UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Venera Berisha-Muharremi

Osteocalcin and type 2 diabetes mellitus

DISSERTATION

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Clinic for Internal Medicine - Department of Endocrinology,

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To whom it may concern

It is my hope that this Thesis will contribute to the future research on the function of osteocalcin in the pathways and regulation of metabolism, and to help in revealing longitudinal association on ucOC and parameters of diabetes control.

Contents

Abbreviations

American Diabetes Association	(ADA)
Anti-glutamine acid decarboxylase	(anti-GAD)
Blood glucose	(BG)
Blood pressure	(BP)
Body mass index	(BMI)
Bone ɣ-carboxyglutamic acid protein	(BGP)
Crosslaps	(croosL)
Diabetes mellitus	(DM)
Diabetes self-management education	(DSME)
Dipeptidyl-peptidase-4	(DPP-4)
Fasting blood glucose	(FBG)
Fasting plasma glucose	(FPG)
Gene encoding a receptor-like protein: OST-PTP	(Esp gene)
Genome wide association studies	(GWAS)
Gestational diabetes mellitus	(GDM)
Glucagon-like peptide-1	(GLP-1)
Glukoza u plazmi	(GUP)
Glycosylated haemoglobin	(HbA1c)
Homeostasis model assessment	(HOMA)
Homeostasis model assessment for estimate insulin sensitivity	(HOMA S%),
Homeostasis model assessment for estimate state of beta cell function	(HOMA B%)
Homeostasis model assessment for insulin resistance	(HOMA-IR)

Impaired fasting glycaemia	(IFG)
Impaired glucose tolerance	(IGT)
Insulin	(ins)
Insulin-dependent diabetes mellitus	(IDDM)
International Association of Diabetes and Pregnancy Study Groups	(IADPSG)
International Diabetes Federation	(IDF)
Knock-out mice (null) for osteocalcin	(OC_/_ mice)
Maturity-onset diabetes of the young	(MODY)
Mice with deletion of the Esp gene	(Esp _/_ mice)
Neutral Protamin Hagedorn	(NPH)
Non-insulin dependent diabetes mellitus	(NIDDM)
Oral glucose tolerance test	(OGTT)
Osteocalcin	(OC)
Osteotesticular protein tyrosine phosphatase	(OST-PTP)
Parathyroid hormone	(PTH)
Total osteocalcin	(TOC)
Type 1 Diabetes mellitus	(T1DM)
Type 2 Diabetes Mellitus	(T2DM)
Undercarboxylated osteocalcin	(ucOC)
World Health Organisation	(WHO)

1 INTRODUCTION AND OBJECTIVE OF THE STUDY

1.1 Diabetes mellitus

1.1.1 Definition and history

Diabetes mellitus (DM) comprises a group of common metabolic disorders of multiple aetiologies that share the phenotype of hyperglycaemia together with disturbances of carbohydrate, fat and protein metabolism resulting from defects of insulin secretion, insulin action or both. Chronically elevated blood glucose (BG) concentrations give rise to its main symptom of passing large quantities of sweet-tasting urine (*diabetes* from the Greek word meaning 'a siphon', as the body acts as a conduit for the excess fluid, and *mellitus* from the Greek and Latin for honey)[1].

The characteristic clinical presentation is thirst, polyuria, blurring of vision and weight loss. Often, symptoms are mild or absent and mild hyperglycaemia can persist for years with tissue damage developing, although the person may be totally asymptomatic.

It is the complications of diabetes which make it a major public health problem. Absolute deficiency of insulin leads to ketoacidosis and coma with a considerable mortality. Hyperglycaemic hyperosmolar state is even more common and serious in elderly people with type 2 diabetes and comorbidities [1,2].

Long term hyperglycaemia affects the microvasculature of the kidney, eye and nerve as well as the large arteries, leading to atherosclerosis. Diabetes is the most common single cause of end-stage renal failure, the most common cause of blindness of working age, and the consequences of neuropathy make it the most common cause of non-traumatic lower limb amputation. Mortality from ischaemic heart disease and stroke is 2-4-fold higher than in non-diabetic age- and sex-matched population[2].

Diabetes mellitus is a disease of antiquity. A polyuric state was described in the Ebers Egyptian papyrus and as long ago as 600 BC two main types were distinguished. The most famous description was made by Arateus the Cappadocian who talked of the melting down of flesh into urine and of the end being speedy. He was the first to use the term "diabetes". Over the ensuing centuries sporadic descriptions were noted, with Maimonides in Egypt pointing

out its relative rarity. It was attributed to a salt-losing state although the sweetness of the urine had long been known[2].

The obvious breakthrough came in the 17th century with the demonstration of excess glucose in the urine and later also in blood. The presence of excess ketones was shown in the 19th century. Himsworth suggested division in insulin-resistant and insulin-sensitive type in 1936[3]. Development of radioimmunoassay for insulin[4] allowed the unequivocal demonstration of insulin deficiency or absence, in those with juvenile-onset (today type 1) diabetes while levels were apparently normal or elevated in those with maturity-onset (today type 2) diabetes. Autoimmune aetiology of type 1 emerged in focus in 1970s[5].

1.1.2 Diagnosis and classification

Current diagnostic criteria for diabetes according to WHO[6] and ADA[7] are at least two of the following in a person with no current acute comorbidity (Table 1):

- A random plasma glucose level ≥11.1 mmol/L (200mg/dL) in someone with symptoms of diabetes
- A fasting plasma glucose level ≥7.0 mmol/L (126 mg/dL)
- HbA1c>6.5% (48 mmol/mol)
- A plasma glucose level ≥11.1 mmol/L (200mg/dL) 2 hours after a 75 g load of glucose given by mouth (the oral glucose tolerance test – OGTT)

Use of a fasting plasma glucose (FPG) of 7mmol/L or higher to define diabetes (Table 1) originated from epidemiological studies in the 1990s, which showed that the risk of microvascular complications increases sharply at FPG threshold of 7 mmol/L. Lately, the notion of a clear glycaemic threshold separating people at high and low risk of diabetic microvascular complications has been brought to question. Part of the rationale for switching to glycosylated haemoglobin – HbA1c>6.5% (48 mmol/mol) as a diagnostic test is that moderate retinopathy is rare below this threshold. Also, greater standardisation of HbA1c assays has led to a recommendation that HbA1c>6.5% be used as a diagnostic cut-off for diabetes[8].

Table 1 Classification of diabetes and glucose intolerance according to ADA fasting and WHO 2-h glucose criteria

	Blood sample		
	Plasma	**Capillary**	**Whole**
Fasting blood glucose (mmol/L)			
Normal	<6.1	<5.6	<5.6
Impaired fasting glycaemia	6.1-6.9	5.6-6.0	5.6-6.0
Diabetes	≥7.0	≥6.1	≥6.1
2-h blood glucose			
Normal	<7.8	<7.8	<6.7
Impaired glucose tolerance	7.8-11.0	7.8-11.0	6.7-9.9
Diabetes	≥11.1	≥11.1	≥10.0

During the natural history of diabetes, the disease passes through intermediate categories of hyperglycaemia, so called – prediabetes. These stages are impaired glucose tolerance (IGT), defined as a plasma glucose of 7.8-11.0 mmol/L (140-200 mg/dL) 2 hours after glucose load in an OGTT and impaired fasting glycaemia (IFG), which is a different category based on fasting glucose levels of 6.1-6.9 mmol/L (110-125 mg/dL) according to WHO or 5.6-6.9 mmol/L (100-125 mg/dL) according to ADA.

Impaired glucose tolerance and impaired fasting glycaemia are intermediate metabolic stages between normal glucose homeostasis and the stage of diabetes. They do overlap, however, are not the same. They both are risk factors for developing future diabetes and cardiovascular disease. Various interventions to prevent overt diabetes in individuals with prediabetes have been studied[9]. Lifestyle modification (diet, exercise and weight loss) seems to be most efficient [2, 3, 5].

The current classification of diabetes is made on the basis of supposed aetiology of the disease (Table 2).

Table 2 Classification of diabetes

▪ Type 1 (β cell destruction, usually leading to absolute insulin deficiency) - Autoimmune - Idiopathic
▪ Type 2 Ranges from predominantly insulin resistant, with relative insulin deficiency, to a predominantly insulin-secretory defect, with or without insulin resistance
▪ Other specific types - Genetic defects of β cell function - Genetic defects of insulin action - Diseases of exocrine pancreas - Endocrinopathies - Drug induced or chemical induced, e.g. steroids - Infections - Uncommon forms of immune-mediated diabetes - Other genetic syndromes sometimes associated with diabetes
▪ Gestational diabetes

There are four categories[8, 10]:

- Type 1 diabetes (caused by pancreatic islet cell destruction)
- Type 2 diabetes (caused by a combination of insulin resistance and β cell insulin secretory dysfunction)
- Other specific types of diabetes
- Gestational diabetes (defined as diabetes that occurs for the first time in pregnancy).

Two major types are type 1 and type 2 diabetes. This classification has replaced the earlier, clinical classification into "insulin-dependent diabetes mellitus" (IDDM) and "non-insulin dependent diabetes mellitus" (NIDDM), which was based on the need for insulin treatment at diagnosis. IDDM is broadly equivalent to type 1 diabetes and NIDDM to type 2 diabetes. One of the most disadvantages of the old classification according to treatment was that subjects could change their type of diabetes.

Type 1 diabetes mellitus (T1DM) is primarily caused by β-cell destruction although some insulin resistance is also present. After the initial stages, insulin is required for survival. In Europeans, more than 90% of type 1 diabetics show evidence of autoimmunity with anti-glutamine acid decarboxylase (anti-GAD), anti-insulin and/or islet cell antibodies and still a growing number of other autoantibodies detectable, which is thought to be due to immunological destruction of pancreatic β cells[11]. In this way, the type 1 diabetes is subdivided into two main types: 1a or autoimmune (in which immune markers suggest autoimmune destruction of β cells), and 1b or idiopathic (where there is no evidence of autoimmunity)[2].

By far the majority of people with diabetes worldwide have type 2 diabetes.

Type 2 diabetes mellitus (T2DM) is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production. Distinct genetic and metabolic defects in insulin action and/or secretion give rise to the common phenotype of hyperglycaemia in type 2 diabetes mellitus. So it is characterized by insulin resistance with relative insulin deficiency (patients secrete insulin, but not enough to overcome the insulin resistance). Typically, they do not require insulin to survive but often will eventually need insulin to maintain reasonable glycaemia control, often after many years[1].

The exact molecular mechanisms underlying type 2 diabetes are not yet known. Major efforts have been made to discover the underlying genetic abnormalities but with only modest success: small scale studies of candidate genes and linkage studies identified few genes causing monogenic type 2 diabetes. In genome wide association studies (GWAS), more than 50 loci are found to be associated with type 2 diabetes[12]. However, combined these loci account for not more than 10% of familiar clustering of type 2 diabetes in Europeans[13]. What is clear is that type 2 diabetes is closely associated with obesity and physical inactivity, and the westernization of lifestyles. The dramatic increase in type 2 diabetes over the past two decades has been closely paralleled by the rise in obesity worldwide. Both obesity, particularly visceral adiposity, and physical inactivity cause insulin resistance which will result in diabetes in those with only a small capacity to increase insulin secretion. The incidence of type 2 diabetes also increases with age, which may probably be related to decrease in exercise and muscle mass; however, the disease is now becoming a problem among younger ages and it is now not uncommon in adolescence and even in children. Sedentary lifestyle and obesity are the main contributory factors, although many have a positive family history of type 2 diabetes[2].

Type 2 diabetes occurs in families so that those with a first-degree relative with diabetes have an almost 50% lifetime risk[2]. There is also marked variation between different ethnic groups. Thus, those of Polynesian, Micronesian, South Asian, sub-Saharan African, Arabian and Native American origin are much more prone to develop diabetes than Europeans. Type 2 diabetes is a diagnosis by exclusion and the prevalence may fall as causes are identified, but this is likely to be a slow process.

The category of **other specific types of diabetes** (Table 3) is a large group of conditions, which includes genetic defects in insulin secretion (such as in maturity-onset diabetes of the

young (MODY) and insulinopathies), genetic defects in insulin action (e.g. syndromes of severe insulin resistance), pancreatitis and other exocrine disorders, hormone-secreting tumours such as acromegaly (growth hormone) and Cushing's syndrome (cortisol). Some cases are caused by the administration of drugs such as glucocorticoids. Some genetic syndromes are sometimes associated with diabetes (e.g. Down's syndrome, Klinefelter's syndrome, and many more).

Table 3 Other specific types of diabetes

Genetic defects of β-cell function	Chromosome 20, HNF4 α (MODY 1) Chromosome 7, glucokinase (MODY 2) Chromosome 12, HNF1 α (MODY 3) Chromosome 13, IPF - 1 (MODY 4) Mitochondrial DNA 3243 mutation Others
Genetic defects in insulin action	Type A insulin resistance Leprechaunism Rabson – Mendenhall syndrome Lipoatrophic diabetes Others
Disease of the endocrine pancreas	Fibrocalculous pancreatopathy Pancreatitis (particularly chronic) Trauma/pancreatectomy Neoplasia Cystic fibrosis Haemachromatosis Others
Endocrinopathies	Cushing syndrome Acromegaly Pheochromocytoma Glucagonoma Somatostatinoma Others
Drug- or chemical-induced	Nicotinic acid Glucocorticoids Thyroxine/triiodothyronine α-adrenergic agonists Thiazides Pentamidine Vacor Others
Infections	Congenital rubella Cytomegalovirus Mumps Others
Uncommon forms of immune-mediated disease	Insulin autoimmune syndrome Anti-insulin receptor antibodies “Stiff man” syndrome Others
Other genetic syndromes	

Gestational diabetes mellitus (GDM) is hyperglycaemia first detected during pregnancy.
There is a certain confusion in terms and criteria. Currently, WHO suggests[14] partial adoption of International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria[15]. Thus, if a nonsymptomatic pregnant woman meets diagnostic criteria of diabetes during pregnancy it is diabetes in pregnancy (IADPSG uses term "overt diabetes"). Gestational diabetes is diagnosed at any time in pregnancy according to IADPSG criteria:

Fasting plasma glucose: 5.1-6.9 mmol/l (92 -125 mg/dl)

1-h post 75g oral glucose load: ≥10.0 mmol/l (180 mg/dl)

2-h post 75g oral glucose load: 8.5 – 11.0 mmol/l (153-199 mg/dl)

After previous acceptance of IADPSG approach to screening and diagnosis of gestational diabetes, current ADA guidelines suggest alternative (either/or) two-step approach with 50g glucose load in nonfasting individual with consequent measurement of BG after 60 minutes: if that is >7.8 mmol/L (140 mg/dL), a 100g OGTT should be performed and interpreted either to Carpenter-Coustan or National Diabetes Data Group [7]. The two-step approach has not been widely used in Europe.
Women with GDM have a high risk of developing later diabetes mellitus, usually type 2. Plasma glucose levels, both fasting and postprandial, are lower than normal in early pregnancy so that raised levels at this stage are almost certainly caused by previously undetected T2DM. Screening for GDM is generally undertaken in 24-28 week.
There is significant morbidity associated with GDM including intrauterine faetal death, congenital malformations, neonatal hypoglycaemia, jaundice, prematurity and macrosomia. Risk factors for GDM include certain ethnic groups, those with previous GDM or abnormalities of glucose tolerance, age, obesity and previous large babies.

The incidence of diabetes is rising owing to the sedentary lifestyle and an ever growing cluster of pre-diabetic syndromes including metabolic syndrome, obesity, and insulin resistance. The number of diabetic population is 382 million, and by 2035 is expected to increase to 592 million people throughout the world. The number of people with type 2 diabetes is increasing in every country[16].

1.1.3 Management of diabetes

The overall goal of diabetes management is to achieve as near normal physiological or ideal values as possible, without detriment to quality of life and, for glucose control in particular, without causing significant hypoglycaemia[17].

Diabetes is a lifelong condition that is, for the majority, currently incurable. It is associated with premature mortality and morbidity from an increased prevalence of cardiovascular disease and microvascular complications affecting the kidney, nerve and eye[2]. High quality randomized trials have shown that improving glycaemic control is associated with a reduction in microvascular complications[18, 19, 20] while a multifaceted approach to cardiovascular risk factors will reduce cardiovascular morbidity and mortality[21].

The person living with diabetes will spend the vast majority of their time managing their diabetes and only an estimated 1% of their time in contact with health care professionals. Therefore, the person with diabetes needs to be supported to take upon themselves much of the responsibility for the management of their diabetes. Given the central role of the person with diabetes and the relatively little contact with health care professionals, it is important that the purposes of the consultation or other contacts with the diabetes health care team are well defined and their aims are made clear so that the patient derives the maximum benefit from the time spent with their diabetes health care team, whether this is in a hospital or in a primary care setting. As well as the clinic visit, diabetes care may also be through phone or email contact or through educational sessions outside a traditional clinic setting.

Much of the focus of care is directed towards minimizing the long-term complications through screening and working together with the person with diabetes to support improved glycaemic control and cardiovascular risk factor management. This provides a challenge for the diabetes team because people often have no symptoms at the time of care and yet are asked to make lifestyle changes and take medications that may place a considerable burden on that individual.

Diabetes education

Diabetes education continues to be a cornerstone of effective diabetes care and supports the philosophy of chronic care models. It is well established that the practice of diabetes self-management education (DSME) is critical to the care and management of people with diabetes. Diabetes educators are being held more accountable for their role in diabetes management. Over time it has become apparent that education standards and a system or

framework describing self-care behaviour could have an important role in supporting people with diabetes to consider behaviour changes that might enhance their quality of life and support better management of their condition. The seven self-care behaviours are: healthy eating; being active; monitoring; taking medicine; problem-solving; reducing risks; and healthy coping[2].

DSME is a comprehensive patient education structure to help achieve the necessary metabolic outcomes and improve the lives of those living with diabetes. Metabolic improvements such as glucose, lipids and blood pressure (BP) in type 2 diabetes care are best achieved with a healthy lifestyle and appropriate use of pharmacologic interventions.

Lifestyle change: Diet

Nutritional management in diabetes aims to assist in optimizing metabolic control and reducing risk factors for chronic complications. This includes the achievement of blood glucose and glycosylated haemoglobin (HbA1c) levels as close to normal as is safely possible and serum lipid concentrations as well as blood pressure values that may be expected to decrease the risk for macrovascular disease. Individual therapeutic needs and the quality of life of the person with diabetes have to be considered when nutritional objectives are defined[2].

Lifestyle change: Exercise

Regular exercise increases insulin sensitivity in individuals with and without diabetes. In individuals without diabetes, plasma insulin levels decrease during low to moderate intensity exercise to compensate for increases in insulin sensitivity. Glucose production and glucose disposal increase in parallel in order to maintain blood glucose homeostasis[2].

Structured supervised diet and exercise interventions can reduce by about 60% the risk of developing type 2 diabetes mellitus in individuals with impaired glucose tolerance[2, 22]. In type 2 diabetes, regular exercise improves glycaemic control significantly [2, 3].

Monitoring diabetes

Glycaemic control should be monitored regularly for all patients with diabetes. The optimal method of determining risk of long-term complications is through HbA1c measurement, although if this is not available, then examination of a series of blood glucose measurements, including fasting tests, may provide some guidance.

Drug therapy

According to the results of key researches on association of glycaemic control and the development of diabetic complications, the target for good glucose regulation is normoglycaemia. However, it was recently shown that aggressive regulation of glucose level in patients with associated diseases (especially cardiovascular) in critically ill patients is not justified, and may even be harmful. It is therefore recommended individually to specify the target control in patients with respect to age, duration of disease, comorbidities (especially cardiovascular) and life expectancy. The general rule is: the younger the patient, the less suffering from diabetes, with fewer complications and comorbidities, the firmer the glucose regulation level. ADA breaks down the target glycaemia presenting recommendations to lower levels for a target HbA1c <7% in most patients, for those younger without complications <6.5%, and for patients with severe accompanying diseases and shortened expected duration of life <8%.

Medications to control blood glucose level

Medications to control blood glucose are traditionally divided into oral anti-diabetics (oral hypoglycaemic agents) and insulin. From the advent of non-insulin injectable drugs (GLP1 mimetics, pramlitid) this classification is not suitable, and it is better to talk about non-insulin medication and insulin.

- ***Insulin***

Insulin was first applied in 1922. The introduction of the highly purified insulin preparations from 1970s significantly reduced the number of adverse reactions associated with impurities in preparations. Insulin in the 1980s was the first peptide for the treatment, obtained by recombinant DNA techniques ("human" insulin). There are no clinically significant differences between highly purified animal and human insulin.

Insulin preparations differ in the length of action. The effect of conventional (animal and human) short-acting insulin starts 30-60 minutes after subcutaneous injection and lasts 6-8 hours. Physiological increase of insulinaemia at a meal is shorter. Short-acting analogues (lispro, aspart and glulizin) have been developed with the aim of better imitation of insulin secretion that occurs during a meal. Insulin and short-acting analogues can be administered intravenously, and for continuous subcutaneous infusion pump. Prolonged action is achieved by crystallization of insulin in the presence of zinc ions (Lente insulin) or by binding to the

neutral protamine suspension (NPH = Neutral Protamine Hagedorn), and pharmacodynamic characteristics of these compositions are the same. NPH insulin showed marked variability that is associated with unpredictable episodes of hypoglycaemia. Analogues of prolonged action, glargine and detemir, were developed with the idea of less variability and predictable basal insulinaemia.

Biphasic, pre-mixed insulins are a mixture of rapid-acting and long-acting insulin in different proportions (typically 30% faster and 70% of extended components). Also, pre-mixed analogue insulins are available.

Insulin in type 1 diabetes

Multiple-dose insulin therapy, the so called basal-bolus therapy, is an appropriate initial approach to reproducing the physiologic insulin profile in patients with absolute insulin deficiency such as those with type 1 diabetes. This consists of a long-acting insulin preparation administered once or twice a day to meet the basal insulin requirement, with the injection of a short-acting insulin preparation with each meal.

The modern approach involves calculating the needs of bolus insulin to the amount of carbohydrates in a meal that gives greater freedom in choosing food. The calculation should include the amount needed for the correction of glycaemia, if it is too high before meals. Substitution therapy with insulin is the most challenging endocrine substitution, which necessarily means a permanent activity of the patient and it is necessary to educate and train for such treatment.

Insulin in type 2 diabetes mellitus

Insulin may be required from time to time and occasionally in the treatment of type 2 diabetes. Most often it is required to be used in intercurrent disease, and sometimes at the beginning of the disease that manifests ketoacidosis (so-called ,,ketosis prone to,, type 2 diabetes) and that needs to be corrected with insulin.

A number of different insulin injection regimens are available for patients with type 2 diabetes who may already be treated with non-insulin-based therapies. These include a once daily injection of a long-acting insulin, a once daily injection of a long-acting insulin with an injection of a short-acting insulin with the main meal, twice a day injections of insulin mixtures, and multiple dose injections. Regardless of the choice of initial treatment schemes (boluses, basal or pre-mixed) after about a year the majority of patients will no longer be able to maintain target glycaemia and it will be necessary to intensify treatment.

Side effects of insulin

The most serious complication of insulin injection for most people is hypoglycaemia. Hypoglycaemia is almost an inevitable side effect of insulin therapy. Patients should be taught about the recognition of hypoglycaemia, the reasons for its creation (the balance of food, exercise and insulin) and how to act in case of its occurrence (extra meal, glucose sweets). The family should be taught about the application of glucagon. With respect to insulin treatment, the fear of hypoglycaemia may be a major barrier to insulin initiation and achievement of tight glycaemic control.

Insulin can also lead to excessive weight gain. This remains a major concern for many patients, particularly for the already overweight patient with T2DM who can no longer be controlled on oral hypoglycaemic agents. Weight gain can be reduced by concomitant advice from the dietitian and an insulin regimen tailored to the individual needs of the patient, that wherever possible provides most insulin when needed (i.e. at meal times). Overaggressive insulin titration regimens leading to low blood glucose and stimulation in appetite can lead to excessive weight gain.

Allergy to insulin is not common, and among them local skin reactions are more frequent, while systemic are rare. These reactions usually occur in those with information on the history of allergy.

Lipohypertrophy at the site of injections is due to frequent injections in the same place. Lipoatrophy is extremely rare with purified insulin preparations. According to epidemiological data, there is a somewhat higher incidence of malignant diseases in people with diabetes. It seems that the said has further increased with insulin treatment, probably because of the possibility that insulin stimulates the growth of existing tumours.

- ***Non-insulin medications***

Metformin

The biguanide metformin is often selected as initial oral antidiabetic drug therapy. It counters insulin resistance and lowers blood glucose through several insulin-dependent and independent mechanisms, notably reducing hepatic glucose production and also increasing glucose uptake by skeletal muscle. It does not stimulate insulin secretion, carries a low risk of frank hypoglycaemia, and does not cause weight gain. Metformin also exerts several potentially beneficial effects on cardiovascular risk factors independently of glycaemic control, with evidence of improved long-term cardiovascular outcomes. Metformin may be

conveniently combined with other classes of antidiabetic drugs. Gastrointestinal side effects including diarrhoea limit the use of metformin. These side effects are usually transient and rarely occur with the gradual introduction of the drug and the application after meals. The maximum daily dose is of 3 g, and usually is 2 g separated twice daily. The rare but serious adverse effect of lactic acidosis excludes the use of the drug in patients with significant renal insufficiency, significant liver disease or any condition predisposing to hypoxia or hypoperfusion including cardiac or respiratory failure.

Few cohort studies showed lower mortality rates to treatment with metformin compared to sulphonylurea derivates. The observational studies found less cardiovascular events with metformin compared to other oral hypoglycaemic agents, and there are indications of antitumour activity and modulation of incretin effect.

Sulphonylureas

These are the oldest drugs to control hyperglycaemia. Sulphonylureas (e.g. gliclazide, glimepiride, glibenclamide/glyburide, glipizide) act on the pancreatic β cells to stimulate insulin secretion.

They bind to the transmembranal complex of sulphonylurea receptors SUR1 with ATP-sensitive Kir6.2 potassium efflux channels. This closes the channels, depolarizes the membrane, opens voltage-dependent calcium channels, and raises intracellular free calcium concentrations.

This in turn activates proteins regulating insulin secretion. The efficacy of sulphonylureas depends on adequate remaining function of the β cells. Sulphonylureas stimulate insulin secretion independent of glucose, the main side effects are hypoglycaemia and weight gain. Hypoglycaemia is the most serious adverse effect and occurs mainly due to poorly supervised treatment of elderly people, especially those with renal failure, particularly with longer acting sulphonylureas. Caution with hepatic and/or renal insufficiency is warranted in accordance with the metabolism and elimination of individual preparations, and interactions with other protein-bound drugs can occur.

Meglitinides

Meglitinides (repaglinide and nateglinide), also known as prandial insulin releasers, are rapid and short-acting insulin secretagogues taken before meals to boost insulin levels during digestion, thereby reducing prandial hyperglycaemia and decreasing the risk of interprandial

hypoglycaemia. They act in a similar manner to sulphonylureas by binding to a "benzamido" site on the SUR1 – Kir6.2 complex. They are conveniently used in combination with an agent that reduces insulin resistance.

Intestinal glucosidase inhibitors

Intestinal glucosidase inhibitors (acarbose and miglitol) competitively bind to glucosidase, thereby slowing down the absorption of carbohydrates. Acarbose is minimally absorbed from the gastrointestinal tract and therefore has no systemic side effects. Common side effects include bloating and flatulence due to fermentation in the gut with too many carbohydrates from the food. It should not be used in patients with severe renal insufficiency (creatinine clearance less than 25 mL / min).

Thiazolidinediones (glitazones)

Thiazolidinediones (pioglitazone and rosiglitazone) produce a slow-onset glucose-lowering effect, attributed mainly to increased insulin sensitivity. They alter the expression of certain insulin-sensitive genes by stimulating the peroxisome proliferator-activated receptor γ, increasing adipogenesis, and rebalancing the glucose-fatty acid (Randle) cycle. Thiazolidinediones can be used as monotherapy or in combination with other classes of antidiabetic agents. They have low risk of hypoglycaemia but often cause weight gain. The potential for fluid retention and an attendant risk of congestive heart failure should be borne in mind, especially in combination with insulin.

Thiazolidinediones are not recommended for individuals at high risk of cardiac disease or women with reduced bone density.

Contraindication of their use is heart failure due to fluid accumulation indicated preferences. They work on many cardiovascular risk factors, but the long-term favourable cardiovascular effects are not unambiguously proven. The use of thiazolidinediones (glitazones) is significantly limited because of the side effects, and because of causing fatal liver failure troglitazone (first drug of this group) was withdrawn from the market. Rosiglitazone was, due to cardiovascular side effects (myocardial infarction), withdrawn from the market in Europe and the US. Also, higher incidence of bone fractures was observed with the use of rosiglitazone and pioglitazone. Pioglitazone was, due to indications of higher incidence of bladder cancer, in 2011withdrawn from the markets of Germany and France, and other European countries and the United States. They listed bladder cancer (active or history) and haematuria of unknown cause as contraindications for use.

Sodium-glucose cotransporter inhibitors (gliflozines)

By inhibition of reabsorption of glucose (and sodium) in renal tubuli these drugs lower BG and are helpful in weight management. Most common side effects are genitourinary infections.

Drugs based on incretin effect

The incretins are a group of gastrointestinal hormones that enhance glucose-stimulated insulin secretion and have an important role in the regulation of postprandial glucose levels. Glucagon-like peptide (glucagon-like peptide-1, GLP-1) is an incretin secreted by the L cells of the small intestine induced by the meal. The physiologic actions of GLP-1 include potentiation of meal-induced insulin secretion, inhibition of glucagon secretion, delay in gastric emptying and suppression of food intake and appetite. GLP-1 coordinates the secretion of insulin and glucagon depending on glycaemia and thus helps glucose level regulation without the risk of hypoglycaemia. In experimental models it promotes the growth of β-cells and prevents cell apoptosis. Degradation and inactivation of GLP-1 is faster under the effect of enzyme dipeptidyl-peptidase-4 (DPP-4) and for therapeutic purposes can be used only continuous infusion. The research is turned in the direction of the synthesis of drugs that would act as a GLP-1, but would be resistant to the enzyme DPP-4 (mimetics), and towards another group of drugs that inhibit this enzyme (DPP-4 inhibitors or blockers). Mimetics therapy achieves ten times higher concentrations compared to DPP-4 inhibitor achieved level and that could be a possible reason for the differences in effects, since mimetics have a pronounced drop in body weight, which is not the case with the DPP-4 inhibitors.

Incretin mimetics (exenatide, liraglutide) are applied as subcutaneous injections, and are suitable for the treatment of patients with type 2 diabetes with a higher degree of obesity. The most common side effect is nausea and vomiting. In some studies there are indications of a higher risk for occurrence of pancreatitis, but this has not been confirmed by a retrospective cohort study and systematic monitoring of side effects. In rodents, a correlation between C-cell hyperplasia and medullary thyroid cancer has been observed, but this is probably due to the effect of a specific type because rodents have distinct expression of the receptor for GLP-1 in C cells of the thyroid. Closely monitoring of the incidence of medullary carcinoma in people with the use of GLP-1 mimetics has not so far given indication to that effect. DPP-4 inhibitors by preventing the degradation of GLP-1 prolong the effect of the incretin, and increase its circulating level. These are oral medications known as gliptins (sitagliptin,

saxagliptin, vildagliptin, linagliptin). They are structurally different, and therefore have a different and specific blockade of DPP-4 isoenzymes. Based on clinical experience, no significant differences have been registered in safety between clinical applications of so far used DPP-4 inhibitors, and neither severe side effects. Indications of frequent incidence of pancreatitis have not been confirmed.

Pramlintide, is an amylin analogue registered for blood glucose control in the US, not in Europe. Amylin is a naturally occurring peptide that is co-secreted with insulin from the β cells of the pancreatic islets. Physiologic actions of amylin include delayed gastric emptying, suppression of postprandial glucagon secretion, and increased satiety. Beneficial effects of parenteral administration of pramlintide, a synthetic amylin mimetic approved for use in insulin-treated subjects with either type 1 or type 2 diabetes, include improved glycaemic control and mild weight loss. It is used for type 1 and type 2 diabetes in combination with the standard insulin therapy. The overall effect is better prandial blood glucose control and lower insulin requirements. The drug is administered by subcutaneous injection, and side effects are hypoglycaemia, nausea, abdominal pain, and headache.

Bromergocriptin and Colesevelam are registered for blood glucose lowering in the US, not in Europe.

Management of type 2 diabetes – Guidelines for pharmacotherapy

Several approaches to the best management of T2DM have been proposed. All algorithms begin with therapeutic lifestyle change (diet and exercise) and are generally focused primarily upon controlling hyperglycaemia.

The American Diabetes Association (ADA) and the German Diabetes Society, whose guidelines with adjustments have been adopted also by the Croatian Society for Diabetes and Metabolic Disorders, recommend to include immediately metformin at diagnosis, of course, if there are no contraindications (Figure 1).

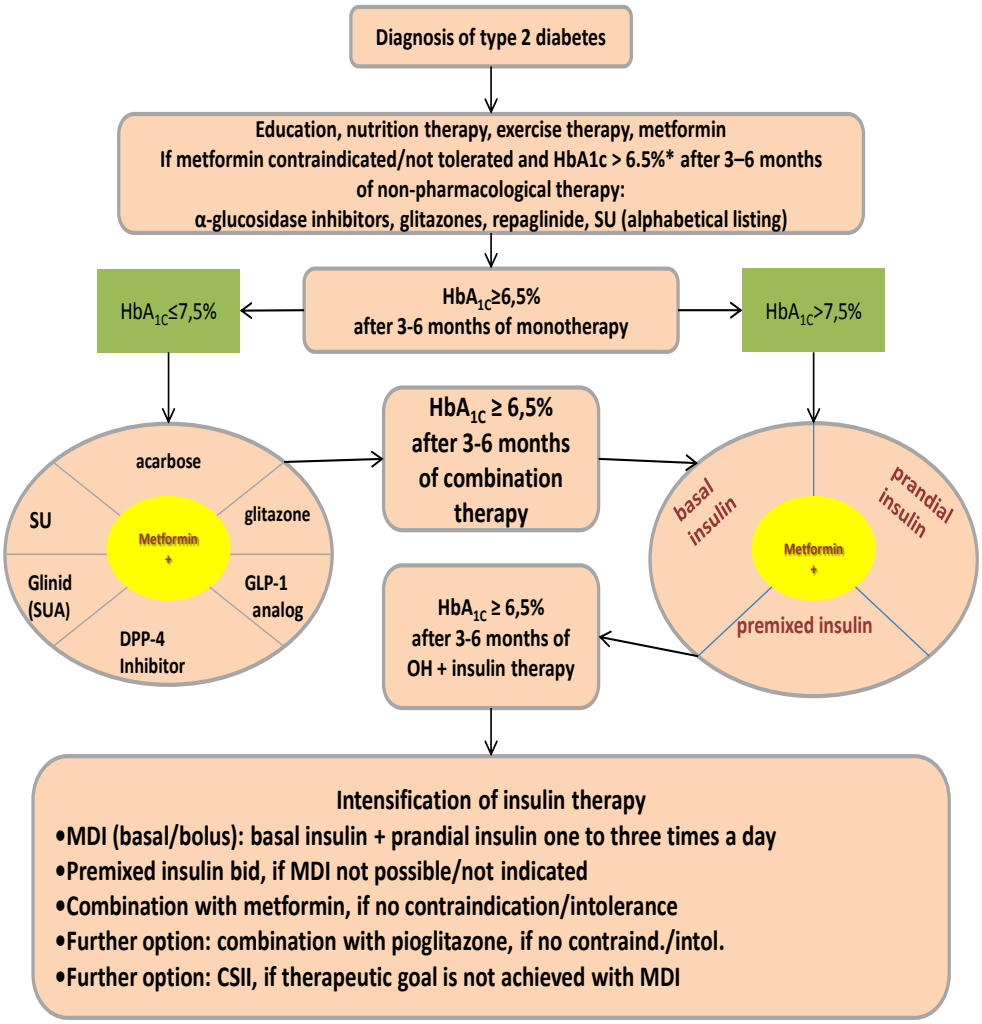

Figure 1 Guidelines for treatment of type 2 diabetes from American Diabetes Association (ADA) and the German Diabetes Society, also adopted and adjusted by the Croatian Society for Diabetes and Metabolic Disorders.

The ADA and the German Diabetes Society recommend to begin immediately the treatment with metformin. When metformin alone is insufficient, the addition of a second drug is recommended. Guidelines of International Diabetes Federation (IDF) distinguish between the conventional and the alternative approach that does not include the initial phase of treatment by pharmacotherapy. The first line of treatment is only basic treatment, which involves a lifestyle change (Figure 2).

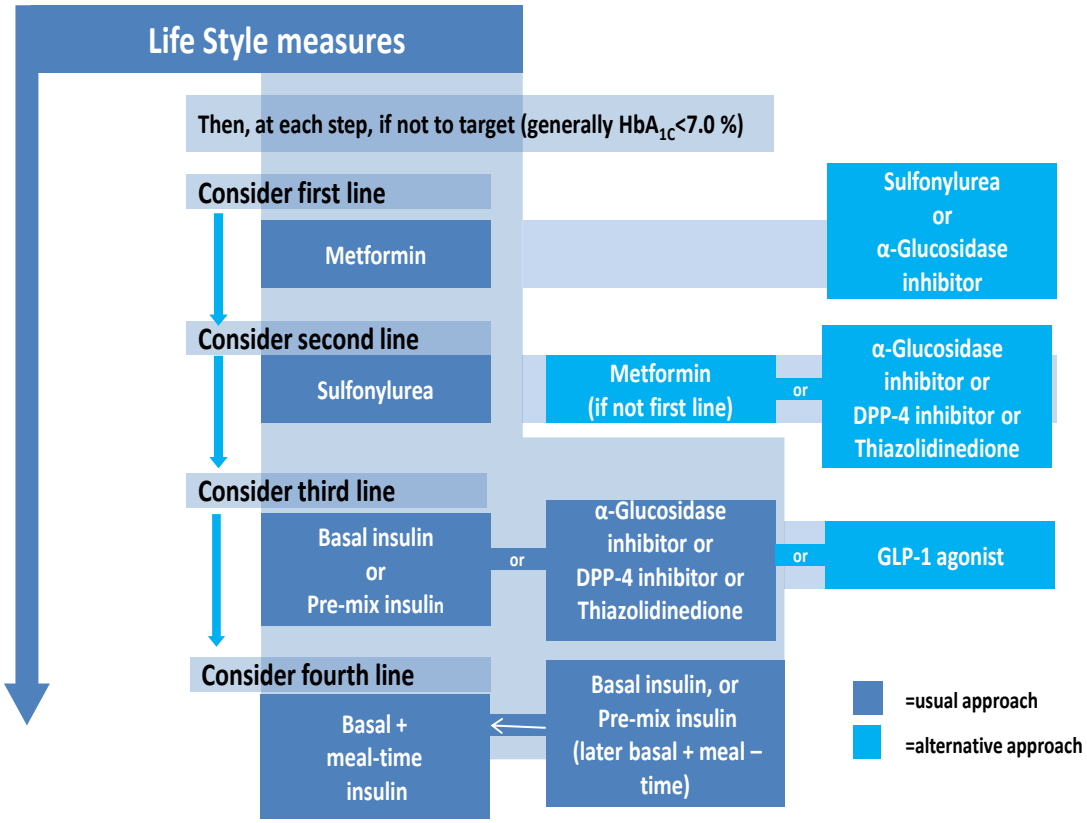

Figure 2 Guidelines for treatment of type 2 diabetes from International Diabetes Federation (IDF).

All societies agree that we should have a proactive attitude, if it is sufficient, to intensify the therapy by combining drugs. If there are contraindications to metformin use, it can be started with the drug from another group as the first line drug. We should interpret with caution reports on the average decline of HbA1c with an individual therapy, which is regularly increasing regardless of the treatment as much as starting HbA1c is greater. Comparison of the effectiveness of drugs regularly conducted by the Agency for Healthcare Research and Quality in Health Care USA shows that all non-insulin drugs have similar effects on HbA1c: about 1%. With sulphonylureas hypoglycaemia is four times more frequent compared with metformin, up to 5 times more in combination. Mean body weight difference is -2.5 kg with metformin compared to sulphonylurea and pioglitazone.

The aim of ideal glucose regulation is HbA1c <6.5%. It is important to emphasize individual approach with regard to the characteristics of the patient, especially comorbidities, complications of diabetes, and age. For some patients a less strict target should be determined (HbA1c <7% or <7.5%). If with metformin alone (or other treatment in case of contraindications) after 3-6 months the target level is not achieved, recommendation is to add a drug from other groups, as the mechanisms of action of various drugs can be combined with each other. After the next 3-6 months estimated effect, if the goal is not achieved, adding an additional medication or insulin is recommended.

1.2 Osteocalcin

Until recently, endocrinologists have looked upon bone as a target for hormones such as sex steroids, parathyroid hormone (PTH), and calcitonin. The skeleton was seen as a mere protective casing for cells and their cytokine products, and perhaps the bone was only thought of as a useful support system. Discoveries in the past decades revealed that bone mass is also regulated by the fat via leptin which acts upon brain and downstream through hypothalamic relay and sympathetic nervous system[23, 24]. These findings suggested, from endocrine point of view, that a feedback mechanism should exist. Indeed, recent studies and pharmacological experiments in mice suggest an entirely new role for bone, by producing osteocalcin that acts as a hormone affecting insulin production and sensitivity, glucose utilization and energy expenditure[25]. These findings have been reviewed recently[26, 27, 28].

Osteocalcin (OC) (also called bone ɣ-carboxyglutamic acid protein, or BGP) is a bone-specific protein secreted by the osteoblasts consisting of 46–50 residues (Figure 1)[29] that undergoes post-translational modification by vitamin- K-dependent ɣ-carboxylation of three glutamic acid residues[30]. Undercarboxylated osteocalcin (ucOC) has fewer than three carboxylated residues, and has a lower affinity for bone. The fully carboxylated and undercarboxylated forms of osteocalcin are both found in serum. Osteocalcin is expressed by mature osteoblasts, binds strongly to hydroxyapatite, stored in bone matrix and released into the circulation, and is a useful marker of bone formation[27].

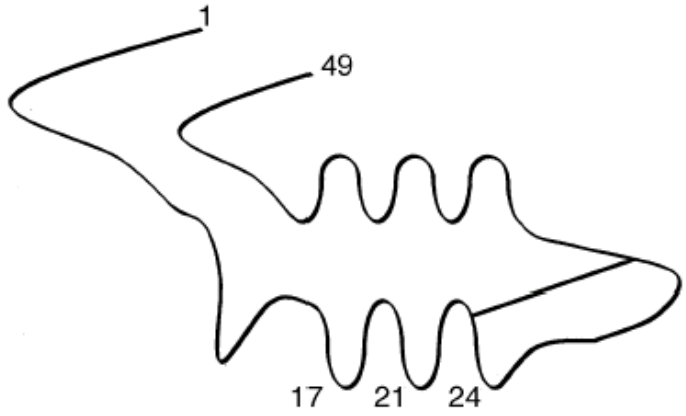

Figure 3 Diagram of the osteocalcin structure. Gla residues are present at positions 17, 21 and 24 and a disulphide bridge is present between residues 23 and 29.

Knock-out mice (null) for osteocalcin ($OC^{-/-}$ mice) show no remarkable bone phenotype but appear mildly hyperglycaemic and have slightly increased visceral fat[31]. On the other side, deletion of the Esp gene (Esp $^{-/-}$ mice), a gene encoding a receptor-like protein: osteotesticular protein tyrosine phosphatase (OST-PTP), results in a phenotype opposite that of the $OC^{-/-}$ mice: increased pancreatic islet size, ß-cell number and circulating insulin levels; increased insulin sensitivity despite hypoglycaemia, decreased visceral fat mass; increased expression of insulin target genes in the liver and muscle, energy expenditure increased, while food intake is not affected[24].

In *ex vivo* experiments, osteocalcin stimulates cyclinD1 and insulin expression in β cells and adiponectin, an adipokine whose overexpression enhances insulin sensitivity[32]. Insulin production and insulin sensitivity is shown to be enhanced by either addition of osteocalcin or overproduction of osteocalcin by osteoblasts. *In vivo* treatment of normal mice with non-ɣ-carboxylated osteocalcin generated by bacterial expression found increased pancreatic ß-cell numbers, insulin secretion, energy expenditure and insulin sensitivity so it has the opposite effect on metabolism as that in the $OC^{-/-}$ mice [33]. So Esp $^{-/-}$ mice are also protected from diabetes, similar to treated mice with non-ɣ-carboxylated osteocalcin while alternately, bone-specific overexpression of OST-PTP resulted in a phenotype identical to $OC^{-/-}$ mice. Therefore, the hypothesis was made that OST-PTP was responsible for inactivating OC through ɣ-carboxylation[25].

In summary, osteocalcin may act as a metabolic hormone whose regulation is not yet elucidated and receptor-like protein osteotesticular protein tyrosine phosphatase (OST-PTP) could be involved in still unclear signalling pathways.

The information about the role of circulating osteocalcin and particularly its undercarboxylated fraction on energy expenditure and the regulation of insulin secretion was derived from studies on mice models in which osteocalcin production was inactivated or increased.

An equivalent to mice OST-PTP in humans has not been identified. However, blood osteocalcin levels were shown to be significantly lower in diabetics than in non-diabetic controls, and the levels were inversely related to fat mass and blood glucose[34, 35]. In postmenopausal women, osteocalcin levels were significantly lower in type 2 diabetic subjects than in controls[36]. In a study on effects of hypocaloric diet and exercise, osteocalcin plasma levels were associated positively with insulin sensitivity and negatively with fasting plasma triglycerides[37]. Serum osteocalcin level is associated with glucose metabolism and

atherosclerosis parameters in patients with type 2 diabetes[38, 39]. In gestational diabetes, higher level of osteocalcin in comparison with pregnancies with normal glucose tolerance is observed[40]. In poorly controlled diabetics, after only a month of treatment and glycaemic control, increase of osteocalcin level was seen while serum adiponectin was not significantly different before and after glycaemic control, but baseline level of adiponectin seems to predict the beneficial bone reaction[41]. Osteocalcin level seems to increase after improved glycaemic control in type 2 diabetes[42, 43].

Most of these earlier studies[41, 42] were focused on the effect of diabetes on bone remodelling and level of osteocalcin on humans. The role of osteocalcin in regulation of glucose metabolism remains to be elucidated.

Most clinical studies investigating possible metabolic effects (or associations) with osteocalcin levels did not distinguish total (TOC) and undercarboxylated osteocalcin (ucOC). Experimental data[25, 32] suggest that undercarboxylated osteocalcin (ucOC) is involved in metabolism. Recent data from healthy children suggest the same relationship in humans[44].

Improved glycaemic control appears to increase TOC but does not necessarily have the same effect on ucOC. Additional studies with ucOC as an outcome are needed to clarify the effects of improved glycaemic control on this marker.

2 HYPOTHESIS

Undercarboxylated osteocalcin level is associated with changes in the level of glucose control assessed by HbA1c; i.e. undercarboxylated osteocalcin (ucOC) is involved in glucose metabolism not related to bone turnover. Osteocalcin levels in diabetics and its relation to blood glucose control might reflect this association.

3 AIMS OF THE STUDY

3.1 Main Aims of the study

1. Association of serum ucOC level and HbA1c as a marker of glucose regulation in type 2 diabetes.
2. Changes in serum ucOC with short term (three months) improvement of blood glucose control in terms of HbA1c in persons with type 2 diabetes.

3.2 Specific Aims

1. To assess the relationship of serum TOC and ucOC level with insulin sensitivity assessed by HOMA indexes.
2. To assess the relationship of serum TOC and ucOC level with BMI.
3. To show that changes in ucOC with glucose regulation (if present) are independent of bone turnover.

4 MATERIALS AND METHODS

4.1 Subjects

A total of 57 consecutive type 2 diabetic patients (WHO criteria), male and female, aged 19-79, whose diabetes was treated by diet only, with HbA1c of 7.5 % or over, participated in this study.

Exclusion criteria included: malignant diseases, kidney or liver diseases, bone metabolism disease, glucocorticoid treatment, hormonal contraception, hormonal replacement treatment, and androgen treatment.

The recruited patients were from an outpatient department or hospitalized in the Clinic of Internal Medicine, Zagreb Clinical Hospital Centre.

4.2 Methods

Clinical data, patient history

All patients underwent physical examination, including blood pressure measurement. Measurements of height and weight were taken. Body mass index (BMI) was calculated as weight (kg) per height (m^2).

Assessment of insulin resistance in the basal state was estimated by the homeostasis model assessment (HOMA) for insulin resistance (HOMA-IR), for estimate steady state of beta cell function (HOMA B%) and insulin sensitivity (HOMA S)%[45], calculated from fasting blood glucose and fasting insulin by downloaded calculator[46].

Analytical procedures – Biochemical measurements

Blood was collected after an overnight fasting, at baseline and after three months. Samples were separated and stored frozen until assay. Measurements of the following levels were taken: TOC, ucOC, fasting blood glucose (FBG), fasting insulin, HbA1c and bone turnover marker (crosslaps telopeptide).The used methods for measurement of parameters are

standard recommended biochemical methods („ligand“) automated or semi-automated performed according to the manufacturer’s instructions.

Insulin, HbA1c, osteocalcin, beta-crosslaps were measured on Roche instruments by Roche reagents (Roche Diagnostics, Mannheim, Germany). Undercarboxylated osteocalcin, Takara Bio Inc., Japan.

4.3 Statistical analysis

A descriptive analysis is presented in tables and figures.

Numeric variables were tested for distribution normality using the Shapiro-Wilk’s test. All variables were distributed significantly different than normal distribution. Therefore, non-parametric tests were used for group comparison (Wilcoxon test for dependent samples) and correlations (Spearman rho test).

The level of statistical significance is chosen to be 0.05.

The SPSS ver. 17.01 was used to perform statistical analysis (SPSS ID: 729038).

Calculated sample size for assessment of association correlation of serum osteocalcin and HbA1c level (from the published data, coefficient is -0.3) for power of 80% was 25 per group. From published data, standard deviation of HbA1c in population is 2.5; thus for 1% difference in HbA1c sample size was 50 assuming that at least half of them would reach a 1% decrease in HbA1c.

4.4 Ethics

This study was conducted according to all currently valid and applied guidelines whose purpose is to assure proper conduction and protection of persons included in this research as examinees, including the Basics of Good Clinical Practice and Helsinki Declaration, Health Protection Law of the Republic of Croatia (NN 121/03), and Patient's Rights Law of the Republic of Croatia (NN 169/04).

Identity of healthy examinees and patients remained confidential and protected.

Consent form and subject information

Prior to the beginning of the trial, the investigator presented oral and written information about the study. The investigator had to be ensured that subjects were fully informed about the aim of the study, procedures, potential risks, discomforts, and expected benefits which could come out from these investigations. Also, subjects were informed and agreed that the Health Authority personnel would require the access to data. It was emphasized that participation would be voluntary and that subjects would have the right to withdraw from the trial at any time without prejudice. A freely given, written Informed Consent was obtained from all subjects prior to their admission to the procedures in accordance with the study protocol.

Ethics committee

Prior to the start of the trial, all three documents – research design of the thesis project proposal, informed consent, and agreement letter for participation of subjects, – were submitted to the Ethics Committee of Zagreb Clinical Hospital Centre and of the School of Medicine, University of Zagreb. A written approval for these documents was obtained.

5 RESULTS

A total of 57 patients participated in the study: 21 (36.8%) females, 36 (63.2%) had hypertension. A descriptive analysis is presented in tables and figures. Basal characteristics of patients are shown in Tables 4-5 and Figures 4-7.

Table 4 Basal patient characteristics and parameters at Visit 1

	Mean	Median	Std. Deviation	Range	Minimum	Maximum
AGE [years]	54.4	57.0	10.4	50.0	30.0	80.0
HEIGHT [m]	1.7	1.7	0.1	0.4	1.5	1.9
WEIGHT_1 [Kg]	88.8	88.0	15.3	69.0	66.0	135.0
body mass index (BMI)_1 [Kg/m^2]	29.9	29.2	4.0	20.4	23.2	43.6
glycosylated haemoglobin (HbA1c)_1 [%]	8.5	8.0	1.6	5.7	6.3	12.0
fasting blood glucose (FBG)_1 [mmol/L]	9.5	9.0	1.9	7.9	7.1	15.0
insulin_1 [uU/L]	24.9	18.0	26.9	179.3	3.7	182.9
HOMA B%_1	19.8	15.0	17.5	101.2	3.9	105.1
HOMA S%_1	259.8	218.9	202.0	1036.6	23.0	1059.6
HOMA-IR_1	0.6	0.5	0.6	4.3	0.1	4.3
total osteocalcin (TOC)_1[ug/L]	12.2	11.8	4.6	28.2	6.2	34.4
undercarboxylated osteocalcin(ucOC)_1 [ng/mL]	2.3	2.0	1.9	7.9	0.1	8.0
crosslaps_1 [ug/L]	0.2	0.2	0.1	0.5	0.1	0.6

Figures 4-7 show distribution histograms and normal curve for age (years), BMI (Kg/m^2), HbA1c (%) and undercarboxylated osteocalcin (ucOC, ng/mL) at Visit 1.

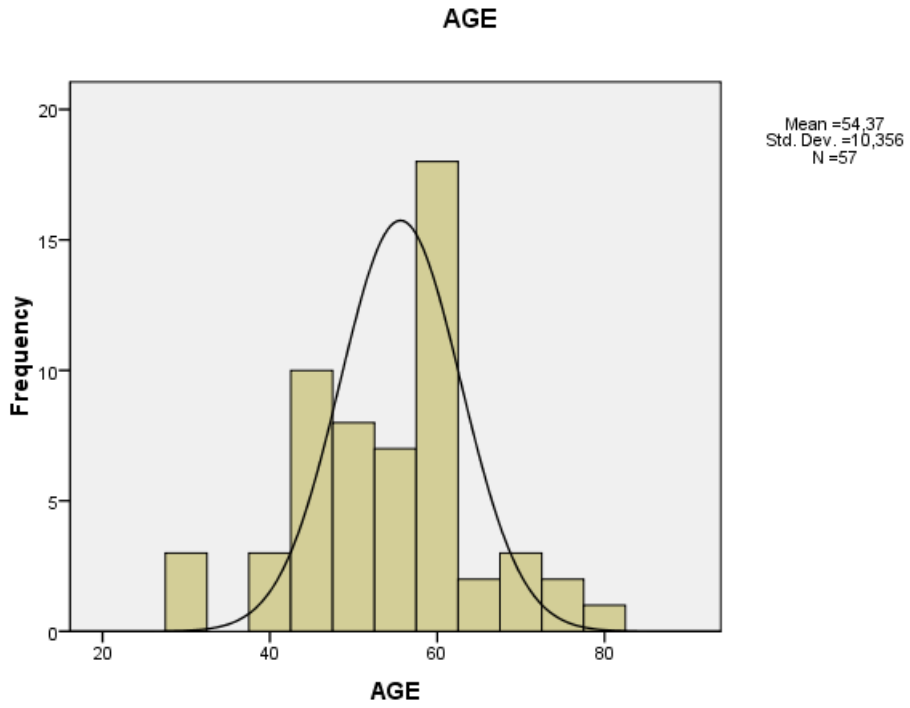

Figure 4 Age of participants: frequency histograms and normal curve

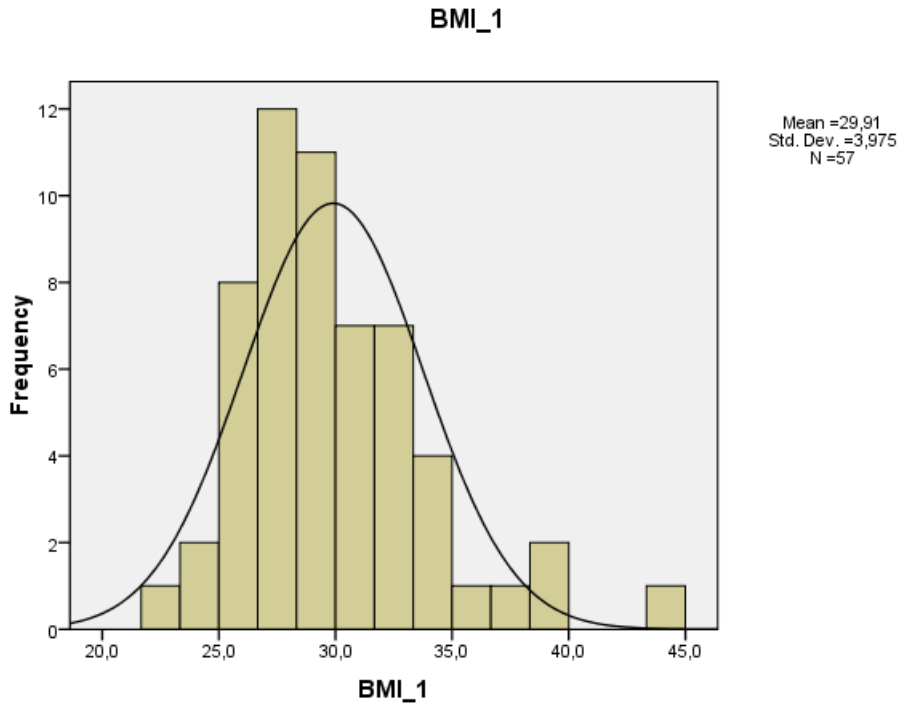

Figure 5 Body mass index (BMI) of participants at Visit 1: frequency histograms and normal curve

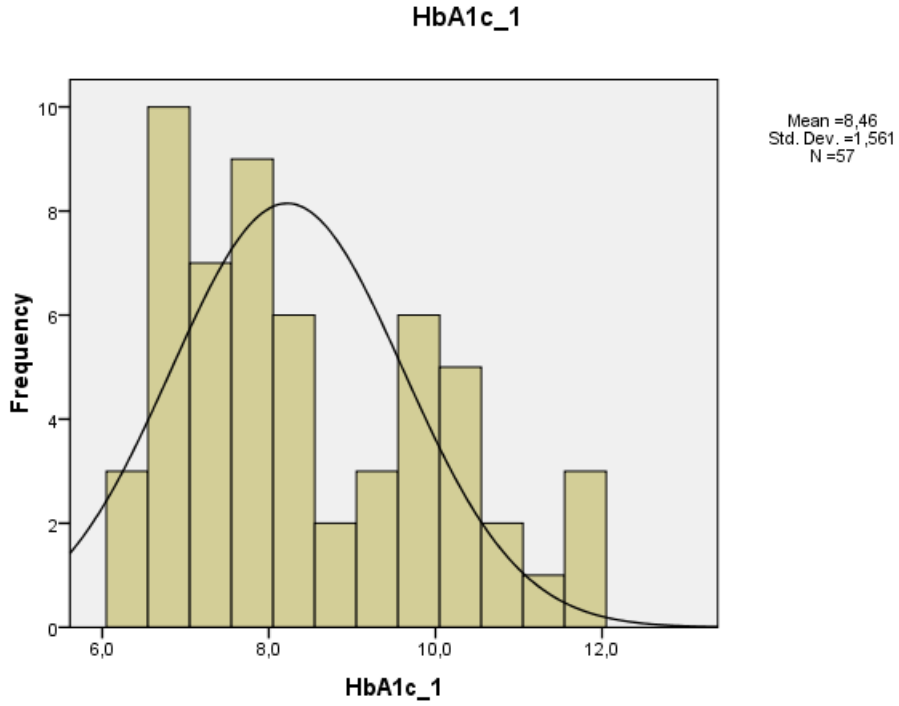

Figure 6 Glycated haemoglobin (HbA1c) of participants at Visit 1: frequency histograms and normal curve

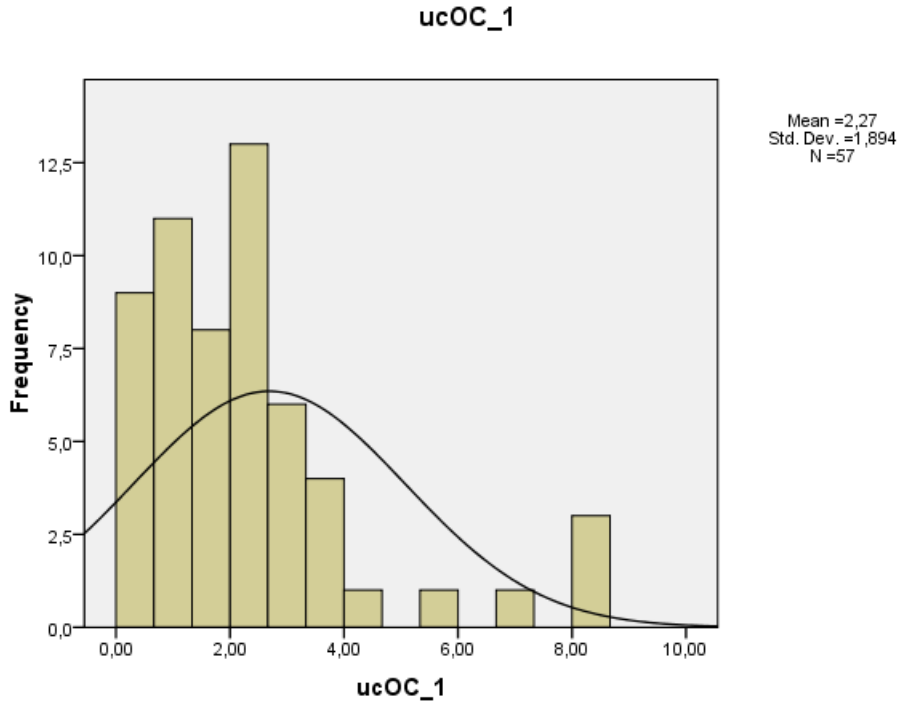

Figure 7 Undercarboxylated osteocalcin (ucOC) of participants at Visit 1: frequency histograms and normal curve

A total of 47 patients completed the study. Parameters at Visit 2 are shown in Table 5.

Table 5 Patient parameters at Visit 2

	Mean	Median	Std. Deviation	Range	Minimum	Maximum
HbA1c_2 [%]	6.9	6.5	1.2	4.9	5.0	9.9
WEIGHT_2 [Kg]	86.8	85.0	14.3	66.0	61.0	127.0
BMI_2 [Kg/m^2]	29.2	28.7	3.5	18.4	22.6	41.0
FBG_2 [mmol/L]	7.2	7.0	0.9	4.0	6.0	10.0
insulin_2[uU/L]	22.6	21.5	17.7	69.0	3.8	72.9
HOMA B% _2	28.0	23.8	16.1	68.1	5.2	73.3
HOMA S%_2	312.1	188.7	256.4	1029.6	56.7	1086.3
HOMA-IR_2	0.5	0.5	0.4	1.7	0.1	1.8
TOC_2 [ug/L]	12.6	12.9	4.3	18.3	7.0	25.3
ucOC_2 [ng/mL]	1.5	1.4	1.0	3.3	0.2	3.5
crosslaps_2 [ug/L]	0.2	0.2	0.1	0.4	0.1	0.5

Numeric variables were tested for distribution normality using the Shapiro-Wilk's test. All variables were distributed significantly different than normal distribution (as presented in Fig 4-7). Therefore, non-parametric tests were used for group comparison (Wilcoxon signed rank test) and correlations (Spearman rho test). The level of statistical significance was chosen to be 0.05.

Correlation of total and undercarboxylated osteocalcin levels with BMI, insulin, HOMA indexes were tested. The only significant correlation was a negative one between BMI and ucOC level at Visit 1 (Spearman's rho -0.295; p=0.026) (Figure 8).

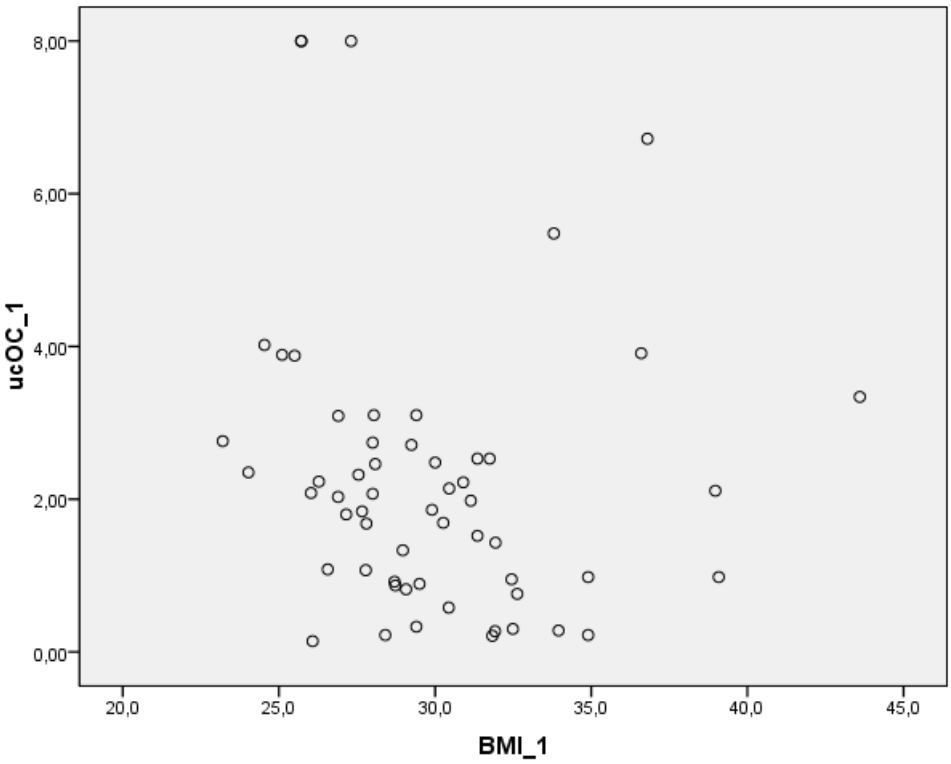

Figure 8 Scatter plot for undercarboxylated OC level and BMI at Visit 1

Average weight, BMI, HbA1c, FBG, insulin, HOMA B%, HOMA S%, HOMA-IR, total and undercarboxylated osteocalcin (ucOC) and crosslaps at Visit 1 and 2 are presented by box-and-whiskers plot ((median, quartiles, range, outliers and extremes) shown in Fig 9-19.

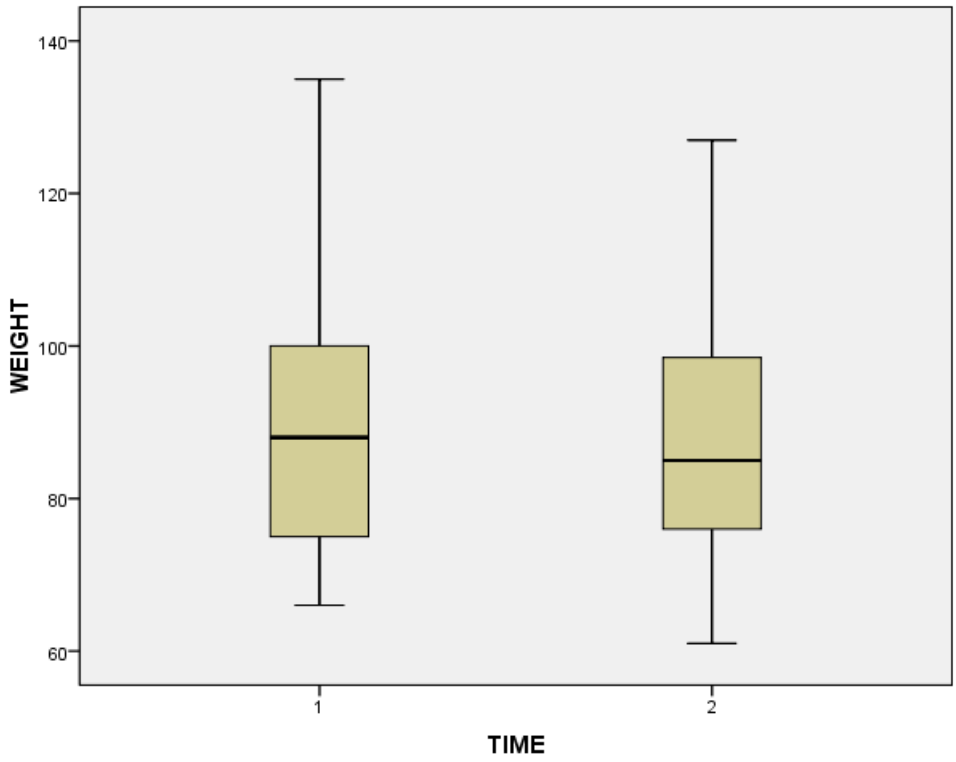

Figure 9 Body weight (kg) at Visit 1 and 2

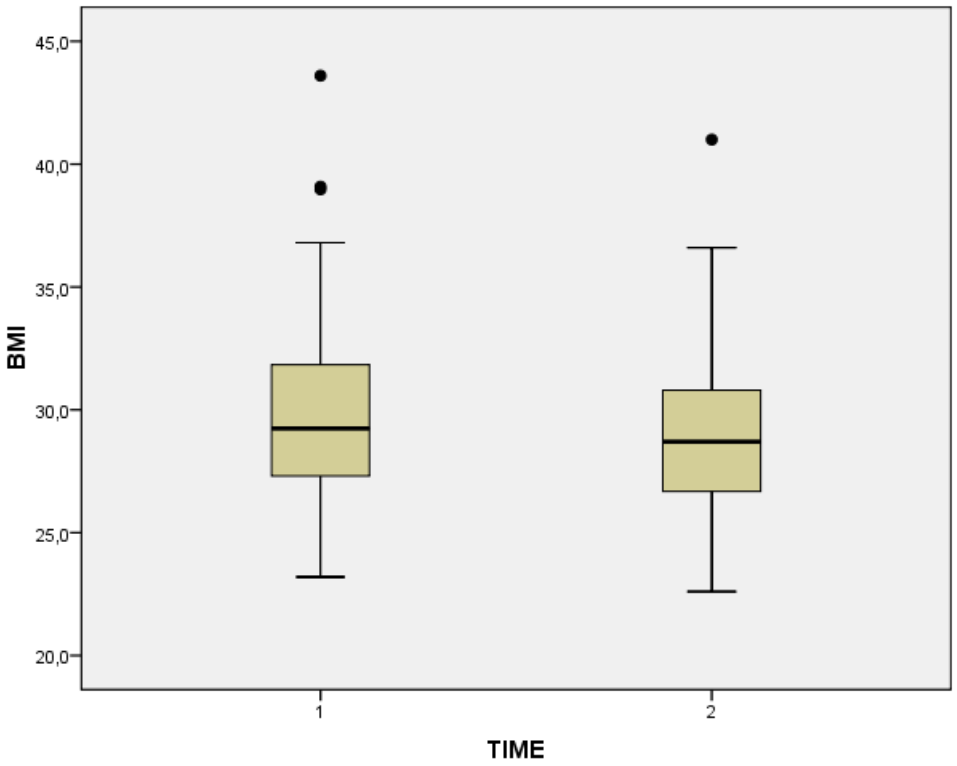

Figure 10 Body mass index (BMI) at Visit 1 and 2

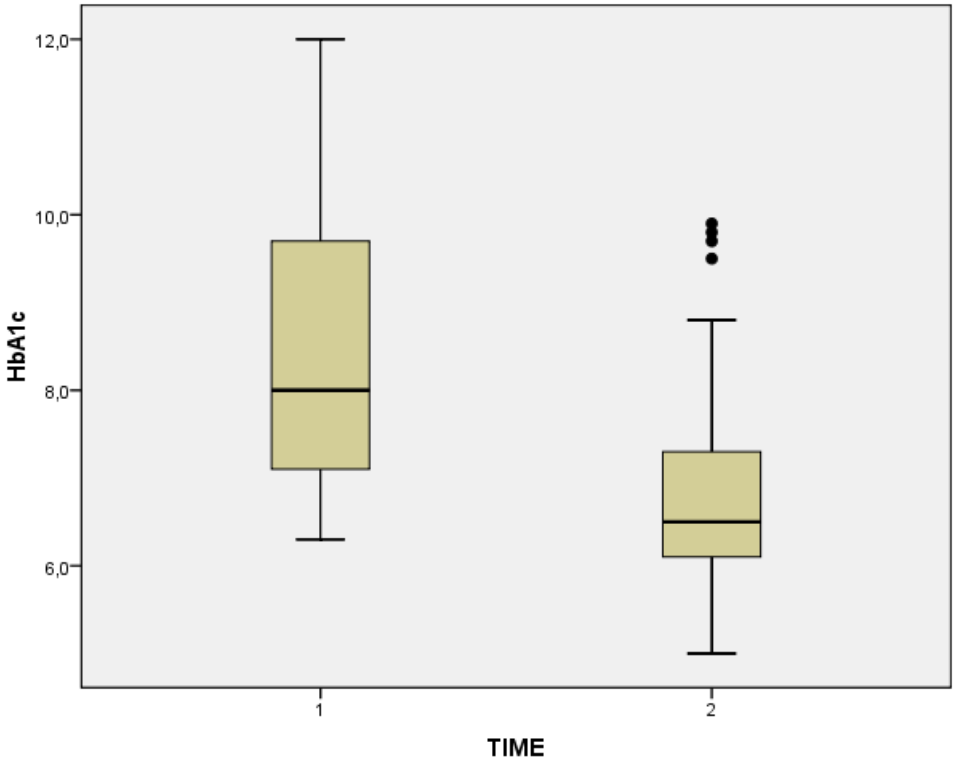

Figure 11 Glycated haemoglobin (HbA1c) at Visit 1 and 2

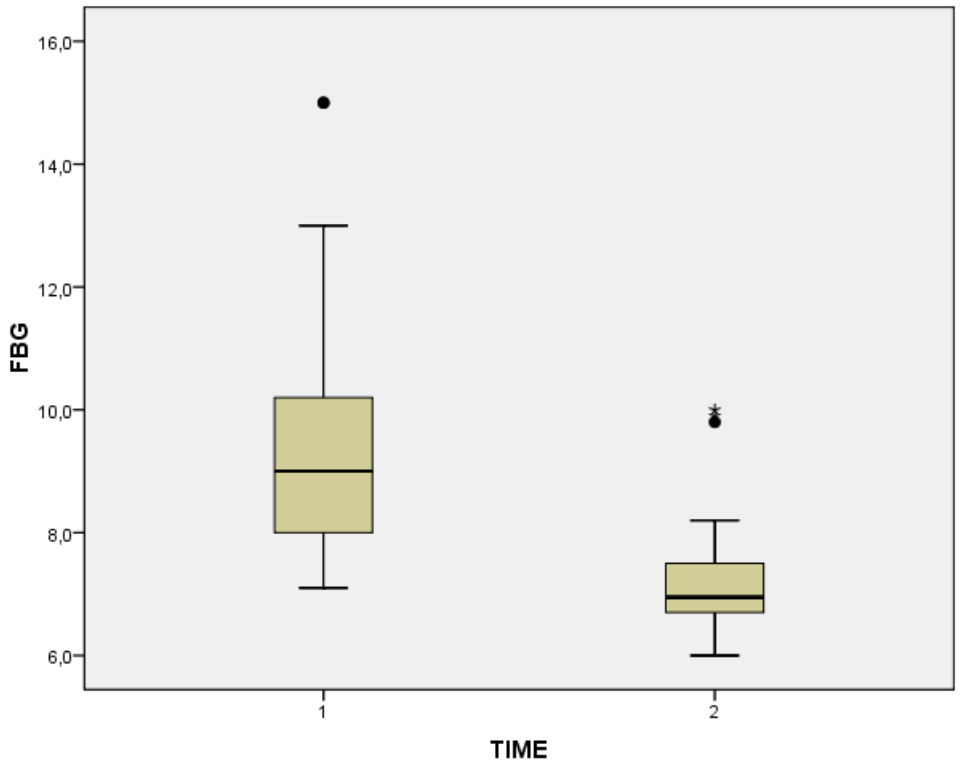

Figure 12 Fasting blood gucose (FBG) at visit 1 and 2

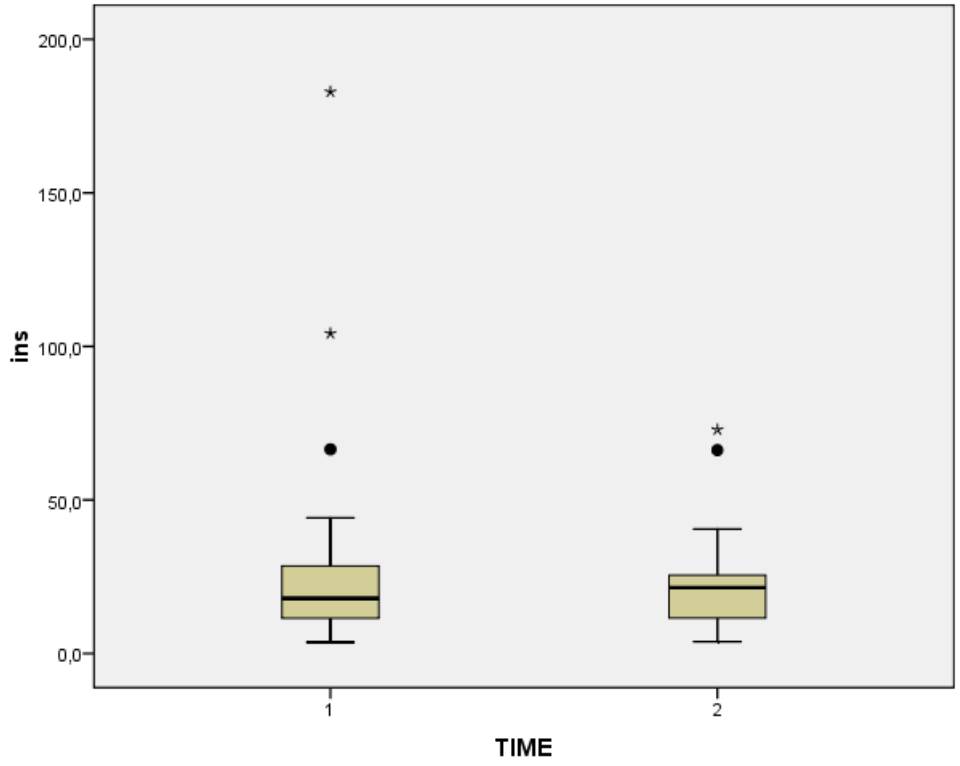

Figure 13 Insulin (ins) at Visit 1 and 2

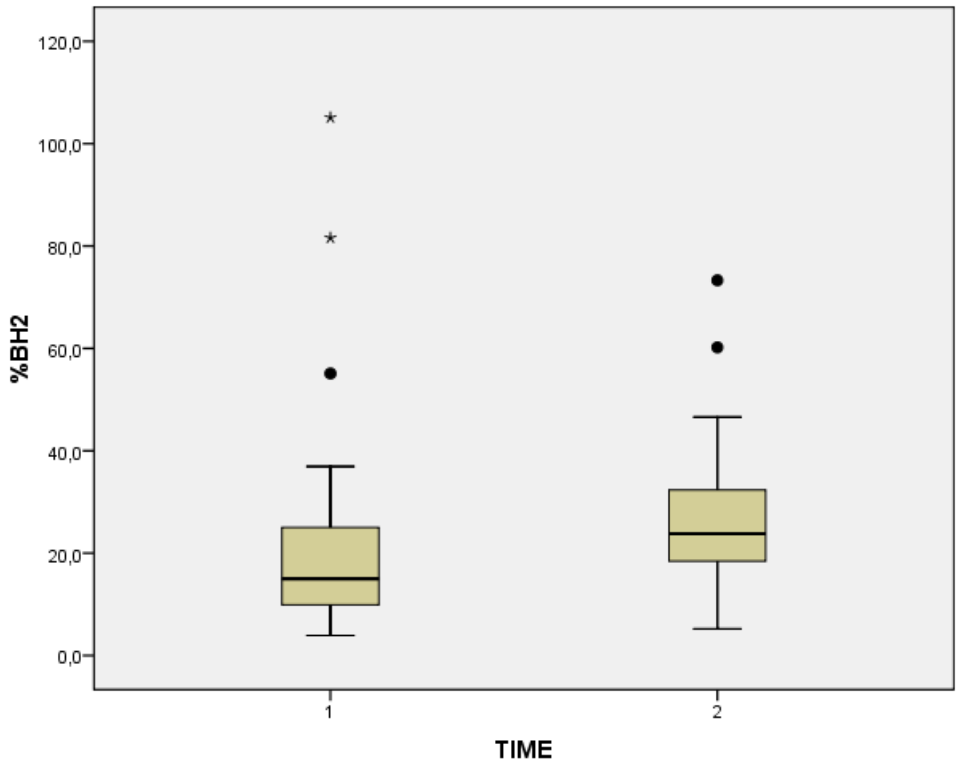

Figure 14 HOMA B% at Visit 1 and 2

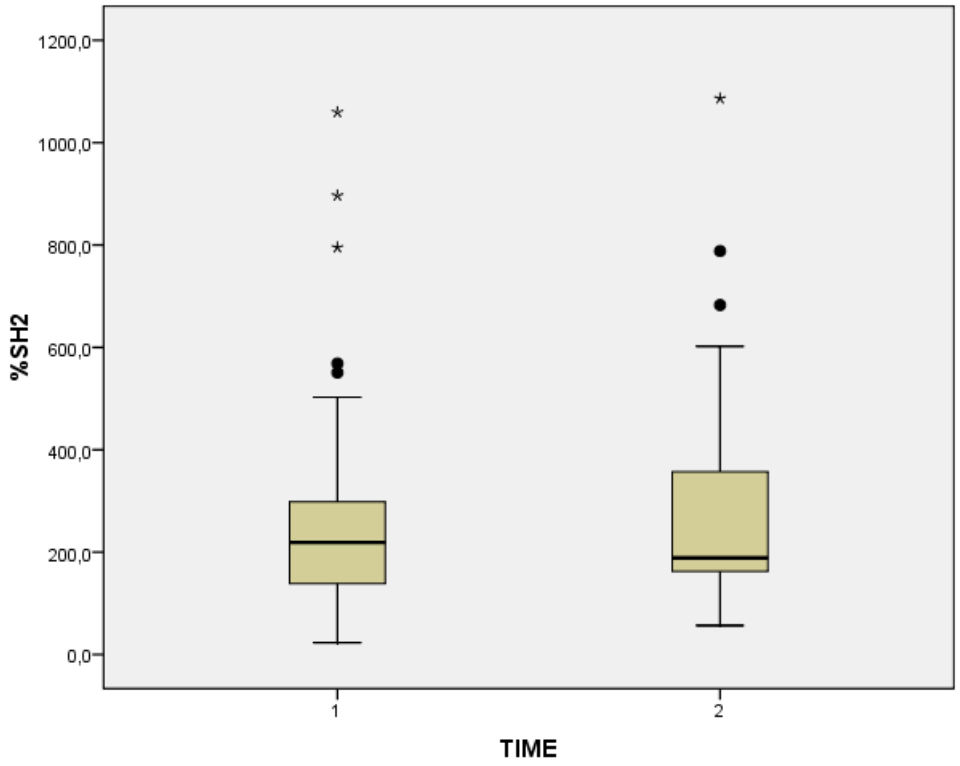

Figure 15 HOMA S% at Visit 1 and 2

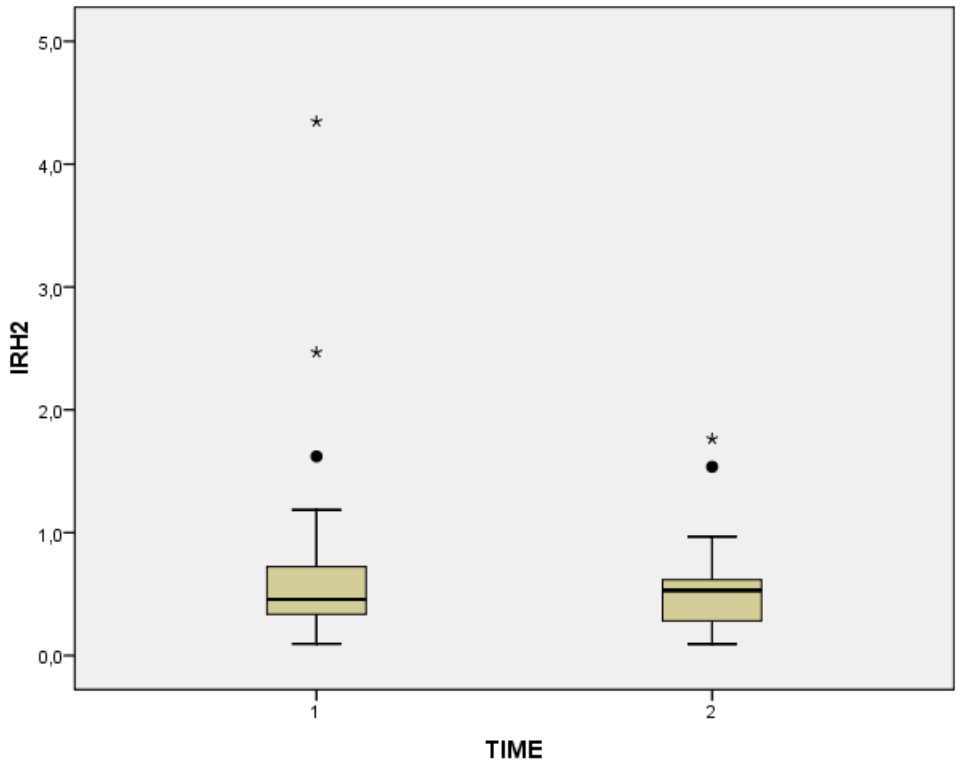

Figure 16 HOMA-IR at Visit 1 and 2

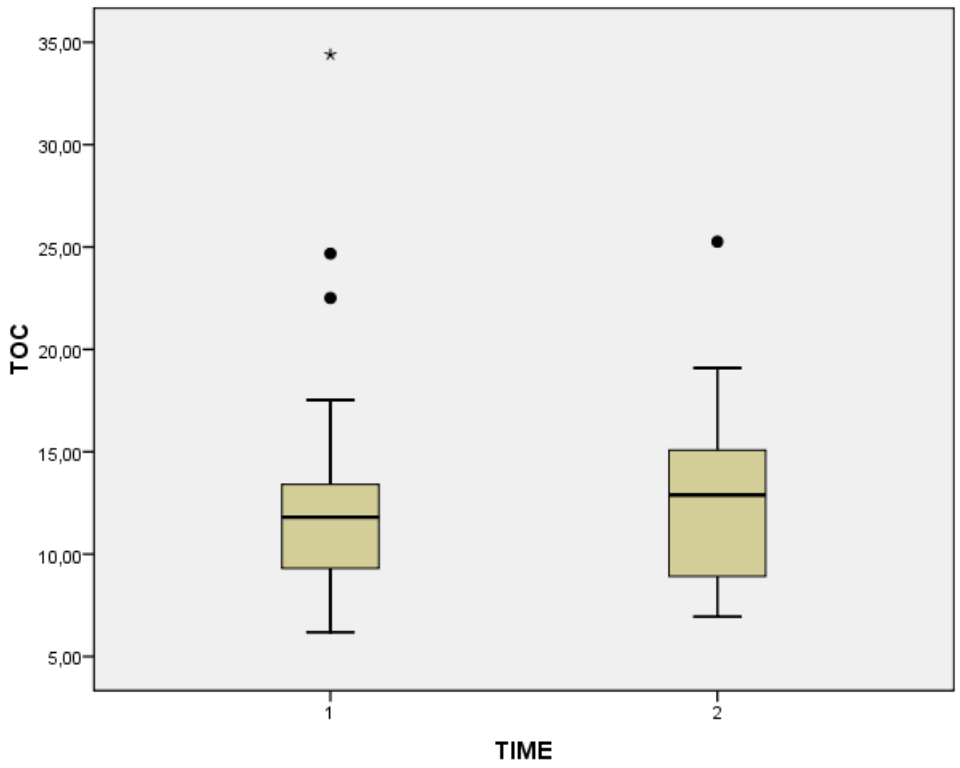

Figure 17 Total osteocalcin (TOC) at Visit 1 and 2

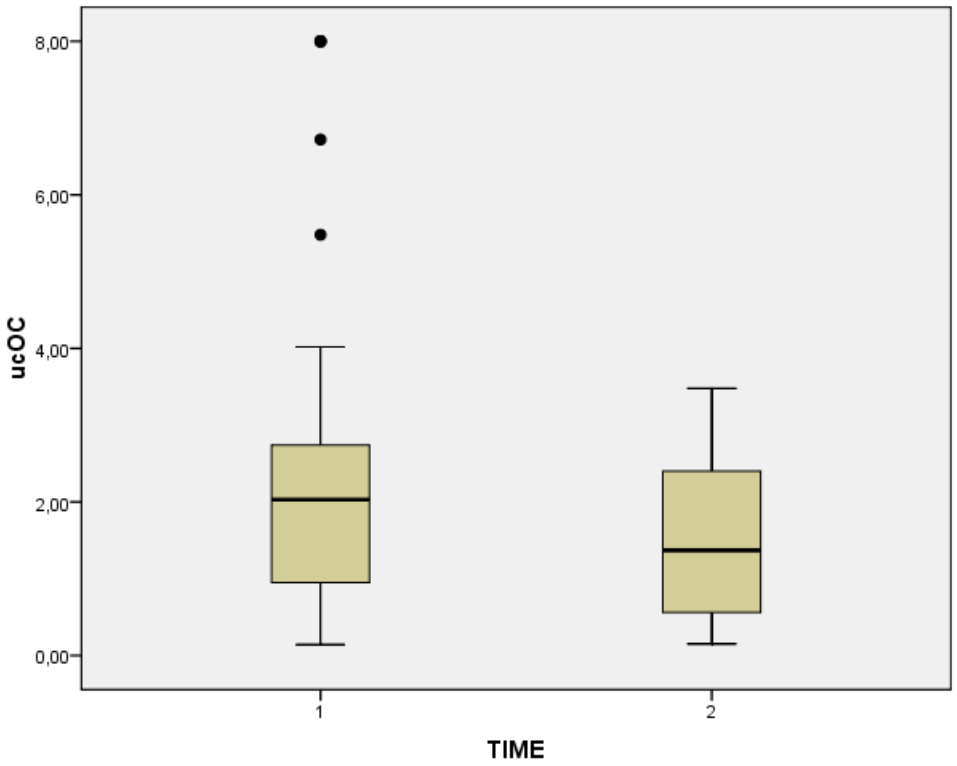

Figure 18 Undercarboxylated osteocalcin (ucOC) at Visit 1 and 2

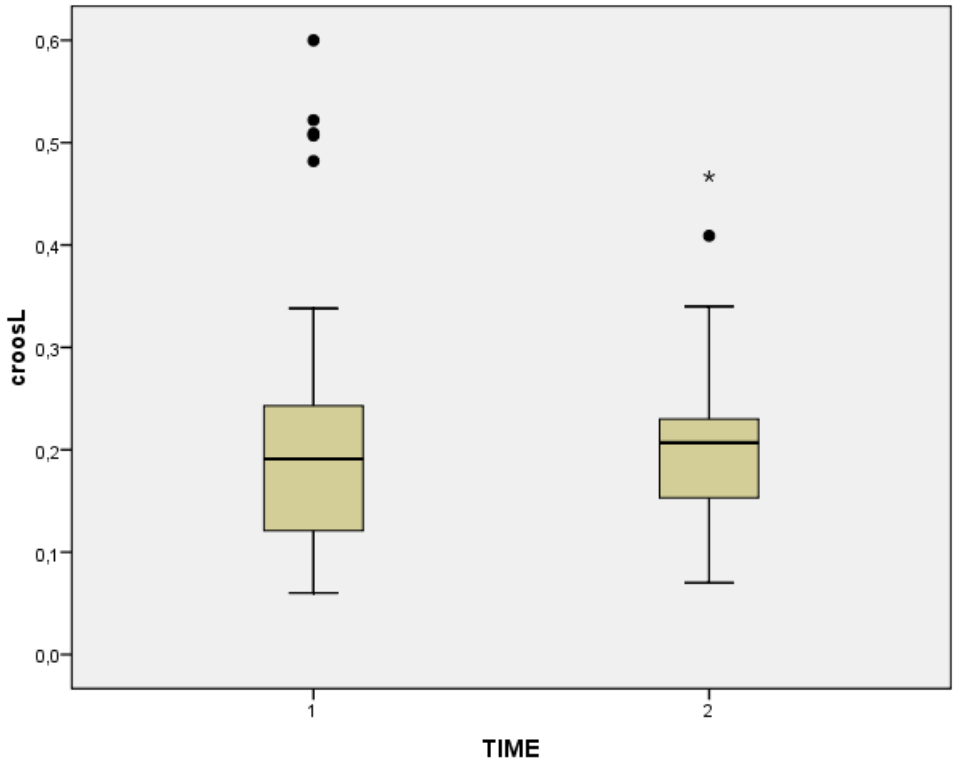

Figure 19 crosslaps (croosL) at Visit 1 and 2

To test significance of changes from Visit 1 to 2, Wilcoxon signed ranks test was performed (Table 6).

Table 6 Weight, BMI, BG regulation parameters, HOMA indexes and total and undercarboxylated osteocalcin levels at Visit 1 and 2

	VISIT 1		VISIT 2		p value
	Mean	Median	Mean	Median	
WEIGHT [Kg]	88.8	88.0	86.8	85.0	<0.001
BMI [Kg/m^2]	29.9	29.2	29.2	28.7	<0.001
HbA1c [%]	8.5	8.0	6.9	6.5	<0.001
FBG [mmol/L]	9.5	9.0	7.2	7.0	<0.001
Ins [uU/L]	24.9	18.0	22.6	21.5	0.693
HOMA B%	19.8	15.0	28.0	23.8	0.001
HOMA S%	259.8	218.9	312.1	188.7	0.606
HOMA-IR	0.6	0.5	0.5	0.5	0.563
TOC [ug/L]	12.2	11.8	12.6	12.9	0.101
ucOC [ng/mL]	2.3	2.0	1.5	1.4	0.465
ucOC/TOC	0.14	0.11	0.09	0.13	0.575

There was a significant change in weight, BMI, HbA1c, FBG and HOMA%B but no HOMA IR (estimate of insulin resistance). Mean ucOC was lower at Visit 2, however the difference did not reach significance as well as the difference of ucOC/TOC ratio.

To test a possible association of difference in HbA1c and undercaboxylated osteocalcin (ucOC) levels between Visits 1 and 2 (Figure 20), Spearman rho test was performed. Correlation coefficient was -0.177; nonsignificant (p=0.431)

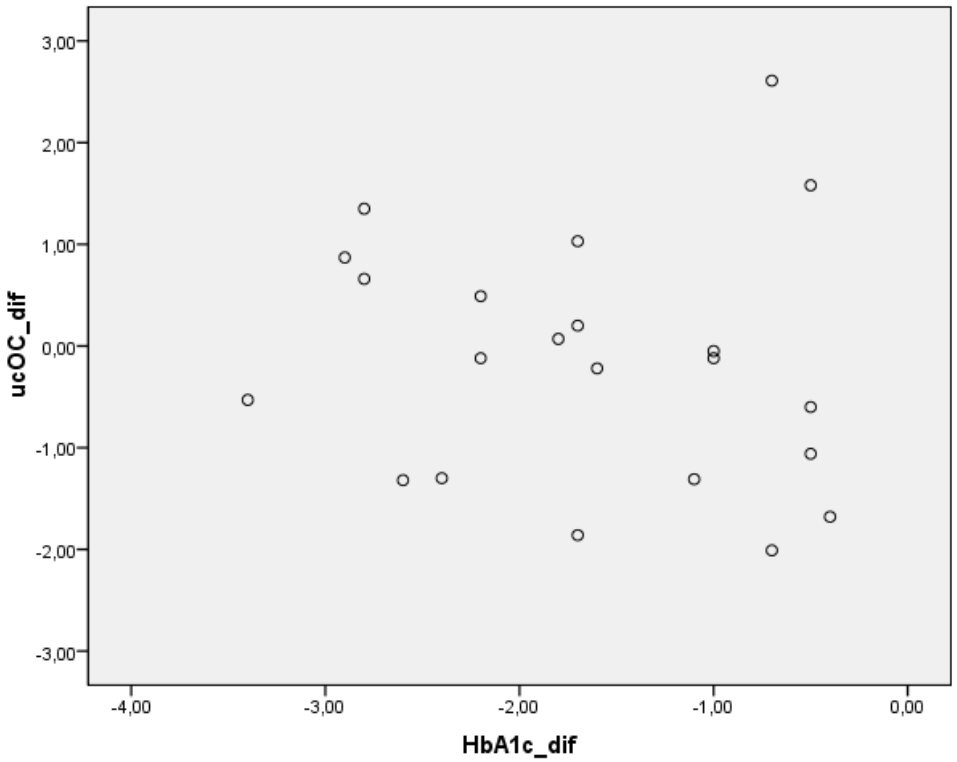

Figure 20 Scatter plot of change in HbA1c vs ucOC between Visits 1 and 2

Median drop in HbA1c between visits 1 and 2 was 1.6%. Median ucOC level in patients with a drop higher than 1.6% was 2.35 ng/mL: no significantly (p=0.22) higher than in those with a lower drop in HbA1c: 1.21 ng/mL

Also, to test a possible association of difference in FBG and undercaboxylated osteocalcin (ucOC) levels between Visits 1 and 2 (Figure 21), Spearman rho test was performed. Correlation coefficient was -0.221; nonsignificant (p=0.324)

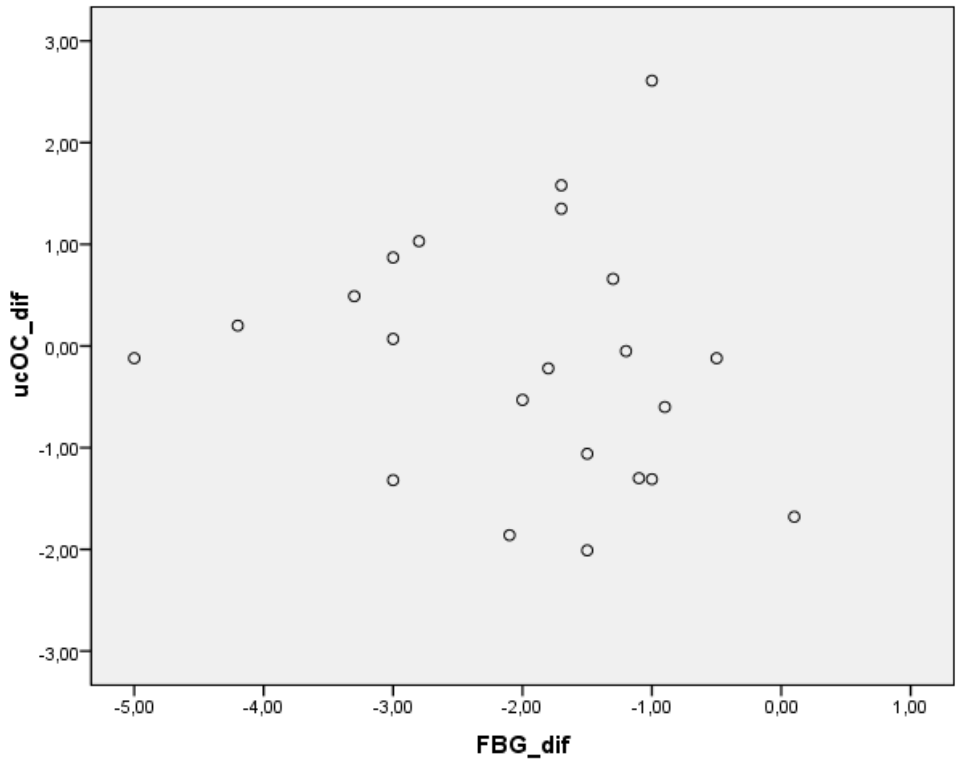

Figure 21 Scatter plot of change in FBG vs ucOC between Visits 1 and 2

Spearman rho test was performed to test a possible association of difference in BMI and undercaboxylated osteocalcin (ucOC) levels between Visits 1 and 2 (Figure 22). Correlation coefficient was 0.216; nonsignificant (p=0.334).

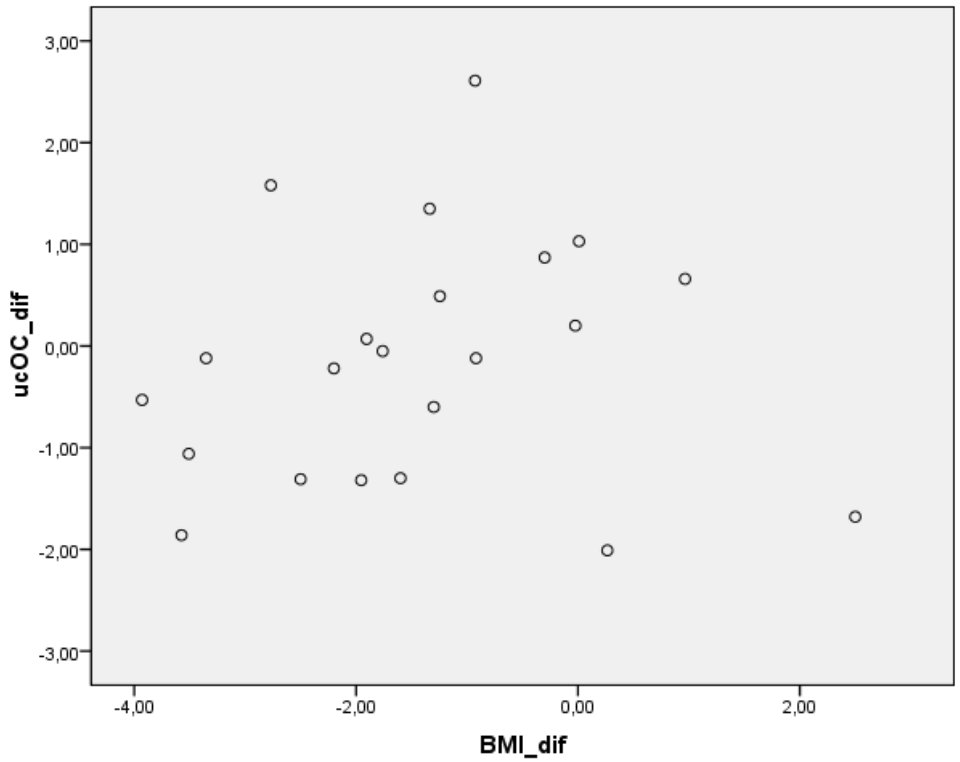

Figure 22 Scatter plot of change in BMI vs ucOC between Visits 1 and 2

6 DISCUSSION

To our knowledge, this study is the first prospective study planned for monitoring the level of the two forms of osteocalcin, TOC and ucOC, in type 2 diabetics, before and after three-month improvement of blood glucose control without antidiabetic therapy in a European population.

Even though we achieved significant change in weight, BMI, HbA1c, FBG and HOMA%B (rough estimate of beta cell function) but not in HOMA IR and HOMA%B (estimate of insulin resistance), we did not find significant change in TOC and ucOC levels with BG levels improvement in three months of follow up with only life style change and no medication for diabetes. Total and undercarboxylated osteocalcin levels did not correlate with BMI, insulin or HOMA IR indexes. The only significant correlation was a negative one between BMI and ucOC level at first Visit.

Mean ucOC was lower at Visit 2, however the difference did not reach significance as well as the difference of ucOC/TOC ratio. We tested a possible association of difference in HbA1c and ucOC levels between Visits 1 and 2 and we found a nonsignificant correlation.

The animal model study conducted by Lee et al. found that OC gene knockout ($OC^{-/-}$ mice) could lead to elevated blood glucose and insulin levels, as well as decreased insulin sensitivity[25], suggesting that OC could in turn regulate glucose metabolism. In the former studies on OC in humans, the total levels of OC were reported lower in patients with T2D and serum OC would increase after blood glucose was controlled[28]. After the discovery of the endocrine properties of ucOC in mice, it was necessary to evaluate the relationship between ucOC and glucose metabolism in humans. Does ucOC have the similar function in human body? The answer is uncertain, the evidence has been only in part confirmed in humans, results remain conflicting and most of these reports were based on retrospective and cross-sectional analyses of studies that were not specifically designed to determine the relationship between osteocalcin and glucose metabolism. To our knowledge, in all studies with type 2 diabetics one of the confounding factors in this issue might be the treatment with antidiabetic drugs.

The first direct evidence in humans showing that OC regulates energy metabolism might be provided by Confavreux et al.[47]. Two patients undergoing surgical resection of an OC-

producing osteoid osteoma have been described. One day after surgery, ucOC levels had decreased and serum glucose levels had strongly increased[48].

Most previous studies have already demonstrated that TOC levels correlate with fasting blood glucose and HbA1c levels[34, 35, 38]. Indeed, only few studies measured both the total and the undercarboxylated form of osteocalcin, which precludes definitive conclusions regarding the role of osteocalcin carboxylation in glucose metabolism. While some did not determine an association between ucOC and the measures of glycaemia[49], another study found inverse correlation of ucOC only with fasting blood glucose[50].

In our study, from two forms of OC, only ucOC was slightly lower at the end, but did not reach significance as well as the difference of ucOC/TOC ratio. Patients reaching higher drop in HbA1c tend to have higher basal ucOC levels, however this also was not significant. Changes in ucOC between visit 1 and 2, did not correlate with the change in HbA1c or FBG. Kanazawa et al.[41] followed the level of TOC and ucOC in type 2 Japanese diabetic patients before and after a month of improvement of blood glucose control. They found an increase of serum TOC, unsignificant increase of serum ucOC and decrease of ucOC/OC ratio. However, unlike in present study, patients (n=50) were on various antidiabetics.

A possible explanation for the lack of concordance among these studies could be in part related to the current limitations in the measurement of circulating undercarboxylated osteocalcin levels and the lack of standardization among the different techniques[51].

Some studies on Caucasian and Asian populations[34, 36, 52] showed that TOC level was significantly negatively correlated with fasting blood glucose, fasting insulin, and HOMA-IR. A Chinese study showed that TOC was not related to HOMA-IR in type 2 diabetics[39]. Mori et al. also found no significant correlation of ucOC with insulin resistance in type 2 diabetic patients[53]. Some studies found a positive correlation of osteocalcin with only HOMA of beta-cell function (HOMA%B) in diabetic patients, before and after glycaemic control[39, 54].

A recent longitudinal study in elderly men, including those with diabetes, showed that the increase in ucOC levels was associated with improvements in the HOMA-IR, and this association was limited to subjects who were not treated with antidiabetic drugs[55]. Therefore, aberrant glucose metabolism, drug interventions or both may make it more difficult to interrupt the correlation between ucOC levels and insulin resistance. In this regard, Iki et al. clearly showed a significant inverse association between ucOC levels and the HOMA-IR in community-based population without apparent health problems[56].

In our study, there was a significant change only in HOMA%B but not HOMA IR, and it did not correlate with TOC or ucOC.

Some studies demonstrated that TOC negatively correlated with BMI[38, 49] as for ucOC are reported no correlations with BMI[49]. In our study, we observed a decrease in BMI between Visit 1 and 2 with only life style change (diet and exercise) and no antidiabetic medication, but not a significant correlation of BMI change with the change in ucOC level.

In our study, ucOC was slightly lower at the end, but there was no association with a TOC or Crosslaps, which might suggest no biological association of this parameter with bone metabolism.

Mouse genetic data have established osteocalcin as a positive regulator of insulin production and insulin sensitivity. There is also a significant body of clinical correlations between low levels of osteocalcin and various measures associated with diabetes. These combined data suggest that raising serum osteocalcin levels could be a novel therapeutic avenue to prevent or treat diabetes. At the present time, the therapeutic potential of osteocalcin has only been tested *in vivo* in two mouse models of metabolic dysregulation. In a first set of experiments, recombinant osteocalcin or vehicle was infused subcutaneously into mice fed a high-fat diet. The mice receiving osteocalcin gained significantly less weight, were less glucose intolerant and were less insulin resistant than mice receiving vehicle[33]. Osteocalcin-treated mice also showed normalised triacylglycerol levels. In a second experiment, mice were pre-treated with gold thioglucose, a chemical destroying neurons of the arcuate nuclei that control satiety, and thus developed hyperphagia. While vehicle-treated mice gained weight and became glucose intolerant and insulin resistant, osteocalcin-treated mice remained normoglycaemic and had normal response to glucose and insulin [33]. Although of very limited scope, the successful outcome of these studies is a strong incentive to further evaluate the therapeutic potential of osteocalcin.

Strengths of the study: treatment-naive newly diagnosed patients who are not prescribed any blood glucose lowering medications.

Limitations of the study: small number of participants.

There is a paucity of published data on undercarboxylated osteocalcin levels in humans and their possible changes in diabetes, so the estimated number might be too low to reveal the trend.

Basic of glucose regulation is in any case a diet and exercise intervention or the life style change, which is considered the basic treatment. For this recommendation there is high-quality evidence. The effectiveness of non-pharmacological therapy is reflected in the fact that it is capable of lowering the HbA1c by about 2 %[57].

When the life style change cannot achieve good glucose regulation (desirable HbA1c), the treatment begins with metformin for which there is a strong recommendation based on evidence of high quality. The ADA and the German Diabetes Society recommend to immediately begin the treatment with metformin at diagnosis point unless a contraindication or intolerance for metformin exists (Figure 1). If a contraindication or intolerance for metformin does exist, the therapy is recommended with a substance that is approved for monotherapy (acarbose, PPAR- γ ligands, repaglinide, sulphonylurea (alphabetical list)), if the HbA1c is still >6.5% after 3-6 months of non-pharmacological treatment.

The guidelines of the International Diabetes Federation (IDF) distinguish normal and alternative approach that does not include the initial phase of pharmacotherapy treatment. First-line treatment is only a basic treatment, which involves a lifestyle change (Figure 2).

In our study, a considerable number of patients achieved the target HbA1c (32 patients at Visit 2 had HbA1c <7%) without introducing drug therapy, which challenges the current guidelines – to prescribe medication from the very beginning.

7 CONCLUSIONS

The present study measured two forms of osteocalcin, TOC and ucOC, in newly diagnosed type 2 diabetic patients, before and after three-month improvement of blood glucose control without antidiabetic therapy.

Even though we achieved significant change in weight, BMI, HbA1c, FBG and HOMA%B (rough estimate of beta-cell function) but not in HOMA IR or HOMSA%S (estimates of insulin resistance), we did not find a significant change in TOC and ucOC levels after three months. Median ucOC was lower at Visit 2, however the difference did not reach significance.

Total and undercarboxylated osteocalcin levels did not correlate with insulin, HOMA IR indexes or BMI.

In our study, ucOC was slightly lower at the end, but there was no association with TOC or crosslaps, which shows that it is not related to bone metabolism.

Although this study did not show changes in osteocalcin levels with blood glucose regulation, it does not exclude them. Sample size, which was calculated from the data from published studies, might be too low. Larger studies possibly with different design are warranted.

The hypothesis of an association between skeleton and energy metabolism could be supported in recent years by experiments in mouse models and by some, but not all, mainly observational data from a modest number of clinical studies. The exact function of osteocalcin, especially its undercarboxylated form in metabolic pathways, remains to be clarified.

8 SAŽETAK (ABSTRACT IN CROATIAN)

Uvod: Od 2007. je objavljeno nešto *in vitro* i *in vivo* ispitivanja koja ukazuju na moguću ulogu podkarboksiliranog osteokalcina (ucOC) u energetskom metabolizmu i glukoregulaciji.

Ciljevi: Cilj studije je ispitati odnos ucOC i kontrole glukoze u plazmi (GUP) u osoba s novootkrivenom šećernom bolešću tipa 2 i promjenu s poboljšanjem glukoregulacije.

Pacijenti i metode: Pedeset sedam osoba s novootkrivenom šećernom bolešću tipa 2 je dobilo savjetovanje o promjeni stila života, nije prepisana medikamentozna terapija za kontrolu GUP na pregledu 1. Zaključni pregled - 2 je bio tri mjeseca kasnije. Uzorci za određivanje parametara glukoregulacije i koštane pregradnje su uzeti na pregledu 1 i 2.

Rezultati: Četrdeset sedam ispitanika je kompletiralo ispitivanje. Trideset dvoje (56%) ispitanika je postiglo ciljnu razinu HbA1c (≤7%). Nije uočena povezanost ucOC sa HbA1c i GUP. HbA1c i GUP su se značajno promijenili (median 8,0 na 6,5%; 9,0 na 7,0 mmol/L resp.; Wilcoxon signed rank test $p<0,001$), ucOC je u drugoj posjeti bio neznačajno niži (2,0 na 1,4 mcg/L; $p=0,465$). Nije uočena povezanost razlika HbA1c i ucOC između 1. i 2. posjete. Indeks HOMA%B, no ne i HOMA IR, je pokazao značajnu promjenu, bez povezanosti s ucOC.

Zaključak: ovo ispitivanje nije dokazalo povezanost regulacije glikemije i razine ucOC, međutim niti je isključuje, pa su potrebna daljnja ispitivanja. Činjenica da je čak 56% pacijenata postiglo ciljnu glukoregulaciju po HbA1c je izazov većini današnjih smjernica za glukoregulaciju.

9 ABSTRACT

Introduction: From year 2007. *in vitro* and *in vivo* studies have suggested a role of undercarboxylated osteocalcin (ucOC) in glucose and energy metabolism.

Aims: The aim of the study was to investigate the relationship of ucOC level and blood glucose (BG) control in newly diagnosed type 2 diabetes and its change with BG control improvement.

Subjects and methods: Fifty seven newly diagnosed type 2 diabetic patients had a consultation about life style changes on visit 1, no BG regulation medication was prescribed. The final visit (2) was three months later. Samples for parameters of BG metabolism and bone turnover were collected on visit 1 and 2.

Results: Forty seven patients completed the study. Thirty two (56%) patients reached the target HbA1c (≤7%). No correlation of ucOC and HbA1c and FBG was observed. Median HbA1c and FBG changed significantly (8.0 to 6.5%; 9.0 to 7.0 mmol/L resp.; Wilcoxon signed rank test $p<0.001$), ucOC was slightly but not significantly lower (2.0 to 1.4 mcg/L; $p=0.465$). No correlation between differences in HbA1c and ucOC between Visits 1 and 2 was revealed. There was a significant change in HOMA%B but not HOMA IR, not correlated to ucOC.

Conclusion: This study failed to prove the relationship between blood glucose regulation and ucOC level. However, it does not exclude it, so further research is needed. The fact that as much as 56% patients achieved the target HbA1c with no medication, challenges most BG control guidelines.

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11 CURRICULUM VITAE

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