 n6) may be a potential mechanism of the insulin-sensitizing effect of a vegetarian diet.

According to the metabonomic research, linoleic and palmitic acids belong to the metabolites which were identified as potential biomarkers for diabetes mellitus (185).

In a cohort of middle-aged normoglycemic men (n = 895) in a Finnish prospective cohort study with follow-up after 4 years, men with a high proportion of linoleic acid in plasma fatty acids, indicating a high intake of dietary linoleic acid, had a lower risk of developing diabetes and showed lower increases in serum insulin and blood glucose over the follow-up period. This is comparable with earlier findings (186) and is also in line with dietary epidemiology (187), which indicated that individuals with a low proportion of linoleic acid or vegetable fat in the diet have an increased risk of developing type 2 diabetes.

Content of linoleic acid in plasma lipids has also been directly related to other features of the metabolic syndrome, particularly plasma lipid concentrations and blood pressure. The increase in the proportion of linoleic acid (18:2n-6) in serum phospholipids corresponded with decreases in serum cholesterol (188) and was inversely related to the incidence of hypertension (189). Low proportion of linoleic acid predicted the development of left ventricular hypertrophy (190). Furthermore, the content of linoleic acid was positively related to an endothelial function index. Endothelial dysfunction may represent a possible link between diet, fatty acid profile in plasma, sustained hypertension and left ventricular hypertrophy (191). In our study, we confirmed a positive association between changes in the content of linoleic acid in serum phospholipids and plasma HDL-cholesterol.

After adjustment for changes in BMI the association between linoleic acid and insulin sensitivity was no longer significant. Nevertheless, the participants in CG who also reduced weight in response to the dietary intervention did not exhibit the increase in the content of linoleic acid. Therefore, the effect of increased content of linoleic acid on insulin sensitivity cannot be explained by weight loss alone. It may be directly linked to specific effects of vegetarian diet and the loss of association after the adjustment for changes in BMI could be explained by a small number of study subjects. But it is also possible that the increase in the content of linoleic acid may be directly related to changes in BMI (since BMI decreased more in VG compared to CG). Also, there is potential for other factors to confound this association (dyslipidemia, oxidative stress, inflammation, endothelial dysfunction etc.).

In CG, changes in insulin sensitivity and visceral fat were associated with changes in docosapentaenoic acid (22:5 n3). An increase in the content of the docosapentaenoic acid in muscle lipids after very low calorie diet was described previously (192). C22:5 n3 was the only long-chain polyunsaturated n3 structural fatty acid that correlated with insulin sensitivity in patients with coronary artery disease (193). It cannot be ruled out, therefore, that C22:5 n3 may play a role in insulin action in the present setting. However, caution is needed in drawing clear-cut conclusions because C22:5 n3 is found in small amounts in serum phospholipids.

There was no significant change in either saturated , monounsaturated, n3- or n6-polyunsaturated fatty acids as a whole in response to dietary intervention. In this regard, there was no significant difference between the groups.

Another factor we studied was the addition of physical exercise. The decrease in the content of arachidonic acid in VG after the addition of exercise is remarkable. Since arachidonic acid is the precursor of the proinflammatory prostaglandins, leukotriens, thromboxane A2, and prostacyclin , its decrease may have beneficial effects o n inflammation, thrombogenesis and vasoconstriction.

The increase in saturated fatty acids in both groups during the exercise training, namely myristic (14:00) and palmitic acid (16:00), is in accordance with some experimental studies showing increase in the content of saturated fatty acids in the membrane phospholipids in response to physical exercise which has been explained by the increase of the membrane stability and reduced lipoperoxidation (194).

The limitations of our study are that 16% of the patients did not complete the study. We also measured all fatty acids only as proportions of total fatty acids.

In conclusion, we showed that vegetarian diet increased the content of linoleic acid in serum phospholipids and its changes were associated wi th changes in insulin sensitivity and visceral fat. The results support the hypothesis that the insulin -sensitizing effect of vegetarian diet may be mediated by changes in fatty acid pattern in serum phospholipids in subjects with T2D. This is in accordanc e with previous

consensus that the changes in fatty acid composition may play a role in the modulation of insulin action in peripheral tissues.

<u>Vegetarian vs. conventional diabetic diet – a 1-year-follow-up (Appendix 5)</u>

In spite of the diminution of benefit 6 months after the end of the intervention, the positive effects of a vegetarian diet compared to a conventional hypocaloric diet were still persisting 1 year after the end of the intervention with regards to body weight and waist circumference, although the patients did not continue in their originally assigned diets and they consumed a comparable diet for 1 year after the end of the intervention (although it is possible that some vegetarian participants may have maintained some of the healthful dietary habits they adopted during the intervention). Blood lipids did not change significantly in either group while the hypolipidemic agents were discontinued in almost one third of the patients originally assigned to a vegetarian diet. HbA1c increased comparably in both groups while oral hypoglycemic agents were increased and insulin therapy was started almost twice as much in patients originally assigned to a conventional diabetic diet.

Our results indicate a partial persistence of the positive effects of a vegetarian diet compared to a conventional hypocaloric diet 1 year after the end of the intervention. This effect cannot be explained by differences in either diet or physical activity. A possible mechanism of this observation could be the so-called metabolic memory (195,196) although we did not measure oxidative stress markers or advanced glycation end products during the post-trial monitoring to support this hypothesis. Our study showed that even a short-term lifestyle intervention elicits marked positive effects in a longer term in patients with type 2 diabetes. One year after the end of a 6-months-intervention, the beneficial effects of a vegetarian diet compared to a conventional diet were partially maintained.

5.5. Summary of main outcomes
<u>Vegetarian Diet Improves Insulin Resistance and Oxidative Stress Markers More Than Conventional Diabetic Diet in Subjects with Type 2 Diabetes (Appendix 1)</u>

43% of EG and 5% of CG participants reduced diabetes medication. Body weight decreased by 6.2 ± 5.8 kg in EG and by 3.2 ± 4.5 kg in CG (interaction group x time p=0.001). Insulin sensitivity increased more in EG (by 30% vs. 20% in CG, interaction group x time p=0.04). Visceral and subcutaneous fat decreased more in EG (interaction group x time p=0.007 and p=0.02, respectively). Plasma adiponectin increased in EG (p=0.02); leptin decreased in EG (p=0.02). Vitamin C, superoxide dismutase and reduced glutathione increased in EG (p=0.002, p<0.001 and p=0.02, respectively). Difference between groups enlarged after exercise. Changes in insulin sensitivity and enzymatic oxidative stress markers correlated with changes in visceral fat.

Improvement in -cell function after Diet-induced Weight Loss is Associated with Decrease in Pancreatic Polypeptide in Subjects with Type 2 Diabetes (Appendix 2)

Both insulin secretion at the reference level and glucose sensitivity increased in weeks 0-12 (by $33\pm54\%$ and by $26\pm53\%$, respectively, p<0.001) and remained unchanged in weeks 12-24. Plasma concentrations of pancreatic polypeptide (PP) decreased in weeks 0-12 (p<0.05) and did not change significantly in weeks 12 -24. Changes in parameters of -cell function correlated negatively with plasma concentrations of PP.

Vegetarian diet improves quality of life and mood more than conventional diet in patients with type 2 diabetes (Appendix 3)

Both diets elicited a positive effect on the quality of life , mood and eating behaviour, however the positive effects of a vegetarian diet were greater.

Beneficial effect of a vegetarian diet on the fatty acid profile in membrane phospholipids in subjects with type 2 diabetes (Appendix 4)

Linoleic acid (18:2n6) increased in EG (p=0.04) while it decreased in CG (p=0.04) in response to dietary interventions. In EG, changes in 18:2n6 correlated positively with changes in MCR (r=+0.22; p=0.04) and negatively with cha nges in visceral fat (r=-0.43; p=0.01).

<u>Vegetarian vs. conventional diabetic diet – a 1-year-follow-up (Appendix 5)</u>

Neither weight nor waist circumference changed significantly in either group. HbA1c increased (p 0.05) similarly in both groups.

5.6. Conclusions

Insulin sensitivity increased more with vegetarian diet. Vegetarian diet led to a greater reduction in visceral fat and greater improvement in plasma concentrations of adipokines and oxidative stress mark ers. Differences between groups enlarged after addition of exercise. After diet -induced weight loss,   -cell function improved in T2D subjects and remained unchanged after the addition of exercise. We demonstrate for the first time that these changes are as sociated with a decrease in PP secretion. Both diets elicited a positive effect on the quality of life , mood and eating behaviour, however the positive effects of a vegetarian diet were greater. We demonstrated that the insulin-sensitizing effect of vegeta rian diet might be related to the increased proportion of 18:2n6 in serum phospholipids. 1 year after the end of the intervention, the positive effects of a vegetarian diet compared to a conventional diet were partially maintained.

5.7. Keywords and abbreviations

Keywords: Beck depression inventory, -cell function, Exercise, Fatty acid composition of serum phospholipids, Follow-up, Gut hormones, Insulin resistance, Linoleic acid, Oxidative stress markers, Pancreatic polypeptide, Quality of life, Vegetarian diet, Visceral fat

Abbreviations:

BMI… body mass index

CG… control group

CI… confidence interval

EG… experimental group

GIP… glucose-dependent insulinotropic polypeptide

GLP-1… glucagon-like peptide-1

group x time… interaction between group and time (ANOVA)

HbA1c… glycated hemoglobin

MCR… metabolic clearance rate of glucose

OWLQoL… Weight-Loss Quality-of-Life Questionnaire

PP… pancreatic polypeptide

P/S ratio… ratio of polyunsaturated to saturated fatty acids

PYY…peptide tyrosine–tyrosine

T2D… type 2 diabetes

WRSM… Weight-Related Symptoms Questionnaire

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5.9. APPENDIX

Appendix 1
<u>Kahleova H</u>, Matoulek M, Malinska H, Oliyarnik O, Kazdova L, Neskudla T, et al. Vegetarian diet improves insulin res istance and oxidative stress markers more than conventional diet in subjects with Type 2 diabetes. Diab et. Med. 2011 May;28(5):549-559 **(IF 2.902)**

Appendix 2
<u>Kahleova H</u>, Mari A, Nofrate V, Matoulek M, Kazdova L, Hill M and Pelikanova T: Improvement in   -cell function after Diet -induced Weight Loss is Associated with Decrease in Pancreatic Polypeptide in Subjects with Type 2 Diabetes. J Diabetes Complicat 2012– *in press* **(IF 2.032)**

Appendix 3
<u>Kahleova H</u>, Hrachovinova T, Hill M and Pelikanova T: Vegetarian diet improves quality of life and mood more than conventional diet in patients with type 2 diabetes . Diabet. Med. 2012 – *in second revision* **(IF 2.902)**

Appendix 4
<u>Kahleova H</u>, Matoulek M, Bratova M, Malinska H, Oliyarnik O, Kazdova L, Hill M and Pelikanova T: Beneficial effect of a vegetarian diet on the fatty acid profile in membrane phospholipids in subjects with type 2 diabetes . Nutr Diab – *submitted, in revision* **(IF 2.89)**

Appendix 5
<u>Kahleova H</u>, Hill M and Pelikánová T: Vegetarian vs. conventional diabetic diet – a 1-year-follow-up. J Am Coll Nutr - *submitted, in revision* **(IF 2.36)**