Sede Amministrativa: Università degli Studi di Padova
Dipartimento di Medicina Clinica e Sperimentale

DOTTORATO DI RICERCA INTERNAZIONALE IN
IPERTENSIONE ARTERIOSA E BIOLOGIA VASCOLARE
XXIII CICLO

# THE EFFECTS OF GLP-1 ON SIRT1 AND ON THE RELATED METABOLIC PATHWAYS IN VASCULAR ENDOTHELIAL CELLS: POTENTIAL EFFECTS ON VASCULAR INJURY IN DIABETIC PATIENTS

**Coordinatore:** Ch.mo Prof. Gian Paolo Rossi

**Supervisore:** Ch.mo Prof. Andrea Semplicini

**Dottoranda**: dott.ssa Luisa Macchini

# Table of contents

**Riassunto**……………………………………………………………………....3

**Summary**………………………………………………………………...........5

**Introduction**……………………………………………………………………...6

Diabetes: epidemiology and complications………………………………………6

Endothelial disfunction and vascular complications in diabetes…………9

Diabetes treatment…………………………………………………………13

Incretin therapy…..………………………………………………………14

The history of GLP-1………………………………………………………17

The biology of GLP-1………………………………………………………18

Synthesis and secretion of GLP-1 in humans……………………........19

Clearance and metabolism of GLP-1………………………...............24

GLP-1 receptor………………………………………………………...26

Physiological actions of GLP-1………………………………………..28

GLP-1 (9-36) actions..........................................................................32

Effects of GLP-1 on vascular function……………………………………...32

SIRT: role in endothelial disfunction……..………………………………...35

Sirtuins………………………………………………………………………37

Biochemical properties of sirtuins………………………………...............38

SIRT 1………………………………………………………………………38

SIRT1 and transcription factors……………………………….............39

Regulation of SIRT1 expression……………………………....................42

**Aims of the study**……………………………………………………………44

**Material and Methods**………………………………………………………44

Experimental protocol…………………………………………….................44

Experiments in HUVEC cells…………………………………....................45

Cell culture…………………………………………………………………..45

Cell vitality……………………………………………………………..46
Gene expression of SIRT1………………………………………………..46
RNA extraction…………………………………………………………46
Quantification of RNA…………………………………….................46
Reverse transcription of RNA………………………………………47
Real-Time PCR…………………………………………………………47
GLP-1 receptor gene expression in HUVEC……………………………..48
Protein expression of AKT/p-AKT, AMPK/p-AMPK, ERK/pERK..........49
Proteins extraction………………………………………………..…49
Protein assay by an assay with BCA……………………….………..49
Western blot.........................................................................................50
Statistical analysis……………………………………………….........51
**Results**…………………………………………………………………...52
GLP-1 receptor expression in HUVEC…………………………........52
Effects of GLP-1 peptides on SIRT1 gene expression under normoglycemia…….………………………………………..……...52
Effects of GLP-1 peptides on SIRT1 gene expression under hyperglycemia ………………………………………………………54
Effects of GLP-1 peptides on activation of p-AKT, p-AMPK, p-ERK……………………………………………………………………...55
Effects of GLP-1 peptides on activation of p-AKT……………………...56
Effect of GLP-1 peptides on AMPK activation…………………………57
Effects of GLP-1 peptides on ERK activation…………………………...58
**Discussion**……………………………………………………….........59
**Conclusions**……………………………………………………………...61
**References**…………………………………………………………….....62

# RIASSUNTO

***Premesse:*** L'introduzione della terapia incretinica nella cura del diabete ha rivelato un potenziale ruolo protettivo di questi farmaci verso le comorlidità cardiovascolari. Il GLP-1 è dotato di effetti protettivi a livello endoteliale e cardiaco, ma i meccanismi molecolari attraverso i quali il GLP-1 esercita tali azioni sono solo in parte conosciuti. Recentemente, è stato dimostrato che SIRT1, una protein- deacetilasi $NAD^+$dipendente, esercita un ruolo protettivo da danno vascolare nel diabete, attraverso la riduzione dello stress ossidativo cellulare. In diversi modelli cellulari e animali di diabete l'espressione genica di SIRT1 è ridotta. SIRT1, può dunque rappresentare un nuovo target terapeutico per la prevenzione delle complicanze macrovascolari nel diabete mellito.

***Scopo:*** Valutare gli effetti di GLP-1 sull'espressione genica di SIRT1, in cellule endoteliali in condizioni di normoglicemia e di iperglicemia. Identificare, inoltre, le vie metaboliche attivate dal GLP-1, nella regolazione di SIRT1.

***Metodi:*** Gli esperimenti sono stati eseguiti *in vitro*, in colture di cellule endoteliali (HUVEC). L'espressione genica di SIRT1 è stata misurata con RTPCR, in cellule HUVEC dopo trattamento con i peptidi di GLP-1, GLP-1(7-37) e GLP-1(9-36)$NH_2$ in condizioni di normoglicemia (glucosio 5.5 mM) ed iperglicemia (glucosio 20mM). È stata successivamente valutata l'espressione proteica e l'attivazione delle vie Akt, AMPK ed ERK mediante western blot.

***Risultati:*** L'espressione genica di SIRT1 è ridotta nelle cellule endoteliali in condizioni di iperglicemia. Entrambi i peptidi di GLP-1 sono in grado di normalizzare l'espressione genica di SIRT1 in condizioni di iperglicemia. In condizioni normoglicemiche non vi è alcun effetto di GLP-1 sull'espressione genica di SIRT1. Inoltre si è visto che i due peptidi stimolano due vie metaboliche differenti: il peptide GLP-1(7-37) attiva la via Akt, mentre il GLP-1(9-36)$NH_2$ attiva la via AMPK.

***Conclusioni:*** La normalizzazione dell'espressione genica di SIRT1 rappresenta un nuovo e promettente target terapeutico per il diabete mellito. La normalizzazione dell'espressione di SIRT1 da parte dei due peptidi GLP-1, apre nuove prospettive per l'impiego della terapia incretinica nella prevenzione delle complicanze macrovascolari nel diabete mellito. L'evidenza di due vie metaboliche differenti attivate dai due peptidi

permette di ipotizzare dei meccanismi indipendenti da parte di GLP-1(7-37) e GLP-1(9-36)$NH_2$ nella regolazione dell'espressione di SIRT1, aprendo la ricerca di nuovi bersagli molecolari per la terapia del diabete con GLP-1

# SUMMARY

***Background:*** Incretin therapy may have a potential protective role in the treatment of diabetes associated with the cardiovascular comorbidities. GLP-1 may have beneficial effects in the endothelium and in the cardiac tissue although the molecular mechanisms by which GLP-1 exerts these actions are still unknown. Recently, it has been demonstrated that SIRT1, a NAD'+ deacetylase protein, has a protective role on vascular damage in diabetes by reducing oxidative stress. In several cells and in animal models of diabetes SIRT1 gene expression is reduced. Therefore, SIRT1 may represent a new therapeutic target in the prevention of macrovascular complications of diabetes.

***Aim:*** To investigate the effects of GLP-1 on SIRT1 gene expression in human umbelical vein endotelial cells (HUVEC) grown at normal and high glucose. Furthermore, we explored the molecular pathways by which GLP-1 may regulate SIRT1 gene expression.

***Methods:*** HUVEC were cultured at 5.5 mM o 20 mM glucose in the presence/ absence of two GLP-1 peptides: GLP-1(7-37) and GLP-1(9-36)$NH_2$. Gene expression of SIRT1 was assessed by RT-PCR real time. Akt, AMPK and ERK phosphorylation were measured by Western Blot.

***Results:*** SIRT1 gene expression was significantly reduced in HUVEC grown at 20mM in comparison to cells grown at 5.5 mM of glucose. The treatment of cells with GLP-1(7-37) or with GLP-1(9-36)NH2 increased SIRT1 gene expression at 20mM glucose but not at 5.5 mM glucose. Furthermore, GLP-1(7-37) e GLP-1(9-36)NH2 enhanced two different intracellular pathways: GLP-1(7-37) stimulated Akt while GLP-1(9-36)NH2 activated AMPK.

***Conclusions:*** SIRT1 is a new and promising therapeutic target for diabetes. The increase of SIRT1 expression by GLP-1 peptides opens new perspectives for the use of incretin therapy in the prevention of diabetic macrovascular complications. Furthermore, the evidence of different GLP-1-activated pathways allows us to hypothesize different mechanisms in the regulation of the expression of SIRT1.

# THE EFFECTS OF GLP-1 ON SIRT1 AND ON THE RELATED METABOLIC PATHWAYS IN VASCULAR ENDOTHELIAL CELLS: POTENTIAL EFFECTS ON VASCULAR INJURY IN DIABETIC PATIENTS

# INTRODUCTION

## DIABETES: EPIDEMIOLOGY AND COMPLICATIONS

As stated in the guidelines developed by the American Diabetes Association (ADA) and the International Diabetes Federation (IDF), diabetes mellitus is defined as a heterogeneous group of disorders, characterized by the common finding of hyperglycemia and glucose intolerance, caused by deficiency in insulin secretion or impaired insulin peripheral action or both (1,2).

Diabetes mellitus is classified based on etiology and clinical features into four main subtypes: diabetes mellitus type 1 (DM1), diabetes mellitus type 2 (DM2), gestational diabetes mellitus (GDM) and other specific types of diabetes (1). Diabetes mellitus type 2 (DM2) is the most frequent in the population, representing 95% of all types of diabetes, while other forms of diabetes are less frequent (3).

The rapidly increasing prevalence of diabetes mellitus worldwide in recent years, is one of the most serious and challenging health problems of 21th century, so that some authors speak of "diabetes epidemic" (4). Data collected and revised in 2010 showed an overall prevalence of diabetes of 6.6% with a total number of people affected, which amounts to 285 million (5). The predictions for 2030 prospect a significant increase in cases of diabetes worldwide, with an estimated prevalence of 7.8% for 2030 and a number of cases of diabetes which amounts to 438 million (5). Recently, (September 2011) the IDF published new data on the epidemiology of diabetes, estimat a total number of people affected worldwide, amounting to 366 million (3). The average health care costs for the treatment of diabetes mellitus is of 465 million dollars representing 11% of total health care costs (3). IDF has also reassessed the forecasts to 2030,

estimating a total number of diabetes mellitus of approximately 552 million with an average health care spending of 595 million $ (3).

The implications of these epidemiological aspects regard also the complications of diabetes and therefore the morbidity and mortality associated with this disease. In fact, diabetes is a major cause of premature and sudden death in many countries (3). About 4 million of deaths a year in the U.S., which represent 6.8% of deaths in individuals aged between 20-79 years (similar to mortality from infectious diseases), are attributable to diabetes mellitus (3). Mortality from diabetes has increased by 5.5% from 2007; this is partly attributable to the increase in the prevalence of diabetes and in part to incomplete therapeutic efficacy in preventing mortality due in particular to the macrovascular complications of diabetes (3).

Complications of diabetes mellitus are classified into acute and chronic, the chronic being divided into microvascular, macrovascular and non vascular (Table 1) (1). Chronic complications of diabetes mellitus, in particular micro-and macrovascular complications are the most frequent and are responsible for the majority of morbidity and mortality associated with this disease. In particular, the macrovascular complications characterized by the development of atherosclerosis lead to increased risk (2-4 fold) of myocardial infarction and stroke which are the leading cause of death in diabetes mellitus (3). It is estimated that approximately 50% of people with diabetes die from the onset of acute cardiovascular events (MI, stroke or sudden death) or from their consequences (3). The Framingham study showed a marked increase in risk, from 2 to 5 times, of developing peripheral vascular disease, heart failure, coronary artery disease, myocardial infarction and sudden death in diabetics (6). The risk for cerebrovascular disease (stroke) is increased 3-fold in diabetics (6).

The American Heart Association has indicated diabetes mellitus as one of major risk factors for cardiovascular disease, placing it in the same category of smoking, hypertension and hyperlipidemia (7). Nevertheless, diabetic patients without previous myocardial infarction have a risk of coronary heart disease and related events as high as non-diabetic patients with previous myocardial infarction. Therefore type 2 diabetes is considered a “coronary heart disease equivalent” (8).

| Table I. DIABETES COMPLICATIONS | | |
|---|---|---|
| ACUTE | | 1. diabetic ketoacidosis<br>2. hyperglycemic hyperosmolar state |
| CHRONIC | MICROVASCULAR | 1. eye disease<br>a) non-proliferative and proliferative retinopathy<br>b) macular edema<br>2. neuropathy<br>a) sensitive, motor, mono-polyneuropathy<br>b) autonomic<br>3. nephropathy |
| | MACROVASCULAR | 1. coronary artery disease<br>2. peripheral vascular disease<br>3. cerebrovascular disease |
| | NON- VASCULAR | 1. gastrointestinal<br>2. genitourinary<br>3. dermatologic<br>4. infectious<br>5. cataract<br>6. glaucoma |

The increase in mortality and morbidity is related to hyperglycemia synergism with other cardiovascular risk factors: dyslipidemia, hypertension, obesity, smoking, low physical activity, abnormal platelet adhesion, alterations of the coagulation cascade, increased the oxidative stress and inflammation (9). Several studies have shown that after controlling

for all the known cardiovascular risk factors, DM2 increases the rate of cardiovascular mortality twice in men and four times in women (10).

Hyperglycemia, which is common to for definition all types of diabetes, represents the main pathogenetic factor for the onset and development of complications in diabetes mellitus (9). Endothelial dysfunction, representing the first step toward the progression of vascular damage in diabetes mellitus, is at the base of both microvascular and macrovascular complications (11-13).

## ENDOTHELIAL DYSFUNCTION AND VASCULAR COMPLICATIONS IN DIABETES

The endothelium is an active monolayer, essential for the maintenance of vascular homeostasis, localized on the inner surface of blood vessels, where it forms an interface between circulating blood and the rest of the vessel wall. In physiological conditions, through the production of chemical mediators, the endothelium regulates several processes including: vascular tone and permeability, balance between fibrinolysis and coagulation, inflammatory activity, synthesis of collagen for the extracellular matrix, platelet aggregation, cell proliferation and interaction with other cells including vascular smooth muscle cells, platelets, leukocytes, monocytes, renal mesangial cells and retinal pericytes. (Fig. 1) (11-15). The regulation of vascular tone is mediated by the balanced production of vasodilatory substances such as nitric oxide (NO) and prostacyclin (PGI2), and vasoconstrictive substances like endothelin-1 (ET-1), prostaglandins and angiotensin II (ANG II) (16). In the clotting process, the endothelium acts by, regulating the fibrinolysis through the production of the tissue plasminogen activator activator (t-PA) and its inhibitor (PAI-1) and by limiting the activation of the coagulation cascade by secretion of thrombomodulin, protein C and antithrombin III (17). The endothelial actions on inflammation involve the expression of adhesion molecules such as LAM, ICAM and VCAM for leukocytes and monocytes, which migrate from the bloodstream into the extra-vascular site (17-19). Finally, the balanced production of substances such as PDGF (plated-derived growth factor) and ANG II promotes or inhibits the growth and differentiation of vascular smooth muscle cells (20). All of these physiological properties of the endothelium are more or less altered in endothelial dysfunction.

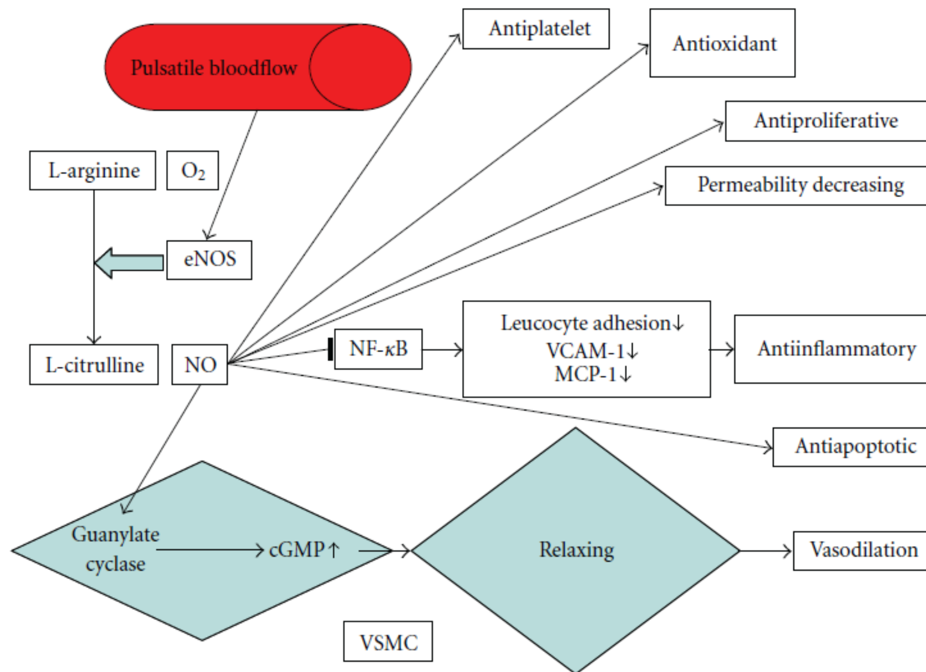


***Figure 1****: Properties and production process of NO (nitric oxide) as important factor in endothelial function.*

The term endothelial dysfunction identifies a condition in which endothelium activities capable to maintain this vasculature homeostasis are compromised (21). This results in an increase of vascular tone mainly due to reduced production of nitric oxide and vessel smooth muscle cell hypertrophy, in an increased synthesis of adhesion molecules for leukocyte which leads to vascular inflammation, in alterations of the coagulation process resulting in increased risk of thrombosis, and in atherosclerosis (22). In diabetes mellitus, endothelial dysfunction is an important pathogenetic factor for vascular complications, and represents the first step for the onset and development of micro-macrovascular (atherosclerotic) complications (9). Numerous studies have demonstrated the relationship between the presence of endothelial dysfunction and subsequent development of diabetic retinopathy, diabetic nephropathy and atherosclerosis in both type 1 and type 2 diabetes (12, 23).

Hyperglycemia is the principal responsible element for endothelial dysfunction in diabetes mellitus, although this is still a multifactorial process to which other concurrent causes may contribute including ageing, obesity, insulin resistance, smoking, hypertension, dyslipidemia and inflammation (13,24,25). The molecular mechanisms by

which hyperglycemia causes endothelial dysfunction are different and some are not yet fully clarified (Fig.2) (14)

Hyperglycemia causes microvascular complications (microangiopathy), increasing cellular oxidative stress and consequent development of endothelial dysfunction through: increased susceptibility to cell damage from free radicals (reactive oxygen species) because of the reduction of cellular redox potentials mediated by activation of the polyol pathway, of hexosamines and the of PKC (protein kinase C), and increased production of free radicals related to the activation of the mitochondrial p66 protein by AGEs (advanced glycosylated end-products), derived from non-enzymatic glycation of proteins induced by hyperglycemia (13,14, 26-31)

Diabetic macroangiopathy recognizes hyperglycemia as its main pathogenic factor, by determining endothelial dysfunction through the induction of vascular senescence process, mediated in part by the same pathogenetic mechanisms of microangiopathy (32).

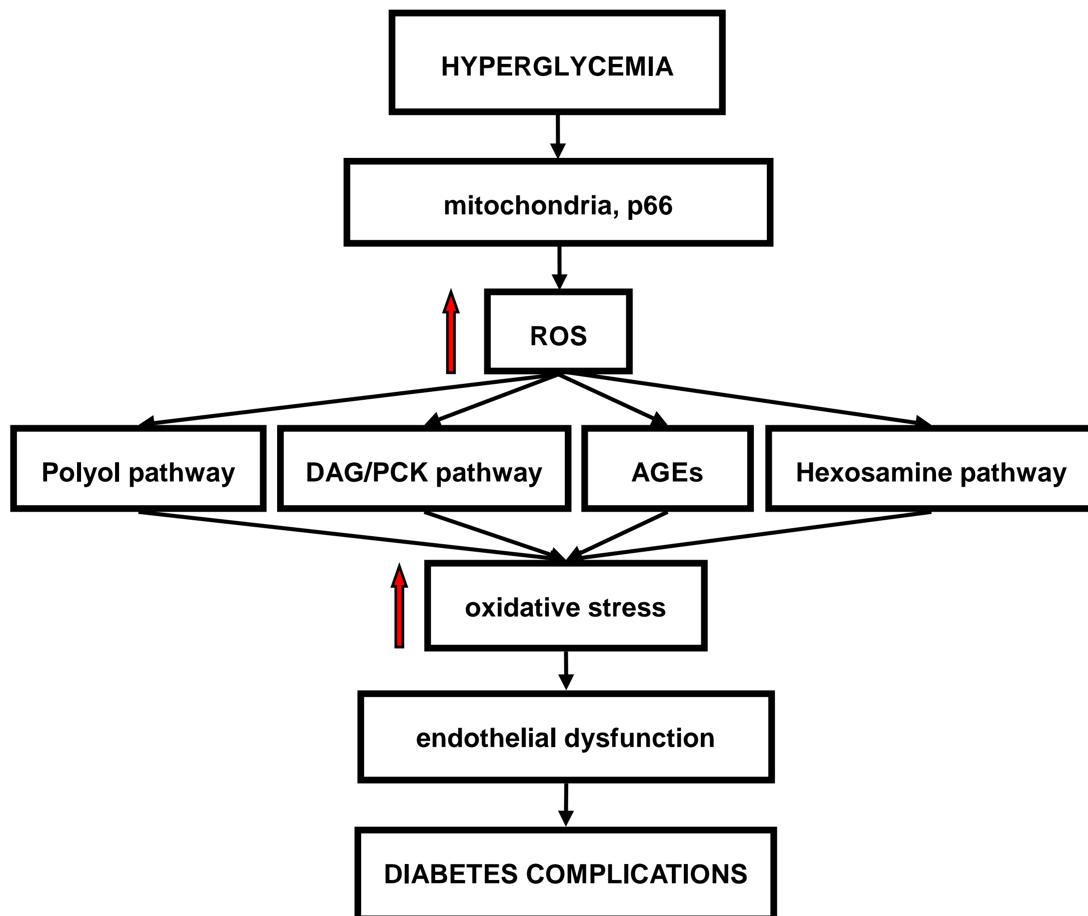


***Figure 2***. *Possible pathogenic mechanism through which hyperglycemia induces endothelial dysfunction*

Currently, AGEs appear to be the main elements that can induce cell and vascular damage causing both micro- and macrovascular complications (33). The currently identified AGEs are 3: pentosidine, pyrraline and carboxymethyl-lysine through their storage and their action on vessels determine the onset of vascular damage in diabetes (33). AGEs cause vascular aging (progressive stiffening and loss of elasticity of the arteries), changes in extracellular matrix, accelerated atherosclerosis, increased endothelial permeability, a procoagulant state and reduced production of nitric oxide (NO) (33). AGEs also interact with monocytes-macrophages through a specific receptor inducing the production of growth factors and cytokines involved in atherogenesis (33). The infiltration of the vascular wall by monocytes represents one of the earliest stages of atherosclerotic lesions (33)

The reduced production of nitric oxide has the most important role in endothelial dysfunction in diabetes mellitus (34). In fact, NO, besides vasodilatory action, presents a set of properties that improve endothelial function and in particular it is able to inhibit platelet aggregation and the proliferation of smooth muscle cells. It decreases the vascular permeability and inflammation by inhibiting the expression of NF-kappaB and adhesion molecules for monocytes (VCAM-1and monocyte chemotactic protein-1 (MCP-1)) and it reduces cellular oxidative stress (34-37).

It was found that insulin is an important stimulator for the activation of eNOS (endothelial nitric oxide synthase). Insulin binding to its receptor induces insulin receptor substrate-1 (IRS-1) phosphorilation and consequently activates eNOS, resulting in increased production of NO, by stimulation of the PI3K/Akt pathway (38-41). This mechanism is strongly impaired in individuals with type 2 diabetes because of insulin resistance that limits this action (42,43). Some studies have demonstrated that infusion of insulin stimulates vasodilation in humans and increases blood flow to peripheral tissues, but in diabetic patients this effect is reduced (44,45). In diabetes mellitus, another pathway involved in endothelial dysfunction is
compromised: the AMPK pathway, whose activation inhibits endothelial cell apoptosis and inflammation through inhibition of NK-kB transcription (46).

Hyperglycemia, besides determining the formation of AGEs, is responsible for the non-enzymatic glycation of laminin and collagen IV resulting in a reduction of the adhesion of endothelial cells, thereby altering the structural integrity of the vascular wall (33). Furthermore hyperglycemia promotes the oxidation of LDL, which contributes

together with the above mentioned events to the development of macrovascular complications (33).

## DIABETES TREATMENT

The cornerstones of diabetes treatment are represented by diet, exercise and pharmacological therapy (1). Because the complications of diabetes mellitus are related to hyperglycemia, the main goal of diabetes therapy is maintaining optimal blood glucose levels, to delay the onset and slow the progression of micro-and macrovascular complications, reducing mortality and morbidity related to the disease (9). However, it appears difficult to achieve a normoglycemic condition with therapy, but several studies have shown that at any level of hyperglycemia, improved glucose control reduces the risk of complications (1). Monitoring of therapeutic efficacy is based on the measurement of blood glucose and glycated hemoglobin (HbA1c), which reflect respectively the current glycemic status and glycemic profile of the last 2-3 months (9,10,33).

Drug therapy of diabetes is currently based on a series of drugs with different mechanisms of action that can be used individually or in combination with each other. Beside pharmacological therapy, it is also very important to maintain a healthy lifestyle through constant physical activity and an appropriate dietary intake. Guidelines for the treatment of diabetes indicate, in fact, as first line therapy for type 2 diabetes lifestyle changes, which must always support all other treatment protocols of diabetes mellitus (1). Drug therapy of diabetes mellitus is based on the use of two main categories of drugs: insulin and its analogues and oral anti-diabetics (ADO) (1,10,33). Oral antidiabetic agents are numerous and are classified on the basis of the mechanism of action in several categories: biguanides, sulfunilureas, thiazolidinediones and other PPAR-α/γ agonists, amylin agonist, incretin mimetics and incretin agonists, dipeptidyl peptidase-4 (DPP-4) inhibitors, inhibitors of the sodium/glucose co-transporter and activators of glucokinase (47). These drugs act by stimulating insuline secretion (sulphonylureas and glinides), lowering insulin-resistance (metformin and glitazones) or slowing glucose absorbtion from bowel lumen (acarbose), but no one has been demonstrated to be able to stop the progressive decline of beta-cell function, neither to lower the elevated glucagon levels (especially the post prandial ones), which are important in the pathogenesis of type 2 diabetes.

Currently, there is no evidence that tight glycemic control results in a reduction of macrovascular complications and consequent mortality. Extended follow-up of UKPDS and DCCT studies demonstrated that intensive glycemic control reduced the long-term incidence of myocardial infarction and death from cardiovascular disease only in those patients who received intensive glucose-lowering therapy very early after diabetes diagnosis, and in contrast to blood pressure and lipid-lowering interventions a reduction of CV mortality cannot be seen before 10-20 years after the start of the glucose-lowering intervention. Several international trials (ACCORD, ADVANCE, VADT) showed that careful adjustment of blood sugar and glycated haemoglobin is more effective in counteracting microvascular damage than in preventing major cardiovascular events. (24,48-51). The latter objective requires a more comprehensive approach to the whole constellation of modifiable risk factors both specific for diabetes and common to atherothrombosis· (52). This approach includes lifestyle changes (such as dietary changes and smoking cessation, physical exercise ) and treatment for associated comorbidities such as hypertension, obesity and dyslipidemia, if present (1). In spite of the benefic effects of a broad-spectrum therapy and the numerous advancements in glucose-lowering medications in recent years, there remains a substantial incidence of CVD in optimally treated patients with type 2 diabetes. This represents the current limit in diabetes care and at this regard, the study of new drugs potentially able to reduce the occurrence of macrovascular complications is fundamental to improve mortality associated with diabetes.

## INCRETIN THERAPY

Glucagon-like peptide-1, and synthetic GLP-1 receptor agonists represent promising new areas of research and therapeutics in the struggle not only against type 2 diabetes but also against the cardiovascular morbidity and mortality associated with type 2 diabetes (53). Patients with type 2 diabetes have a low concentration of GLP-1 in response to the meal and for this reason treatment with incretin mimetic drugs is specifically indicated in these patients.(54-55)

Native GLP-1 cannot be used for therapeutic purposes because of poor bioavailability due to its unfavorable pharmacokinetic profile. Thus, active GLP-1 metabolites have a short half-life of a few minutes undergoing rapid enzymatic degradation by their primary endogenous inactivator, DPP-4 (56).

The general principle, on which incretin therapy is based, is enhancement of GLP-1 action. For this purpose two types of drugs have been developed: the GLP-1 homologous (exenatide, liraglutide) and DDP-4 inhibitors (sitagliptin, vildagliptin and saxagliptin) (57). The GLP-1 homologous are proteins with different percentages of homology with GLP-1 (53% exenatide, 90% liraglutide), which are resistant to DPP-4 through changes in the amino acid sequence (57). In addition to the action on glycemic control, these drugs mimic all the other physiological actions of GLP-1 shown in detail later on.

Homologues of GLP-1 are further subdivided into incretin mimetics and analogues. GLP-1 analogues are those peptides which closely resemble the GLP-1 amino acid sequence (e.g. liraglutide), whereas mimetics are compounds with alternative structures that seek to mimic the actions of GLP-1 (e.g. exendin-4, small molecule agonist Boc5) (58-59).

Exenatide, is a synthetic version of a protein, exendin-4, derived from the Heloderma Suspectum (Gila monster) lizards' venom. Exendin-4 is a highly potent GLP-1R agonist both in vitro and in vivo. Alignment of native GLP-1 and exendin-4 amino acid sequences demonstrates 53% amino acid identity. Exendin-4 contains a glycine residue at position 2, which confers resistance to cleavage by DPP-4, and a half-life of 2-4 hours allowing a twice-daily administration therapeutic scheme.(58)

The efficacy and tolerability of exenatide for the treatment of type 2 diabetes was assessed by three phase III clinical trials: exendin-4, at a dose of 5 or 10 mg twice daily, added to patients not achieving optimal glucose control with either metformin, a sulfonylurea, or both, for 30 weeks, was effective in lowering HbA1c in all three treatment groups by about 0.9%. Approximately 34%–46% patients achieved a HbA1c less than 7% after addition of exenatide. Exenatide-treated subjects displayed progressive dose-dependent weight loss. (-2.8 +/- 0.5 kg [10 microg], -1.6 +/- 0.4 kg [5 microg]; $P < 0.001$ vs. placebo) The most frequent adverse events were gastrointestinal in nature, with mild to moderate nausea being the principal side effect noted which, on the other hand lessens over time. Nevertheless weight reduction is not correlated with nausea induction. The incidence of mild to moderate hypoglicemia was increased compared to placebo only in patients receiving concomitant sulfonylurea therapy and not in those in which exenatide was added only to metformin (60-62); in fact one of the advantages of GLP-1 over conventional antidiabetic therapies, such as sulphonylureas, is that its insulinotropic

actions are dependent on ambient glucose concentrations, thus mitigating the risks of hypoglycaemia.

A long-acting release formulation of exenatide, 2 mg administered once weekly, has been developed. Exenatide-LAR, constitutes a microsphere-encapsulated suspension of exendin-4 administered once weekly via subcutaneous injection, and a preliminary phase II trial has indicated that it is effective in patients with type 2 diabetes. (63). Other research efforts are also being invested in the direction of GLP-1-based therapies which may be administered via alternative, more desirable routes, such as inhalation or oral administration (64, 59).

Liraglutide, recently approved by FDA on the basis of the positive results of the phase III clinical trial LEADTM (Liraglutide Effect and Action in Diabetes), is a GLP-1 analogue obtained as an acetylated recombinant molecule; liraglutide is 97% homologous to native human GLP-1; it presents only two changes: the binding of palmytic acid to a lysine in position 26 and the substitution of lysine with arginine in position 34. These changes cause pharmacokinetic modifications. The additional 16-carbon fatty acid chain causes non-covalent binding to albumin, which slows absorption from the injection site and protects the molecule from degradation by the enzyme dipeptidyl peptidase-4, allowing for protraction of action, extending half-life to 10-14 h, with plasma concentration enough elevated for 24 h to allow once-daily subcutaneous injection ( 65).

Clinical trials of liraglutide in patients with type 2 diabetes demonstrated that liraglutide, given at a dose of 1.2 and 1.8 mg/day, when used in addition to other antidiabetic therapies, resulted in improved levels of both fasting and postprandial glucose and in reductions in the mean glycated hemoglobin concentration of 0.8 to 1.4 percentage points as compared with placebo. When compared as monotherapy with a sulfonylurea, liraglutide was associated with a lower risk of hypoglycemia. Other benefits include greater weight loss than that achieved with some active controls and the absence of a need to adjust the dose for patients with renal impairment, improvement in β-cell function and a reduction in plasma glucagon concentration (66-68).

In addition to the important effects on intermediary metabolism, GLP-1 receptor agonists have important actions on the cardiovascular system such as reduction of blood pressure, increase of myocardial contractility and improvement of endothelial function. (69-71). In particular an interesting anti-atherogenic potential emerged, as it has been shown that these drugs suppress the inflammatory response mediated by macrophages in the plaque and reduce the occurrence of cardiovascular events (72). Very recently two

new incretin mimetic drugs still under study have been introduced: albiglutide and lixisenatide (47). These compounds have been shown to maintain a similar activity on glycemic control to the other incretin mimetics, with promising effects on cardiovascular system disease observed in vitro and in vivo in animals. They reduce apoptosis of human cardiomyocytes in vitro and reduce ischemic damage in isolated perfused rat heart (47) This important result is interesting for the potential use of these drugs in preventing macrovascular complications, cause of death in individuals with diabetes.

The DPP-4 inhibitors prolong the physiological action of GLP-1 by inhibition of the metabolizing enzyme. This class of drugs has proved equal to the homologous of GLP-1 in glycemic control, reporting same effects in this regard. In addition sitagliptin has interesting anti-inflammatory effects, visible through the reduction in plasma levels of CRP (C reactive protein), TNF-alpha and IL-6. A further effect of siptagliptin on cardiovascular level is the activation in animal models of reverse cholesterol transporter in foamy macrophages, thereby reducing the risk of cardiovascular events (47). Two recent DPP-4 inhibitor drugs, alogliptin and linagliptin, also determine an improvement in lipid profile, reducing the circulating levels of LDL and an improvement in vascular function through antioxidant activity and an increase in vasodilation (47).

In order to better understand the potential of these drugs in the prevention of complications and mortality associated with diabetes it is necessary to analyze and know in detail the biology of GLP-1.

## THE HISTORY OF GLP-1

In 1902, W.M.Bayliss and E.H.Starling showed for the first time the existence of a factor (which they called "secretin"), contained in the acid extract of the intestinal mucosa, which can stimulate through the blood pancreatic secretion (73). In 1930 La Barre and Still purified the "secretin" factor identifying two components: one with stimulatory activity for the exocrine pancreatic secretion and the other, with insulin secretagogue properties (74). In 1932, La Barre coined the term "incretin" to define the substances extracted from the intestinal mucosa with glucose-lowering action, and without stimulatory capacity for exocrine pancreatic secretion, hypothesizing its use in the treatment of diabetes in humans (75). In later years, research on incretins, suffered a significant slowdown due to the conflicting results of various unsatisfactory experiments

(76). Following the development of insulin radioimmunoassays for the study of glucose tolerance, a series of studies demonstrated that oral glucose administration enhances insulin secretion to a greater extent than that seen with isoglycemic iv loading in normal human subjects (77). This gut-associated potentiation of insulin secretion was attributable to one or more humoral or neural factors, called incretins that potentiated insulin secretion following enteral nutrient ingestion (78). Respectively in 1970 and 1981 sequences of the major incretins were identified: GIP (glucose-dependent insulinotropic polypeptide, originally called gastric inhibitory-polypeptide because of its inhibitory activity on acid gastric secretion) and GLP-1 (glucagon-like peptide-1, so called for its homology to glucagon of about 50%) (76). In 1985 the insulinotropic effect of GLP-1 was shown, and the increased activity of the truncated forms of GLP-1 (GLP-1 7-36NH2 and GLP-1 7-37) compared to the intact form (GLP-1 1-36NH2 and GLP-1 1-37) emerged (79-80). From 1987 the first studies, designed for the perspective of incretin therapy for patients with diabetes, were started (76).

It has been recently demonstrated that the incretin effect accounts for as much as 70% of insulin release in health persons and that it is significantly impaired in type 2 diabetic patients (54-55).

## THE BIOLOGY OF GLP-1

The incretinic effect depends mainly on the secretion of two peptidic hormones, released by gastrointestinal endocrine cells, both belonging to the glucagon-related peptides super family: GIP (glucose-dependent insulinotropic polypeptide) produced predominantly in duodenal K cells and in the proximal small intestine, identified in 1970 and GLP-1 (glucagon-like peptide-1) produced in L cells in the distal small bowel and colon, identified in 1983 (81-83). The intestinal enteroendocrine L cells, are polarized cells, forming an open-type epithelium, which are directly in contact with luminal nutrients through the apical pole and are connected to a neurovascular network through the basolateral surface (84). Plasma levels of GIP and GLP-1 rise rapidly within minutes of food intake, with peaks being achieved within approximately 30 min of nutrient ingestion. The precise mechanisms by which GIP and GLP-1 stimulate insulin secretion only at elevated levels of plasma glucose remain unclear (85) (fig 3). Despite the similarities in their effects on glucose-dependent insulin secretion, on promotion of

pancreas beta cell proliferation and inhibition of apoptosis, the other targets of these hormones demonstrate little overlap.

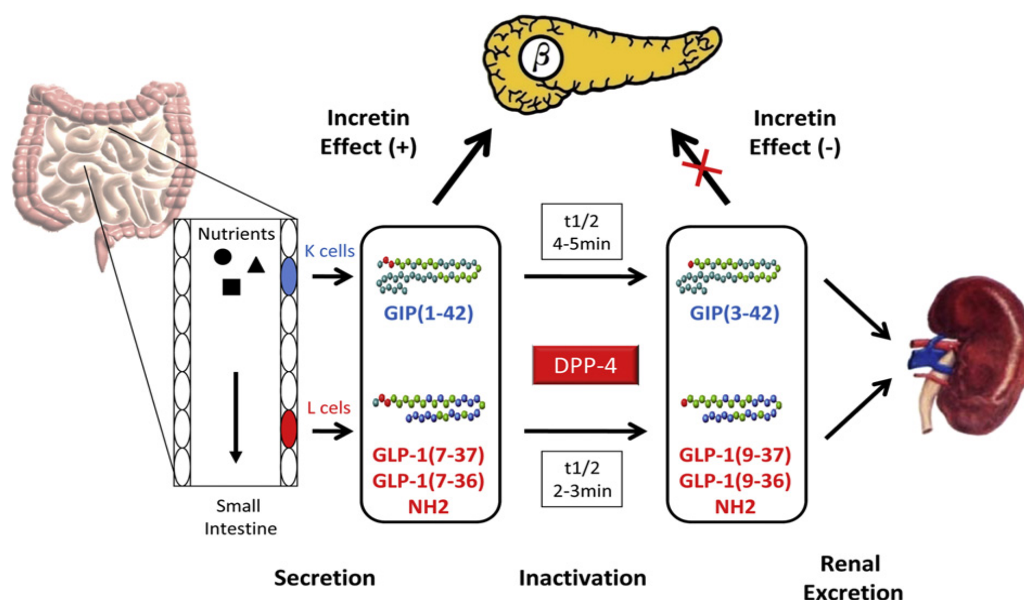


***Figure 3**. Biological cycle of incretin hormones: secretion and metabolism of GIP and GLP-1. GIP is secreted by K cells of proximal bowel; GLP-1 is secreted by L cells of the distal gut. GIP and GLP-1 are then rapidly processed by proteolytic enzyme DPP-4 and their metabolites are excreted by the kidney. The bioactive forms of incretin hormones, GIP (1-42), GLP-1 (7-36) amide and GLP-1 (7-37) have an insulinotropic effect on pancreatic beta-cells, while their metabolites GIP (3-42), GLP-1 (9-36) amide and GLP-1 (7-37) lose their function.*

Hence, GIP increases fatty acid uptake and lipogenesis by adipocytes, leading to suggestions that GIP may contribute to obesity. In contrast, GLP-1 appears to be largely beneficial to glycemic control, via additional actions on glucose sensors, inhibition of glucagon release, gastric emptying, appetite, gastrointestinal fat absorption (84).

## Synthesis and secretion of GLP-1 in humans

Preproglucagon is transcribed on proglucagon gene, which is located on the long arm of chromosome 2 and consists of 6 exons and 5 introns, with the entire GLP-1 coding sequence localized only in exon 4 (86) and is expressed in pancreatic α-cells, intestinal L-

cells an duodenal K-cells, in the nucleus tractus solitarius in the hindbrain (which is the nucleus of the vagus nerve) and in magnacellular neurons of the hypothalamus. The transcription of the gene leads to the formation of a single mRNA, which is structurally identical in the three cell types in which it is expressed (86-88). The transcript is then translated into a single precursor protein of 180 amino acids that is processed differentially in different cell types due to tissue-specific expression of prohormone convertase enzymes (PC) (89,90). In gut L cells it is processed to glicentin, oxyntomodulin, GLP-1, GLP-2, IP-2 (fig.4). Products derived from the processing of proglucagone, with the exception of IP-2 for which no functions have been identified, have different effects. Physiological actions of glicentin are not well defined yet, but it was found that it exerts trophic effects in the small intestine of rodents (91). Oxintomodulin inhibits gastrointestinal motility and secretions, it stimulates the exocrine pancreatic secretion and intestinal glucose uptake, it promotes satiety and regulates heart rate (92-95).

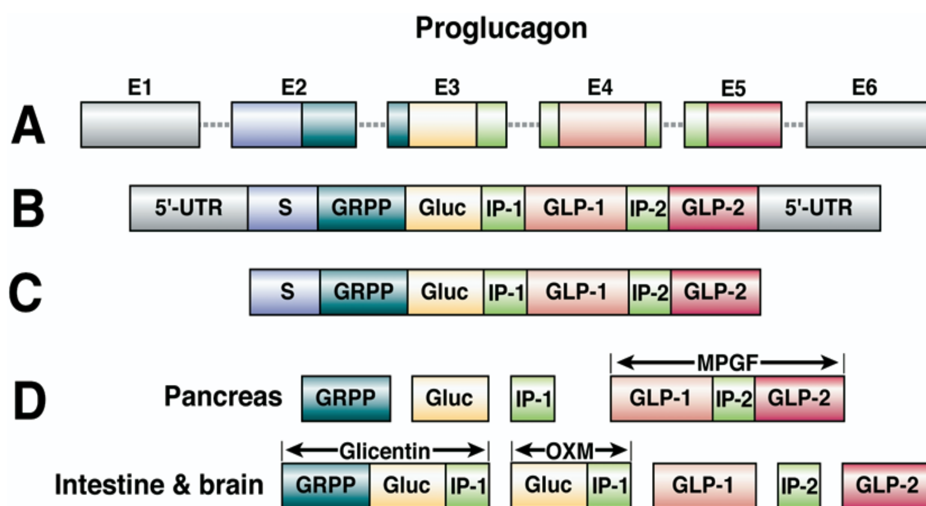


***Figure 4.*** *Structure of the proglucagone gene (A) mRNA (B) and protein (C). Proglucagone tissue-specific post-translational processing (D). Generation of: GRPP (Glicentin-related polypeptide), Gluc (glucagon), IP-1 (intervening peptide-1) and MPGF (major proglucagon fragment) in pancreas. Generation of GLP-1, GLP-2, OXM (oxintomoduline), Glicentin and IP-2 (intervening peptide-2) in the gut and nervous system*

GLP-2 stimulates cell proliferation and inhibits intestinal cells apoptosis, it up-regulates intestinal glucose, transport, it improves intestinal barrier function, it inhibits gastric acid secretion, gastric emptying and food intake, it reduces bone reabsorption and promotes the survival and proliferation of neurons (96-99). The GLP-2, unlike GLP-1, has no stimulatory function for insulin secretion from pancreatic β-cells (84).

Proglucagons' peptide sequence is identical in mouse, rat, and human (100,101) and the derived products, are highly conserved throughout the evolution of animal species. Sequence analysis performed in numerous species indicated that there is complete conservation of the GLP-1 amino acid sequence in mammals. The high degree of conservation of the glucagon and GLP sequences during evolution indicates the importance of the physiological processes regulated by these hormones. The conservation of GLP-1 also reflects the fact that essentially the entire amino acid sequence of GLP-1 is required for full biological activity (102).

Four isotypes of GLP-1 are produced and secreted into circulation from the intestinal enteroendocrine L cells under the action of prohormone convertase-1 and 3 (PC1 and PC3) enzymes (Fig.5). The convertases initially process proglucagon, resulting in the formation of two whole chain GLP-1 peptides: GLP-1 (1-36) NH2, derived from proglucagone 78-107 and GLP-1(1-37 ) derived from proglucagone 78-108. Then, the action of PC 1/3 on GLP-1 peptide whole chain generates two isotypes of GLP-1 with truncated chain: GLP-1(7-36) NH2, derived from GLP-1 (1-36) NH2 and GLP-1(7-37) derived from GLP-1(1-37) (90). The whole chain isotypes of GLP-1 are inactive, while the truncated chain isotypes are the biologically active forms of GLP-1 (84).

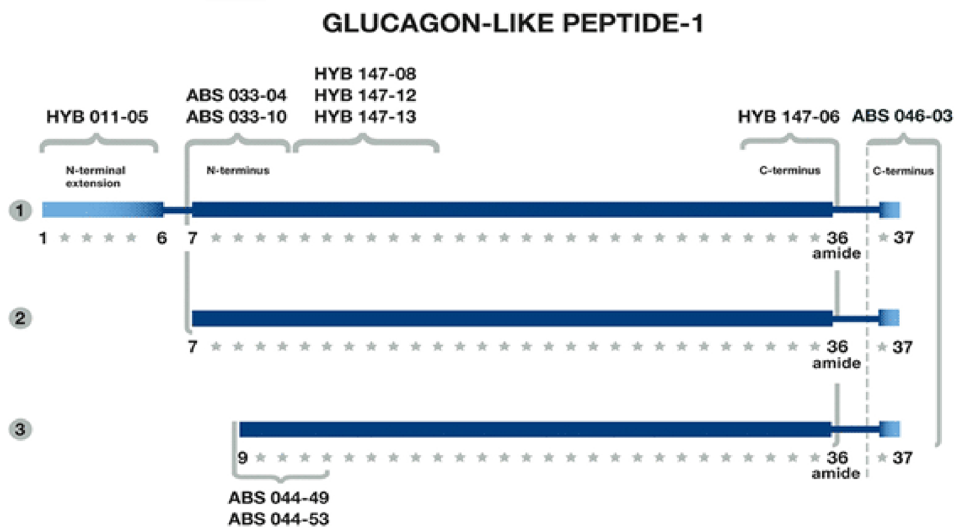


***Figure 5.*** *Isotypes of GLP-1 secreted by intestinal enteroendocrine L cells and their metabolites.*
*(1) GLP-1 non-trunkated forms: GLP-1 (1-36) and GLP-1 (1-37)*
*(2) bioactive forms of GLP-1: GLP-1 (7-36) amide, GLP-1 (7-37)*
*(3 )metabolites of GLP-1:GLP-1 (9-36) amide, GLP-1 (9-37).*

The addition of an amide group to the peptide GLP-1 (1-36) NH2 and GLP-1 (7-36) NH2, is mediated by the enzyme peptidylglycine monooxygenase, which adds the amide group to arginine residue present in the C-terminal extremity of the two peptides, but the function of this process remains unknown (103). In humans, the major isotype of circulating GLP-1 is GLP-1 (7-36) NH2, which constitutes about 80% of GLP-1 secretion, and the remaining 20% is represented by GLP -1 (7-37) isotype (104).

GLP1-1(7-37) and GLP-1(7-36) are equipotent in humans and rapidly stimulate glucose-dependent insulin secretion by binding to the GLP-1 receptor (GLP-1R)( 105).

Extensive studies have shown that secretion of GLP-1 is regulated by a variety of dietary nutrient, neural and endocrine inputs. The result is a biphasic mechanism of release, with both hormonal and neural mediation of early GLP-1 release (15-30 min), and direct nutrient contact with L cells mediating later GLP-1 secretion (30-60 min) (84).

The primary stimulus for GLP-1 secretion is enteral nutrient ingestion. Secretion of GLP-1 largely depends upon the specific nutrient composition of the meal. In particular, meals rich in carbohydrates are the primary physiological stimulus for secretion of GLP-1(106). Furthermore, the release of GLP-1 can be enhanced by a number of nutrients, among which the most important are: glucose and other sugars, fatty acids and triglycerides, essential aminoacids and fibers (107,108). It has been reported that a particular caloric threshold or nutrient delivery rate must be reached in order to trigger significant secretion (109). Although high concentrations of glucose have been demonstrated to stimulate GLP-1 secretion from isolated rat intestinal cell cultures, it is unlikely that glucose normally acts directly on L cells because under normal feeding conditions the majority of glucose is absorbed before reaching the ileum. In addition, the rapid GLP-1 secretory response to oral glucose suggests that glucose must activate the release of GLP-1 by means of other then a direct effect on L cells. Otherwise, meals that contain fat and proteins increase GLP-1 secretion in humans acting directly on L cells. (84). In addition to nutrients, neuro-hormonal stimuli also regulate the secretion of GLP-1. Many studies have demonstrated the role of the autonomic nervous system in the early stage of secretion of GLP-1, which through the action of neurotransmitters such as acetylcholine, the incretin hormone GIP and GRP (gastric releasing peptide), stimulate the secretion (84)

The importance of the vagus nerve (the fundamental component of the sympathetic nervous system which innervates, duodenum, jejunum, ileum and colon) in mediating the release of GLP-1, was established in experiments in rats. It was shown that

complete sub-diaphragmatic vagotomy blocks the early GLP-1 secretion, while the electrical stimulation of the celiac branches of the vagus nerve increases the secretion in rats (110). In humans, administration of atropine, a non-specific muscarinic antagonist, decreases early secretion phase of GLP-1 following stimulation with oral glucose and subsequent studies in rats have shown that the antagonists of muscarinic receptors M1 and M2 (to which acetylcholine binds) completely inhibit the secretion of GLP-1 in the early phase (111-113). Experiments performed in rats also show a possible role of catecholamines. In fact, the administration of beta-agonists in the isolated and perfused gut determines an increase of secretion of GLP-1 (84).

Of the endocrine peptides tested for effects on L cells, GIP has been found to stimulate GLP-1 release. It has been demonstrated in rats that physiological doses of GIP, act through the nervous system (either vagal or myenteric) to indirectly stimulate the secretion of GLP-1, rather than acting directly on the intestinal enteroendocrine L cell. The GRP is an important component of the non-adrenergic/non-cholinergic branch of the vagus nerve, as well as the enteric nervous system, and the candidate transmitter in these pathways stimulating the release of GLP-1 in vivo in rats. Most likely, therefore, the ingestion of nutrients in rats, causes a few minutes later, the GRP mediated release of GIP by the duodenal K cells, which via the vagus nerve and myenteric plexus indirectly stimulates early secretion of GLP-1 (114). At present in humans the stimulatory action of GIP and other gut peptides (GRP) on GLP-1 secretion has been excluded, so that the most probable mechanism appears to be nerve stimulation mediated by acetylcholine and other mediators not yet identified (115).

However, since the intestinal enteroendocrine L cells are present throughout the small intestine, it is possible that the early secretion of GLP-1 is caused by direct contact of nutrients with L cells located in the proximal small intestine (82,83).

Recently other mechanisms of control of GLP-1 release emerged. First of all it has been shown in vivo in rats and in vitro in humans that the hormone leptin, through the presence of its receptor in the intestinal enteroendocrine L cells, stimulates the secretion of GLP-1, identifying a leptin resistance at the base deficit of incretin secretion in obese rats (116). Very recently, the molecular mechanism underlying the long-known ability of bile acids to stimulate GLP-1 secretion has been elucidated, with the discovery that intestinal L cells express the G protein-coupled bile acid receptor (TGR5; also known as GPR131); the available evidence to date implicates TGR5 agonism as a feasible approach to incretin therapy. (117)

In vitro and in vivo studies in humans have shown an inhibitory action on the release of GLP-1 by insulin, somotostatin and the neuropeptide galanin (84).

## Clearance and metabolism of GLP-1

After secretion from enteroendocrine L cells, GLP-1(7-36) amide is rapidly degraded, by its primary endogenous inactivator dipeptidyl peptidase-4 (DPP-4) to its N-terminally truncated metabolite GLP-1(9-36). DPP-4 is a serine protease that specifically cleaves dipeptides from the amino terminal region of oligopeptides or proteins containing proline or alanine amino acid residues at position 2 (84,118). GLP-1 contains a residue of alanine at position 2, which serves as a substrate for the action of DPP-4. The enzyme is expressed in many tissues and cell types including: kidney, lung, liver, intestine, spleen, testicle, pancreas, central nervous system, adrenal medulla and the surface of lymphocytes and macrophages (84). In addition, DPP-4 is present on the surface of endothelial cells, including the vascular system supplying the intestinal mucosa, which is adjacent to the site of secretion of GLP-1 (119). As a result, more than half of the GLP-1 product that enters the portal circulation, is inactivated by DPP-4 before reaching the systemic circulation (119). DPP-4, besides being present in the membrane bound form on cell surfaces, also exists in a soluble form in the blood stream (120). In rats, more than 50% of an intravenous bolus of GLP-1 is converted into its N-terminal metabolites by DPP-4, approximately two minutes after administration, whereas in the DPP-4 knock-out rodents, GLP-1 is not metabolized (121,122). DPP-4, by cleaving the N-terminal fragment of the bioactive forms of GLP-1, leads to the formation of two metabolites: GLP-1 (9-36) NH2, derived from the metabolism of GLP-1 (7-36) NH2 and GLP-1 (9-37) derived from the metabolism of GLP-1 (7-37) (Fig. 6) (121,123,124). GLP-1 (9-36)$NH^2$ has a 1000-fold lower affinity for the GLP-1 receptor (GLP-1R) and is characterized by a complete lack of insulinotropic activity (125,126).

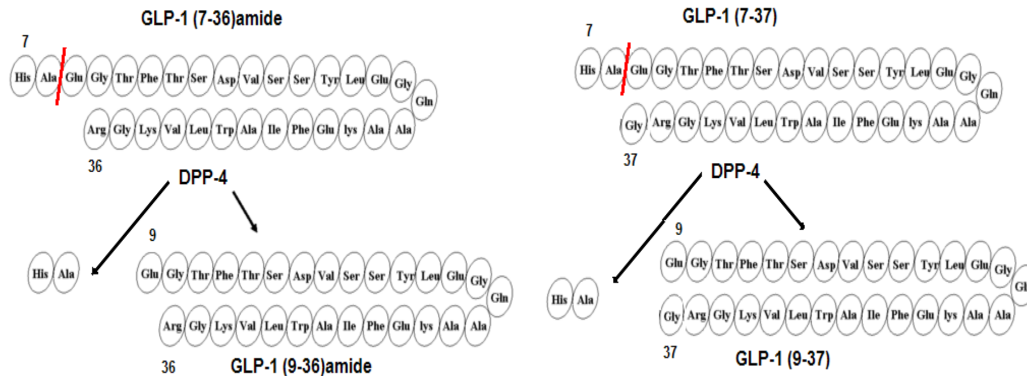


***Figure 6.*** *GLP-1 metabolism*

In addition to the importance of DPP-4 for inactivation of GLP-1 and GIP, both peptides are also rapidly cleared from the circulation via the kidney through a variety of mechanisms including: glomerular filtration, tubular reabsorption and catabolism (127). In rats, bilateral nephrectomy or ligation of the ureters, is associated with an increased half-life of GLP-1 and GLP-1 levels are increased in patients with chronic renal failure (127,128). In patients with chronic renal failure, the concentration of metabolites of GLP-1 is increased compared to healthy individuals, while the concentration of the bioactive forms of GLP-1 remains similar in both categories. This shows the importance of renal function for elimination of the metabolites of GLP-1 (128,129).

The glucose lowering activity of GLP-1 is of relatively short duration, given the GLP-1 half-life in circulation of 2 minutes (84). Plasma levels of bioactive forms of GLP-1 in fasting in humans, are in the range 5-10 pmol/L and increased approximately 2-3 times after the meal, with a peak absolute value dependent both on the amount and of the composition of nutrient intake (54,104,130,131). The postprandial peak of the GLP-1 bioactive forms is reduced in obese subjects and in subjects with type 2 diabetes (54,132-134). Since the elimination fraction is similar in healthy, obese and type 2 diabetes subjects, the decreased levels of GLP-1 observed in obese subjects and in subjects with type 2 diabetes is caused by reduced secretion of GLP-1 in the last two categories (135). It has been shown that the decrease in the secretion of GLP-1 in obese subjects can be attributed to leptin resistance, while the factors responsible for the phenomenon in individuals with diabetes have not yet been identified (84,116).

Numerous studies in animals and humans have shown that inhibition of DPP-4, prolongs the half-life of bioactive forms of GLP-1 (84).

## GLP-1 receptor

GLP-R is a heptahelical transmembrane-spanning G protein–coupled receptor that consists of 463 amino acids functionally associated with adenylate cyclase through the stimulatory G protein subunit ($G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_o$) (136-138). (figure 7). The human GLP-1R has 90% sequence homology to the rat GLP-1R, and its gene is localized to chromosome 6p21 (139). The GLP-1R, comprises eight hydrophobic domains, seven of which span the membrane, with a further extracellular N-terminal domain, linked by hydrophilic intra-and extracellular loops (84).

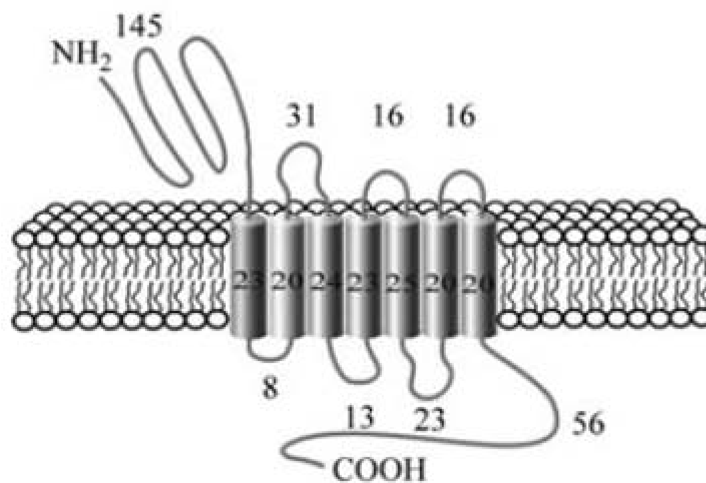


***Figure 7.*** *Illustration of the structure of the glucagon-like peptide-1 receptor (GLP-1R) including the number of amino acid residues in each segment.*

GLP-1R is expressed in the pancreatic islets beta-cells and in several tissue including gut, lung, kidney, heart, endothelium and multiple regions of the peripheral and

central nervous system, including hypothalamus and autonomic nuclei that control cardiovascular functions (84).

The specific cellular localization, relative abundance, and functional importance of the GLP-1R in cardiovascular tissues have not been fully defined. Ban et al showed that GLP-1R is expressed throughout the mouse cardiovascular system, with absence in cardiac fibroblasts and particular abundance in the endocardium and, remarkably, demonstrated that some cardioprotective and vasodilatory actions of GLP-1 are independent of the known GLP-1R (140,141).

Although numerous studies suggest the functional existence of a second GLP-1 receptor, only a single GLP-1R coupled to glucose homeostasis has yet been identified. Evidence supports a role for the GLP-1 inactive metabolic product GLP-1(9–36) amide in regulation of cardiovascular function, but a separate receptor for this peptide has not yet been discovered( 140,141). This presupposes the existence of other receptors for GLP-1 or the presence of non-receptor mediated actions by the peptides.

GLP-1 has been demonstrated to bind to the receptor in an orthosteric manner. However, recent studies employing both a small molecule GLP-1R agonist and molecular modeling of the three-dimensional structure of the receptor, have also identified a non-allosteric activation site (59,142).

The GLP-1R has an extremely high affinity for GLP-1 (7-37, 7-36) and similar agonists (exendin 3, exendin 4) which selectively binds at nanomolar concentrations, while other peptides of the glucagon superfamily, such as glucagon, PACAP, GIP and VIP, bind either poorly or not at all.

It is well established that activation of the GLP-1R stimulates the production of cyclic AMP via the action of adenylate cyclase. cAMP signaling may then be further amplified and diversified via the activation of several downstream factors, such as protein kinase A (PKA) and cAMP regulated guanine nucleotide exchange factors (GEF) (143).

Activation of the GLP-1 receptor can trigger at least 2 downstream pathways, generation of the second messenger cAMP (cAMP) followed by activation of protein kinase A (PKA), and the indirect activation of epidermal growth factor receptor followed by phosphoinositide 3-kinase (PI3K) and Akt signaling

Activation of GLP-1R is known to be associated with inhibition of voltage-dependent potassium channels, thereby generating cellular depolarization, elevation of intracellular calcium, phosphorylation of the cyclic AMP response binding element (CREB) and activation of a number of kinases, including AMPK and extracellular signal-

regulated kinase (ERK)1/2, protein kinase C, phosphoinositide 3-kinase (PI3K), mitogen activated protein kinase (MAPK) (p38 and JNK) and protein kinase B (144-148). The different activated metabolic pathways are responsible for several cellular effects that are the expression of the physiological actions of GLP-1 in various organs and tissues.

## Physiological actions of GLP-1

The physiological actions of GLP-1 reflect the functions of organs in which specific GLP-1 receptors are expressed. These organs include the pancreatic islets, stomach, lung, brain, kidney, pituitary gland, cardiovascular system, kidney, and small intestine (84). A wide range of biological actions for GLP-1 have been reported in several experimental systems from in vitro cell lines to human subjects (table II).

In pancreatic islets bioactive GLP-1 peptides act through receptor mechanism, on beta-cells, alpha cells (glucagon producing) and delta-cells (producing somatostatin). The actions on beta-cells include increase of glucose dependent insulin secretion and biosynthesis, inhibition of apoptosis, stimulation of proliferation, increase in beta-cell mass and increased in glucose cellular sensitivity (79,80,149). The molecular mechanisms at the basis of the insulin secretagogue function are the activation of adenylate cyclase and the production of cAMP that induces: membrane depolarization by inhibition of KATP channels, increased intracellular $Ca^{2+}$, activation of two metabolic pathways: a PKA-dependent and a PKA-independent mediated by the EPAC2 enzyme and finally exocytosis of insulin granules (fig. 8). Whereas the increase in insulin biosynthesis is mediated by activation of two major promoters of the insulin gene, NFAT, and Pdx-1 (by both PKA-dependent and independent pathways) which increase gene transcription and mRNA stability (84, 150-152).

The anti-apoptotic and pro-proliferative processes appear mediated by PI3K/Akt metabolic pathway, which results in activation of MAPK and inhibition of FOX01 with a consequent increase in Pdx-1 and up-regulation of cyclin D1 (153-155). GLP-1 inhibits glucagon secretion in alpha-cells, and stimulates the secretion of somatostatin in pancreatic δ-cells through the activation of metabolic pathways not entirely understood (156,157).

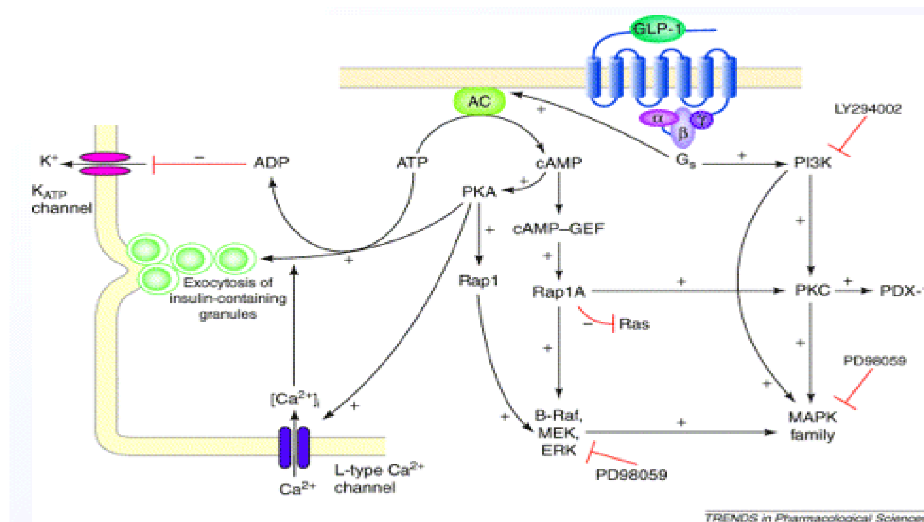


***Figure 8.*** *Metabolic processes activated by the binding of GLP-1 to its receptor (GLP-1R) in pancreatic beta-cell*

In addition to its insulinotropic actions on pancreatic beta-cells, GLP-1 inhibits glucagon secretion, gastric emptying and small bowel motility, regulates gastric acid secretion through mechanisms partly directly receptor mediated and partly indirect mediated by the vagus nerve, and induces satiety acting on the appetite control centers located in the hypothalamus, thereby reducing postprandial glycemia and favoring weight loss (158-162).

As for the nervous system, GLP-1 improves memory and learning, stimulates afferent sensory nerves, and has neuroprotective functions (84,140).

GLP-1 receptors are expressed at high density in rat lung membranes and on vascular smooth muscle. The treatment of rat trachea and pulmonary artery with GLP-1 results in inhibition of mucous secretion and relaxation of smooth muscle; GLP-1 receptor mRNA is detected in type II pneumocytes (84, 163) and stimulates the secretion of surfactant from these cells.

| ***Table II. GLP-1 (7-36)amide and GLP-1 (7-37) physiological actions*** | |
|---|---|
| **Tissue-System** | **Effect** |
| ***PANCREAS*** | - *Stimulation of insulin biosynthesis and secretion*<br>- *Proliferation of beta-cells*<br>- *Inhibition of beta-cells apoptosis*<br>- *Increased glucose sensibility of beta-cells*<br>- *Inhibition of glucagone secretion*<br>- *Stimulation of somatostatin secretion.* |
| ***CNS/PNS*** | - *Decreased appetite and body weight*<br>- *Neuroprotection.*<br>- *Improvement of memory and learning* |
| ***GASTROINTESTINAL SYSTEM*** | - *Inhibition of acid gastric secretion*<br>- *Inhibition of gastric emptying*<br>- *Inhibition of bowel motility* |
| ***LIVER*** | - *Inhibition of glucose production* |
| ***MUSCLE*** | - *Increase in glucose uptake* |
| ***ADIPOSE TISSUE*** | - *Increase in glucose uptake*<br>- *Lipolytic and lipogenic effects* |
| ***CARDIOVASCULAR SYSTEM*** | - *Increase in heart rate and blood pressure*<br>- *Improvement of endothelial function*<br>- *Cardioprotection.* |
| ***LUNG*** | - *Relaxation of smooth muscle*<br>- *Inhibition of mucous secretion*<br>- *Stimulation of surfactant secretion* |
| ***KIDNEY*** | - *Natriuresis* |
| ***PITUITARY GLAND*** | - *Stimulation of TRH, LH, ACTH secretion* |

Several experimental findings suggest that GLP-1 activates hormone secretion from the anterior pituitary gland, where GLP-1 receptors have been detected (140).

GLP-1Rs have been reported to be widely expressed in the heart and vasculature of both rodents and humans, with specific localization in vascular smooth muscle, cardiomyocytes, endocardium and coronary endothelium/smooth muscle (163,164). Nevertheless GLP-1 has been demonstrated to have cardiac and vascular actions that include effects on blood pressure (165-167), contractility and cardiac output (168-172), cardioprotection (141,173,174) and endothelial function (175-178).

The administration of GLP-1 to rats results in increases in arterial blood pressure and heart rate. These actions of GLP-1 on the cardiovascular system have been attributed to actions of GLP-1 receptors in the nucleus tractus solitarius, which is involved in the central control of cardiovascular function (165). Furthermore, GLP-1 activates c-Fos expression in the adrenal medulla and in neurons in autonomic control sites in the rat brain, and rapidly activates tyrosine hydroxylase transcription and catecholamine production in brainstem neurons (166). Several studies have demonstrated that the infusion of GLP-1 into Dahl salt-sensitive rats attenuated the development of hypertension, with reduction of proteinuria and improvement in endothelial and cardiac functions (167). Consistent with the cytoprotective actions of GLP-1 in the endocrine pancreas, several different groups using various experimental models have reported that acute GLP-1treatment exerts beneficial effects after ischemia and successful reperfusion. Thus, GLP-1 reduced infarct size in the isolated perfused rat heart and in vivo animal models (141,168,171). GLP-1 exerts beneficial effects on cardiac function also in human subjects following myocardial infarction and angioplasty or bypass grafting (170,172). A 72 hours infusion of GLP-1 in patients with acute myocardial infarction and an ejection fraction less than 40% resulted in significantly improved left ventricular ejection fraction and improved regional and global wall motion scores, in association with a trend toward earlier hospital discharge (170).

Finally, the presence of GLP-1 receptors on vascular endothelial cells, and the evidence that GLP-1 can improve endothelial cell dysfunction (141,175-178), suggests that GLP-1 may modulate vascular endothelial cell function as discussed later.

Even if the presence of GLP-1Rs in skeletal muscle, liver, and adipose cells is debatable there are numerous reports of physiological anabolic (glycogenic and lipogenic) actions of GLP-1 on these tissues (179, 180). In particular, it inhibits the release of hepatic glucose, increases glucose uptake in muscle and fat cells and it stimulates

lipogenesis in adipose tissue (180-185). These effects are probably mediated through indirect extra-receptorial mechanism.

## GLP-1 (9-36) actions

Accumulating evidence indicates that GLP-1 metabolically inactive breakdown product, GLP-1(9–36), may play an active beneficial role acting on liver and in the cardiovascular system. GLP- 1 (9-36) amide suppresses hepatic glucose production through not yet identified mechanisms, exerts antioxidant cardioprotective actions and reduces oxidative stress in vascular tissue by a GLP-1 receptor independent mechanism in subjects with type 2 diabetes and stable coronary disease. GLP-1 has beneficial effects on flow-mediated vasodilatation by an increase of nitric oxide production. These effects seem to be mediated by the activation of cAMP and the K+-channel opening and independent of alterations in glucose or insulin concentrations (141,168,186-188).

# EFFECTS OF GLP-1 ON VASCULAR FUNCTION

First of all, the beneficial effect of GLP-1 bioactive peptides on the endothelium is related to the peptides hypoglycemic action. In fact, the lowering of blood glucose reduces the damage to endothelial cells caused by hyperglycemia by inhibition of the polyol pathway, inactivation of the protein kinase C (PKC) and reduction of advance glycation end products formation (AGEs) that lead to a decrease of cellular oxidative stress, increased synthesis of nitric oxide, reduction of endothelial permeability, inflammation, pro-coagulant and pro-aggregating activity (14). Existence of GLP-1 bioactive peptides effects on the endothelium unrelated to hypoglycemic action, resulting in improvement of endothelial function through the stimulation of the synthesis of vasodilators, primarily nitric oxide, and the inhibition of the expression of adhesion molecules, was also demonstrated. In a number of small trials in humans, as well as in preclinical and in vitro studies, both native GLP-1 and GLP-1 receptor agonists have demonstrated to improve vascular function in vivo in humans. Using the flow-mediated dilatation technique, Nystrom et al. (176) observed that GLP-1 intra-venous infusion improved flow mediated vasodilatation in type 2 diabetic patients with established coronary artery disease, without any effects in healthy subjects. There was no effect on

endothelial independent vasodilatation or on blood pressure. Furthermore, the authors demonstrated that GLP-1 receptor is expressed in human coronary artery endothelial cell cultures (176). Similarly, Basu et al., using venous occlusion plethysmography, demonstrated that acute treatment with intravenous infusion of GLP-1 increased endothelium-dependent blood flow in the resistance vessels (arterioles and small arteries) in response to acetylcholine (which stimulates muscarinic receptors on the endothelial cells that evoke nitric oxide (NO) release through stimulation of endothelial isoform of the NO synthase enzyme)), in the forearm of healthy non-diabetic human subjects at least in part, via the nitric oxide pathway and that this effect is modulated by KATP. There were no alterations in endothelium-independent responses to GLP-1 implying upregulation of endothelial nitric oxide synthase enzyme system as a possible mediator of its effects (189).

Study models of ex vivo isolated rat vessels demonstrated that GLP-1 has a direct vasorelaxant action on pulmonary (175, 190), mesenteric artery and aorta (141,167,186) and femoral artery (177). Golpon et al. (175) in fact demonstrated that GLP-1 induced a dose-dependent and time-reversible endothelial-dependent relaxation of preconstricted pulmonary artery rings and it also reduced vascular tone in the isolated, perfused and ventilated rat lung. The authors suggested that this effect was NO dependent, since it was abolished by L-NAME, a nitric oxide inhibitor (175). Yu et al. showed that chronically administered GLP-1 improves vasodilator response in preconstricted aortic rings in a salt-sensitive rodent model (167). Green et al. (186) demonstrated that GLP-1 causes significant concentration-dependent relaxation of isolated rat aorta through mechanism leading to stimulation of cAMP and subsequent opening of K-ATP channels. The GLP-1 agonist exendin-4 has the same effect (though weaker than that of GLP-1) (186). Furthermore it was demonstrated that GLP-1 dose dependently relaxes rat femoral arteries and this effect is NO and endothelium independent and occurs specifically through its receptor binding as exendin 9-39 significantly inhibits GLP-1-induced relaxation in rat femoral artery (177).

The mechanisms through which GLP-1 influences cardiovascular function are complex and incompletely understood. Some studies have suggested that the vasorelaxant actions of GLP-1 are both endothelium and nitric oxide dependent (141,175), whereas others have indicated that these effects may occur via endothelium independent pathways, through activation of KATP channels and cyclic AMP (177,186) mediated via the classical GLP-1R, as they were found to be abolished in the presence of the GLP-1R

antagonist, exendin (9–39) (177). However, it has recently been reported that both exendin (9–39) and metabolically inactive GLP-1(9–36) are by themselves capable of causing vascular relaxation (141,186). Indeed, relaxation responses to both native GLP-1 and GLP-1(9–36) were found to persist in gene-modified mice lacking the GLP-1R, indicating that there may be more than one type of GLP-1R in the cardiovascular system or that GLP-1 is capable of exerting receptor-independent effects (141).

Beside the measurement of FMD in humans and of vasoreactivity in animal isolated vascular models, in vivo and in vitro studies on the expression of biomarkers associated with vascular dysfunction have been conducted to investigate the role of GLP-1 and GLP-1 receptor agonists on endothelial function. Courreges et al demonstrated that liraglutide reduces the expression of such markers of vascular inflammation as high-sensitivity C-reactive protein, plasminogen activator inhibitor-1, and brain natriuretic peptide in type 2 diabetes patients (67). Arakawa et al., showed that GLP-1 receptor agonist, exendin-4, reduced monocyte/macrophage accumulation in the arterial wall by inhibiting the inflammatory response in macrophages, and that this effect may contribute to the attenuation of atherosclerotic lesion by incretins (72). Liu et al. demonstrated that the treatment with GLP-1 and liraglutide, inhibited TNF or hyperglycaemia-mediated induction of PAI-1, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 mRNA and protein expression in a human vascular endothelial cell line and this effect may involve the modulation of NUR 77 nuclear orphan receptor. (191, 192). In recent study it was demonstrated that GLP-1 dose-dependently inhibited RAGE gene expression, and decreased reactive oxygen species generation and subsequently reduced vascular cell adhesion molecule-1 mRNA levels in AGE-exposed HUVEC, and that GLP-1 directly acts on HUVEC via GLP-1R through activation of cyclic AMP pathways (193). Recently, Hattori et al found that liraglutide exerts an anti-inflammatory effect on vascular endothelial cells by increasing nitric oxide production and suppressing NF-κB activation, partly at least through AMPK activation which occurs through a signaling pathway independent of cyclic AMP: in HUVECs liraglutide dose-dependently increased nitric oxide production, caused eNOS phosphorylation, potentiated eNOS activity and restored the cytokine induced downregulation of eNOS mRNA levels, which is dependent on NF-κB activation. The effect of liraglutide on TNFα-induced NF-κB activation and NF-κB-dependent expression of proinflammatory genes was therefore examined: liraglutide dose-dependently inhibited NF-κB activation and TNFα-induced IκB degradation. It also reduced TNFα-induced MCP-1, VCAM1, ICAM1 and E-selectin

mRNA expression (71).  Oeseburg et al. demonstrated that GLP-1 could prevent oxidative stress-induced cellular senescence in endothelial cells involving downstream PKA signaling and  induction of antioxidant genes (HO-1 and NOO1) (194). Finally, GLP-1 agonist exendin-4 stimulates proliferation of the of human coronary artery endothelial cells in vitro (effects that are shared by native GLP-1 (7–36) and its major metabolite GLP-1 (9–36)) and this effect is mediated through PKA-PI3K/Akt-eNOS activation pathways via a GLP-1 receptor-dependent mechanism. In contrast, MAPK (Erk1/2) does not seem to play an important role in the exendin-4-induced cell proliferation, although exendin-4 stimulated phosphorylation of this kinase (195).

Taken together, the data reported sofar suggest that incretine mimetic drugs improve endothelial dysfunction associated with premature atherosclerosis identified in type 2 diabetes patients and could be useful as a starting point for further research in this field.

## SIRT1: ROLE IN ENDOTHELIAL DYSFUNCTION

The activity of SIRT1, a protein belonging to the sirtuin family, recently emerged as being involved in endothelial homeostasis. It was found that SIRT1 improves endothelial function and an increase of its expression exerts protective effects against the development of atherosclerosis and endothelial dysfunction (196-198). SIRT1 promotes endothelium dependent vasodilation and regenerative functions in endothelial and smooth muscle cells of the vascular wall by targeting endothelial nitric oxide synthase for deacetylation, which stimulates the activity of this enzyme and consequently increases endothelial nitric oxide production and the scavenger activity on oxygen reactive species (ROS). If SIRT1 deacetylation is inhibited in endothelial tissue, nitric oxide synthase acetylation predominates, nitric oxide production decreases, and vasodilation is impaired (199).

Furthermore, SIRT1 is involved in the regulation of endothelial inflammatory response and this raises the possibility that targeting of SIRT1 might be a useful strategy for treating the inflammatory component of metabolic diseases. In endothelial cells, knockdown of SIRT1 activates NF-kB inflammatory pathways and increases the inflammatory gene expression induced by TNFα, while SIRT1 activators inhibit TNF-α stimulated inflammatory pathway (200-202). NF-κB is of special interest in endothelial

cells, since it drives the expression of important adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-Selectin, which recruit blood monocytes to atherosclerotic lesions (203). Several studies have demonstrated that SIRT1 regulates the transcriptional activity of Nf-kB by deacetylating RelA/p65 subunit of Nf-kB at lysine 310. This regulation is biologically significant because the activation of SIRT1 inhibits NF-κB transcription and reduces the expression of inflammatory adhesion molecules (201). Since increasing evidence indicates that chronic, low-grade inflammation can cause insulin resistance and metabolic syndrome, it is very important to understand the role of SIRT1 in protection against pro-inflammatory responses in endothelial tissue and whether its expression may be modulated by pharmacological treatment.

SIRT1 exerts also other effects on atherosclerotic plaque macrophages: it deacetylates RelA/p65-NF-kB, it suppresses the expression of Lox-1 (a scavenger receptor for oxydized LDL), it favors the expression of the reverse ABCA-1 cholesterol transporter, preventing the formation of foamy macrophages in the atherosclerotic plaque and it suppresses the expression of endothelial tissue factor (coagulation factor III), and hence it exerts anti-thrombotic properties (204).

SIRT1 is up regulated by caloric restriction or fasting in multiple tissues and an abnormal regulation of SIRT1 expression may play a key role in the pathogenesis of age-associated metabolic diseases. According to several studies, decreased expression of SIRT1 in endothelium, is involved in the onset and development of atherosclerosis in diabetic patients: in conditions of hyperglycemia the gene expression of SIRT1 is reduced through molecular mechanisms which are still unknown (205-209).

We have recently demonstrated that SIRT1 expression is reduced in peripheral blood mononuclear cells from subjects who are insulin resistant, specifically in those who are glucose intolerant, and particularly in those with several components of metabolic syndrome. We also demonstrated how hyperglycemia and saturated fatty acids (palmitic acid) are responsible for the reduction of SIRT1 expression and activation in cultured lymphomonocytes. (210)

Increasing SIRT1 activity inhibits glucose-induced endothelial senescence and dysfunction (206,207,209). These effects were also seen *in vivo*; activation of SIRT1 prevented hyperglycemia induced vascular cell senescence and protected against vascular dysfunction in diabetic mice (211). On the other hand, transgenic mice that overexpress SIRT1 display some phenotypes similar to mice on a caloric restricted diet since they are

leaner than littermate controls and are more metabolically active and show a better glucose tolerance (212).

Overall these data indicate that a defect in the regulation of SIRT1 expression and activation might play a role in both metabolic derangement and cardiovascular complications associated with diabetes and metabolic syndrome and the increased expression of SIRT1 is an exciting therapeutic perspective in the prevention and treatment of atherosclerosis and therefore of macrovascular complications in diabetic patients. Until now, a link between the physiological and pharmacological action of GLP-1 and the molecular target SIRT1 has not been studied.

## SIRTUINS

Sirtuins (SIRT) comprise a unique protein family with nicotinamide adenine dinucleotide (NAD(+))-dependent deacetylase activity that target histonic proteins and several transcription factors. Originally, they were identified as belonging to MAR1(Mating-Type Regulator 1) protein group, able to act on gene suppression and/or activation; later, they were recognized to have an important biochemical and molecular role in Saccharomyces cerevisiae yeast by which the name of SIR2 (Silent Information Regulator 2). Later, Gottlieb and Esposito showed that SIR2 was able to repress the expression of certain genes through deacetylation of the ε-amino group of N-terminal lysine residues of histones and identified the presence of four additional SIR2 homologous genes, which were called HST 1-4 (homologues of SIR2) in S. cerevisiae. The discovery of homologs of SIR2 in yeast and later in bacteria, plants and mammals, showed that SIR2 is a member of a large and ancient family of genes that expresses proteins that are now called "sirtuins". These enzymes have a key role in the regulation of many aspects of cellular metabolism, modulating the transcription of chromatin gene and regulating the activity of several transcription factors such as p53, NF-kB, PGC-1α, FOXO and HSF-1. SIRT are in fact involved in metabolic processes that delay aging and increase lifespan, in cell cycle regulation, in inhibition of apoptosis, in glucose homeostasis and in insulin secretion.

SIRT have gained considerable attention over time in the medical field due to their role as metabolic sensors and as mediators of cell survival under stress conditions, such as the restriction of caloric intake and exercise, in which their transcription is activated. The characterization of the molecular, biochemical and physiological mechanisms involved in

effects of SIRT is important not only for a better definition of their physiological and pathophysiological role but also for the identification of new potential pharmacological targets in metabolic disorders, diabetes, endothelial dysfunction, and in cardiovascular and neurodegenerative disease (213).

## Biochemical properties of sirtuins

In eukaryotes, the family of sirtuins consists of seven proteins called SIRT 1, 2, 3, 4, 5, 6 and 7. SIRT consist of a central catalytic domain of 275 amino acids capable of binding NAD +, highly conserved in eukaryotic cells, and an N-terminal and/or C-terminal sequence of variable length that is responsible for the different biological and functional properties of each of them (214).

Sirtuins are enzymes with deacetylase activity that remov lysine residues from proteic substrates or histones in the presence of cofactor NAD + (nicotinamide adenine dinucleotide). The products of this reaction are nicotinamide (NAM), the protein substrate or deacetylated histone on lysine residues, and the 2'-O-acetyl-ADP-ribose molecule.

NAD + is a coenzyme involved in many biological functions, in oxidation-reduction reactions, in DNA repair, in the reactions of ADP ribosylation, in the immune response and in transcriptional regulation. An increase in cellular levels of NAD + leads to an increase of the action of SIRT1 activating the regulation of glucose-stimulated insulin secretion in pancreatic β-cells (215).

Unlike other sirtuins, SIRT4 has transferase activity (catalyzing ADP-ribosylation of several proteins), whereas SIRT3 and SIRT6 possess both the enzymatic activities.

## SIRT 1

In mammals, seven genes belonging to the family of sirtuins (SIRT1-7) have been identified. In particular, the homologous gene to Sir2 is SIRT1 (Sir2homolog 1), the best understood in terms of cellular activity and function. SIRT1 is localized predominantly in the nucleus, but in response to particular physiological conditions or stimuli, it can be translocated into the cytoplasm through nuclear export signal (NES). The subsequent transport from the cytoplasm to nucleus occurs through nuclear localization signal (NLS). It is expressed in all organs, but particularly in those in which energetic metabolism is

more developed. SIRT1 knock-out mice die prenatally, while those that survive become sterile and present lung infections and pancreatic atrophy (216).

SIRT1 codes for a protein capable to deacetylate nuclear and cytoplasmic factors that control critical cellular processes such as apoptosis and metabolism. It also regulates the production of insulin and glucose, the lipid metabolism and cell survival, mediating the effects of caloric restriction in mammals (217). Therefore, the potential anti-aging role of the gene appears to be valid because involved in a complex protein pattern that includes enzymes involved in cellular repair mechanisms. Thus, caloric restriction promotes cell survival by inducing SIRT1 deacetylase (218).

## SIRT1 and transcription factors

Sirtuins have the capability to deacetylate transcriptional factors, in addition to histones, interfering with gene expression. In most cases, these factors control genes related to growth, cell cycle, apoptosis and energy metabolism.

Several transcription factors are negatively regulated by SIRT1. This leads to an alteration of the cellular metabolic and survival balance, since deacetylation of lysine residues decreases the expression of transcription factors, such as p53, FOXO1, PPARγ, HSF1, Ku70, PGC-1α and NF-kB.

### SIRT1 and p53: inhibition of apoptosis

The p53 protein is involved in cell cycle control and has several potential sites of acetylation of lysine residues in the C-terminal region where SIRT1 may exert its catalytic activity (219). The acetylated form of p53 activates the transcriptional program with increased cell proliferation and apoptosis. In fact, in tumor cells this transcription factor is hyperacetilated. In vitro and in vivo animal models, the overexpression of SIRT1 reduces cell proliferation and inhibits apoptosis by deacetylation of p53, indicating SIRT1 as a potential target for cancer therapy (220).

## SIRT1 e FOXO1: stress resistance and cell survival

The FOXO (Forkhead Box type O) family consists of 4 proteins (FOXO1-4) involved in various cell functions such as cell response to oxidative stress, cell cycle regulation and response to insulin. In response to a cellular stress, increased expression of SIRT1 causes deacetylation of the FOXO transcription factor resulting in increased expression of genes involved in resistance to stress, in cell cycle slowing and in inhibition of apoptosis (221).

The FOXO1 isoform is involved in intracellular signal transduction mechanism of insulin. Insulin, by means of the enzyme Akt, regulates the expression of several gluconeogenic and lipogenic enzymes by regulating the activity of FOXO1 which is found primarily in tissues responsive to insulin, such as the liver, the adipose tissue and β-pancreatic cells. Activation of FOXO1 by acetylation of lysine residues induces its translocation from the nucleus into the cytoplasm, inhibiting activation of gluconeogenic enzymes and favoring the activation of genes involved in glycolysis such as glucose-6-phosphatase (222). Under conditions of prolonged fasting or caloric restriction overexpression and activation of SIRT1 induce deacetylation of FOXO1 that leads back into the nucleus  promoting gluconeogenesis and inhibiting glycolysis. Therefore, the role of SIRT1 is crucial in the regulation of cellular energy metabolism.

## SIRT1 and PPARγ: inhibition of lipogenesis and stimulation of lipolysis

SIRT1 acts as a negative regulator of the adipogenic process. It was shown that in SIRT1 transgenic mice the intracellular level of triglycerides was reduced by 50%, while in SIRT1 knock-out mice the fatty acids amount was significantly increased (223). Under conditions of restricted caloric intake, SIRT1 inhibits PPARγ (peroxisome proliferator-activated receptor γ), a transcription factor essential for the differentiation of adipocytes. In response to caloric restriction SIRT1 binds PPARγ through the PGC-1α co-factor, inhibits the adipogenesis and stimulates lipolysis, i.e. promoting mobilization of fatty acids in white adipose tissue.

### SIRT1 and Ku70: increased DNA repair activity

SIRT1 directly interacts with Ku70, a protein involved in DNA repair, with which it forms a complex. The interaction between SIRT1 and Ku70 leads to an increase in DNA repair and promotes cell survival in response to stress (224).

### SIRT1 and HSF1: protection from cell damage

HSF1 (heat shock factor 1) is a transcription factor that controls the expression of Heat Shock Proteins. Under basal conditions HSF1 is present in cytoplasm as a monomer complexed with several chaperone molecules in particularly Hsp90. Binding to these proteins inhibits binding to DNA and transcriptional activity. Exposure to stress factors, such as temperature and age, leads to an increase in the proportion of denatured proteins present in the cell and a consequent increase in needs of chaperone proteins, so that the dissociation from HSF1 takes place. The detachment from Hsp90 determines exposure of signal residues for the nuclear localization of HSF1, allowing translocation into the nucleus, a process followed by trimerization and phosphorylation of the transcription factor. At this stage HSF1 is able to bind to the promoters of the genes for HSPs and to unlock the RNA polymerase, which undertakes its transcriptional activity. HSF1 has a protective role in the cellular damage associated with protein denaturation. Deacetylation operated by SIRT1 prolongs the binding of HSF1 to Hsp70 (promoter of heat shock proteins) and maintaines it in a competent state for binding to DNA. Conversely, a reduction of SIRT1 expression attenuates the response to heat shock and causes the detachment of HSF1 from the promoter and its complex with cytoplasmic Hsp90. The role of SIRT1 in the regulation of proteins production, in the resistance to insults, in the protection from damage caused by the protein misfolding is therefore essential (225).

### SIRT1 and PGC-1α: decrease of glycolysis in favor of gluconeogenesis

Factor PGC-1α (PPAR-γ co-activator 1α) is a transcriptional coactivator of nuclear receptors that induces mitochondrial biogenesis, promotes the remodeling of the composition of muscle fibers and is involved in regulation of glucose and lipid

metabolism. By deacetylating PGC-1α, SIRT1 represses glycolysis, increases gluconeogenesis, and promotes hepatic glucose output (226).

### SIRT1 and NF-kB: inhibition of apoptosis and regulation of the immune response

The nuclear factor kB, as explained in previous paragraphs, is a dimeric transcripion factor that regulates the expression of genes involved in the immune and inflammatory response, and in cell proliferation and differentiation.

One of the main mechanisms of NF-kB inactivation is deacetylation that occurs through interaction of the factor with co-repressors, HDACs enzymes. These translocate an acetyl group from the five lysine residues of the protein to other substrates. Several studies have demonstrated that SIRT1 regulates the transcriptional activity of Nf-kB by deacetylating RelA/p65 subunit of Nf-kB at lysine 310 leading to its inactivation (204). This regulation is biologically significant because the activation of SIRT1 inhibits NF-κB transcription and reduces the expression of inflammatory adhesion molecules. A recent study has shown that in addition to blocking cell proliferation, SIRT1 acting on NF-kB, sensitizes cells to apoptosis induced by TNF-α (201). In microglial NF-kB is involved in neuronal death induced by peptide β-amyloid, which causes Alzheimer's disease. SIRT1 may thus play also a neuroprotective role by inactivating NF-kB (227).

## REGULATION OF SIRT1 EXPRESSION

The regulation of SIRT1 expression occurs through the control of transcriptional and post-transcriptional mechanisms, activated by different pathways. However, not all the mechanisms of expression regulation have been identified.

### Akt and SIRT1

Akt, also known as PKB (protein kinase B) is a serine/threonine kinase involved in the regulation of various cell processes. There are three isoforms of Akt: Akt1, Akt2, and Akt3, expressed in different cell types. Akt is an enzyme that is activated by phosphatydil inositol-3 phosphate kinase (PI3K). This pathway is usually activated

following activation of various receptors, induced by binding of specific ligands. Once activated, Akt, through phosphorylation of different substrates, contributes to cell cycle regulation and apoptosis, to cell differentiation, to the regulation of cell metabolism and oxidative stress and to the synthesis of extracellular matrix (228).

Recently it has been observed that the activation of Akt results in a increas of SIRT1 by inhibition of miRNA199a-5p. It is actually a pre-microRNAs of 120bp, which generates a microRNA that bind to 3'-UTR untranslated region of SIRT1 mRNA, and inhibits SIRT1 gene expression SIRT1. Activation of Akt through inhibition of this micro-RNA precursor results in increased gene expression of SIRT1 (229). In addition to this control mechanism, the activation of PI3K/Akt pathway regulates SIRT1 gene expression through pre-transcriptional mechanisms. Recently it has been demonstrated that statins increase SIRT1 gene expression in endothelial cells through activation PI3K/Akt pathway (230).

## AMPK and SIRT1

AMP activated protein kinase (AMPK) is a serine/threonine kinase highly conserved during evolution. Structurally it is a heterotrimeric complex composed of a catalytic α subunit, a regulatory β subunit and a γ subunit. There are also two isoforms of the α subunit (α1 and α2) and β subunits (β1 and β2) and three γ subunits, which are differently expressed in cells of various tissues (230-232).

AMPK, when activated in allosteric way, determines the phosphorylation of various substrates, including numerous proteins and transcription factors, by the increase in cAMP and phosphorylation of the α subunit by the kinases LKB1 and CaMKKβ (233). In particular, the main role of AMPK is the regulation of cell metabolic and energy processes by stimulation of the production of energy through the catabolism of glucose and lipids, inhibition of metabolic pathways with energy consumption and by synthesis of proteins, fatty acids and cholesterol. Furthermore, AMPK plays a key role in cells, by maintaining homeostasis under conditions of cellular stress (234-235). Several studies have shown that a reduced activation of AMPK is involved in a series of diseases, characterized by insulin resistance, including obesity, metabolic syndrome and diabetes (235-238). It was also demonstrated that AMPK has an important anti-inflammatory role through inhibition of NF-kB pathway. Recently it was shown that this process is mediated by activation of SIRT1 by AMPK through a mechanism not fully known, but probably

due to direct phosphorylation of SIRT1 protein by AMPK. This mechanism is altered in particular in endothelial dysfunction induced by diabetes and hypertension (234).

### ERK and SIRT1

The extracellular regulating kinase (ERK 1 and 2) belongs to the family of mitogen activated protein kinases (MAPK) and are important regulators of signal transmission within the cells. They are specific serine/threonine kinases involved in the regulation of several cellular processes. Their activation is mainly induced by activation of stimulatory G protein-coupled receptors. ERK1 and ERK2, when activated, phosphorylate a number of molecular targets including several cytoplasmic proteins and nuclear transcription factors thereby regulating various cells activities including: apoptosis, cell cycle and expression of various genes. It has been demonstrated that ERK can be activated by different metabolic pathways including the PI3/Akt and the AMPK pathway (239-241). A link between ERK and the molecular target SIRT1 is currently unclear.

# AIMS OF THE STUDY

Aim of the present study is to verify, in vitro, in endothelial cells (HUVEC), the effects of treatment with GLP-1(7-37) and GLP-1 (9-36) NH2 peptides on gene and protein expression of SIRT1 in conditions of normoglycemia and hyperglycemia. Another objective was to identify the GLP-1 dependent metabolic pathways that are involved in SIRT1 activation.

# METHODS

## EXPERIMENTAL PROTOCOL

HUVEC cells ($1x10^{6}$ cells) were incubated with GLP-1 (7 -37) and GLP-1 (9-36) NH2 (Sigma) at concentration of 2.33 mM in the presence or the absence of the inhibitor of DPP-4 (valine-pirolidide) at 24.1 mM concentration, under conditions of

normoglycemia and hyperglycemia for 6h, 12, 24h and 48h. The peptides GLP-1 (7-37), GLP-1 (9-36) NH2 and the DPP-4 were kindly provided by the Department of Chemistry, University of Padua (Prof. L.Toniolo). During incubation with GLP-1 peptides, the cells were placed in quiescence (3% FBS) in complete medium. After treatment with different peptides, the cells were immediately collected for extraction of RNA or proteins.

## EXPERIMENTS IN HUVEC CELLS

### Cell culture

The experiments were performed in human endothelial cells (HUVEC) extracted from umbilical vein, obtained from Clonetics Inc. (SanDiego, USA) (Fig. 9). The cells were cultured in complete medium (PromoCell, Heidelberg, Germany), containing 10% fetal bovine serum (FBS Sigma), 0.02% of Supplemental Mix / Endothelial Cell Growth Medium (Promo Cell, Heidelberg, Germany) and 1% penicillin and streptomycin (Sigma). The cells were maintained in an incubator at 37° C in a humidified atmosphere, with 5% CO 2 in the presence of normal levels of glucose (5.5 mmol/L). They were commonly used between the third and sixth passage.

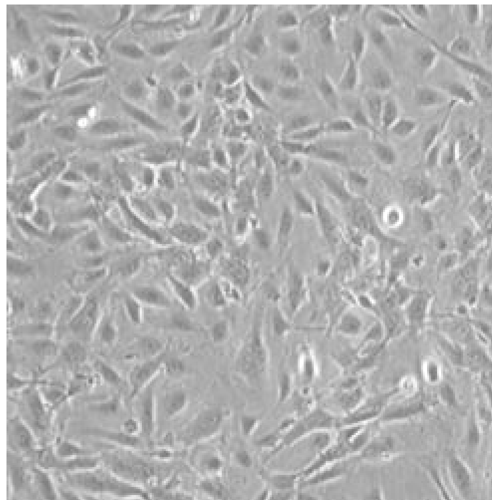

***Figure 9***. *HUVEC image at microscope*

## Cell vitality

Endothelial cell vitality was measured before and after treatment with GLP-1 peptide by Trypan Blue. This chromophore is negatively charged and is unable to pass through the intact cell membrane, but through that of dead cells, which turn into blue. This allows the easy discrimination of alive cells (which excluded the color) from those death, by simple observation under a microscope. Usually, cellular vitality in our experiments corresponds to 80-90%.

## GENE EXPRESSION OF SIRT1

### RNA extraction

RNA was extracted and purified from endothelial cells using the RNeasy Mini kit (QIAGEN), by separation on silica columns and elution using Eluition buffer (QIAGEN).

### Quantification of RNA

RNA concentration (mg/l) was determined by spectrophotometric analysis (NanoDrop 2000C, Thermo Scientific) at λ of 260 nm. The absorbance value at λ of 260 nm allows us to quantify total RNA extracted using the formula:

44 microgram/ml x A260 x dilution factor.

The quality of RNA was determined by electrophoresis on Agilent microchip (RNA6000Nano kit). Values greater than 8 R.I.N. were considered optimal for subsequent analysis.

## Reverse transcription of RNA

The reverse transcription reaction is used to amplify the double stranded DNA from the extracted single-stranded RNA. During the RT phase (reverse transcription) the enzyme reverse transcriptase synthesizes a single-stranded DNA molecule (cDNA) complementary to the template RNA. The RT reaction was performed using the iScript cDNA Synthesis Kit (Bio-Rad). The temperature gradient of the reaction consists of a first phase at 25 ° C for 5 minutes, a second at 42 ° C for 30 minutes to activate the reverse transcriptase and a third at 85 ° C for 5 minutes to inactivate the reverse transcriptase

## Real-Time PCR

The quantification of gene expression of SIRT1 was obtained by Real Time-PCR using specific primers and a fluorescent probe (SYBR-Green). This fluorescent probe has the particularity to emit fluorescence only when bound to double-strand of the amplification product of the oligonucleotidic sequence (300 bp) of the gene of interest. Consequently the increase of fluorescence signal will be proportional to the amount of amplification product obtained by the PCR reaction that corresponds to the value of the threshold cycle (Ct). In fact, in real-time PCR reaction, monitored in real time for 40 cycles, a sigmoidal curve is obtained, where the first 10-15 cycles correspond to values of fluorescence given by the background noise (background), then between 15-25 cycles the reaction has an exponential proceeding and then it flexes and reaches the plateau (30-40 cycles). The value of Ct, used to quantify the product of the PCR reaction, corresponds to Ct where the fluorescence increase results to be significantly higher than baseline levels (background) measured during the first cycles of amplification.

The Real-Time PCR was performed using the i-Cycler iQ5 Real Time detection system (Bio-Rad). The Real-Time PCR reaction was performed using iQ SYBR Green Supermix Kit (Bio-Rad) and the specific primers for the genes of interest. The PCR reaction is carried out with a total of 20 µl containing 10 µl of PCR-SYBR Green PCR Master Mix 2x (Biorad), 2 µl of forward primers (400 nM), 2 µl reverse primer (400 nM), 2 µl H2O and 4 µl of cDNA (equivalent to 1/5 of the reverse transcription). The thermal

profile of the reaction includes a reaction cycle at 95 ° C for 5 min (to activate the enzyme Taq polymerase) and 40 cycles consisting of 15 seconds at 95 ° C (for denaturation) and 1 min at 60 ° C (for the hybridization and extension phase ). The oligonucleotide sequences of the primers for SIRT1 and the housekeeping gene β-actin were designed in our laboratory using the Primer3 (Whitehead Institute, Cambridge, Massachusetts, USA) and Operon (Operon Technologies Inc., Alameda, California, USA) software. The specificity of the primers was verified by agarose gel electrophoresis and by melt-curve analysis that allows to obtain the temperature at which half of the complementary molecules are hybridized. This allows to verify the presence of primer dimers or the presence of nonspecific products in PCR reaction. After verifying that the choice of primers was optimal, calibration curves for each gene at scalar concentrations (dilution 1:2) of total RNA were made. This has allowed to obtain the efficiency of the PCR reaction that must be approximately of 100%. Using the calibration curves, the slope values, the intercept of the regression line (r) and coefficient of correlation of the points were obtained. The slope value is then used to calculate the efficiency of the PCR reaction:

$$E(eff)= 10 -1/slope$$

The quantification of the expression of the gene of interest is of relative type because it is normalized for a housekeeping gene and is calculated using the following mathematical formula:

$$expression= 2-\Delta\Delta Ct$$

where -ΔΔCt= [(Ct (SIRT of a sample) – Ct (β-actin of a sample)] – [(Ct (SIRT of a normalizer) – (Ct ( β-actin of a normalizer )].

Normalizer is defined as a sample with expression value of 1, in our case the β-actin.

## GLP-1R RECEPTOR GENE EXPRESSION IN HUVEC

The determination of gene expression of the GLP-1R receptor in HUVEC cells was performed by PCR and the amplification product (278 bp) was detected by 1%

agarose gel electrophoresis. PCR reaction was performed using High Fidelit PCR System kit (Roche) using the primers:

sense (forward) GAGGAACCGGAACAACATTG

antisense (reverse) CCAAGTGACTGGCAGGAGAT.

## PROTEIN EXPRESSION OF AKT/P-AKT, AMPK/P-AMPK, ERK/PERK

### Protein extraction

After incubation with GLP-1 peptides HUVEC cells were lysed with 150 µl of hypotonic buffer containing: 20 mmol HEPES, 2mmol EGTA, 10 mmol glycerophosphate, 1mmol DTT (ditiotritol), 2mmol vanadium, 10 mmol fenilmetilsulfonil fluoride (PMSF), 1 mmol leupeptin, 1 µg/ml aprotinin. The cells are detached at the bottom of the plate by means of a "scraper", collected and stored at -80 ° C.

Then the protein lysate was sonicated in lysis buffer and the homogenate was centrifuged at 800 x g for 10 min at 4°C to remove cell debris. All this must occur at temperatures around 4 ° C to avoid protein degradation.

### Protein assay by an assay with BCA

To determine the total concentration of protein in a solution of lysate an assay with BCA (bicinchoninic acid, Euroclone) was used. This method is extremely sensitive and precise, it can reveal quantity of protein less than 0.5 µg/ml. It is based on the chemical principle for which under alkaline conditions copper ions $Cu^{2+}$ form a complex with peptide links of proteins and are reduced to $Cu^{+}$ (fig 10).

Bicinchoninic acid under alkaline conditions is a highly sensitive, stable and specific reagent for Cu+, and forms with it a purple colored compound, whose intensity is proportional to the amount of protein. The change in intensity is determined by spectrophotometric absorbance at 562 nm. The concentration value is then extrapolated from a calibration curve previously constructed using samples of bovine serum albumin of known concentration.

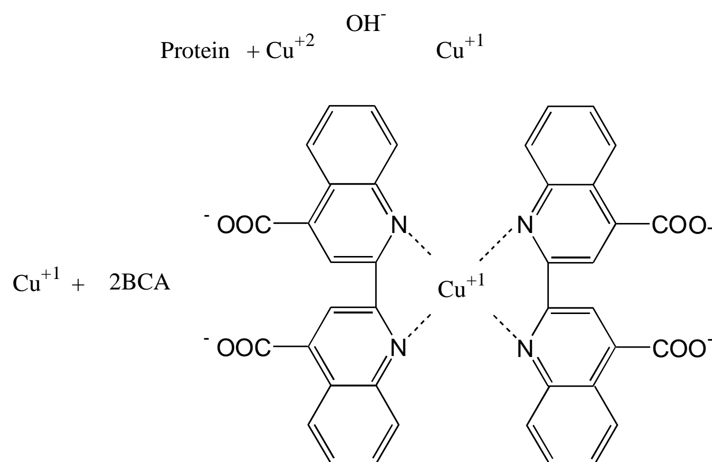


***Figure 10.*** *Bicinchoninic acid reaction*

## Western blot

Protein expression and activation of Akt, AMPK and ERK was measured by polyacrylamide gel electrophoresis and Western blot. Proteins (50-200μg) were denatured in Laemmli buffer, containing dithiothreitol (DTT) and SDS in 1:9 ratio, at 100 ° C for 5 minutes. The protein separation was performed by polyacrylamide gel electrophoresis SDS-PAGE 10%, with the electrophoresis buffer containing 0.12 M TRIS, 1.23 M glycine, 0.01 M SDS. The proteins were then transferred through electroblotting (for 180 minutes at 100 V at 4 ° C) on a nitrocellulose membrane using a Transblot device (Elettrofor, Padua, Italy) with the blotting solution containing 48 mM TRIS, 39 mM glycine, 0,037%, SDS and 20% Methanol (vol/vol). The proteins, once transferred to a membrane, were incubated with a solution of PBS containing 0.05% (vol/vol) Tween (T-PBS) and 5% albumin, overnight at 4 ° C. The membranes were again incubated overnight with primary antibodies anti-Akt, anti-ERK1/2, anti-AMPK (dilution 1:2000, Santa Cruz Biotechnology) and anti-p (phospho) Akt, anti-p (phospho) ERK1/ 2, antip(phospho) AMPK (Cell Signaling Biotech) and then with secondary antibody (Amersham) conjugated to horseradish peroxidase.

The detection of antibody binding with the protein under examination was made by chemiluminescent reaction (ECL) using the Versa-Doc Imaging System 3000 (BioRad) (Figure 11). The densitometric analysis of the bands corresponding to the protein in question was analyzed using the software Quantity One (Biorad) that allows the

measurement of the protein in examination expression. The primary antibody was then removed from the membrane by incubation at 50 ° C for 30 minutes in a solution containing 0.76 g Tris base, 2 g SDS, 700 µl β-mercaptoethanol at pH 6.8 and incubated again with primary antibody anti-GAPDH (glyceraldehyde-3-phosphatodehidrogenase, 1:5000, Chemicon). The densitometric analysis of the expression of GAPDH was used to normalize the expression of the protein under consideration and to correct any errors in sample loading. The results were expressed as the percentage of control, which was set as equal to 100%.

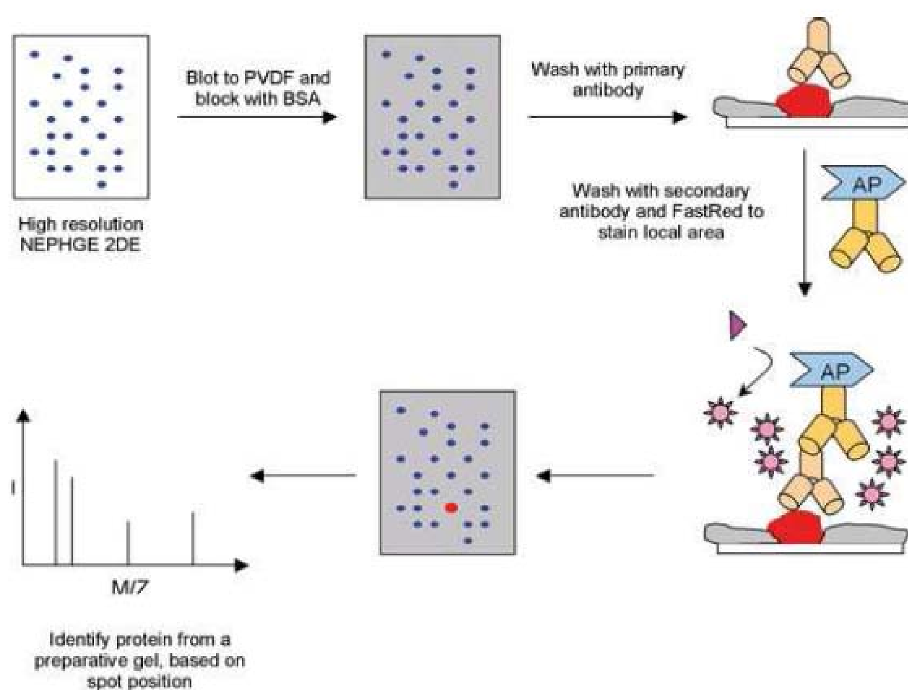


***Figure 11.*** *Chemiluminescent detection of western blot*

## STATISTICAL ANALYSIS

All the quantitative results were expressed as the mean ±SD. The statistical analysis was performed by SPSS18 software package. Comparison of numerical variable data was conducted with analysis of variance (ANOVA) and statistical significance was set at $P<0.05$

# RESULTS

## GLP-1R-RECEPTOR EXPRESSION IN HUVEC

The presence of GLP-1R receptor in endothelial cells HUVEC was documented by RT-PCR with specific primers for receptor mRNA and 1% agarose gel electrophoresis. Figure 12 shows the presence of a single band corresponding to the amplification product of 278 bp obtained by PCR reaction of GLP-1 receptor RNA .

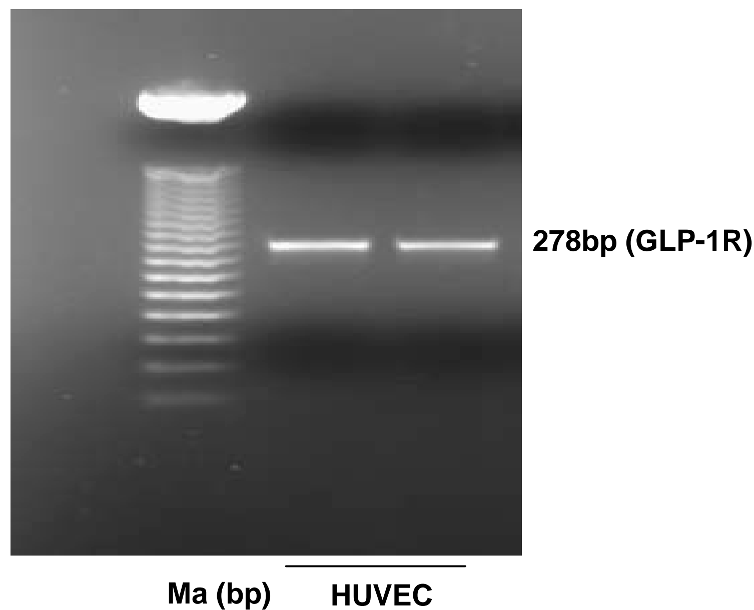


***Figure 12***. *Electrophoresis on 1% agarose gel of GLP-1R. The first left column (Ma) represents the marker, while the other two columns represent the presence of a band at 278 bp indicating the presence of GLP-1R in endothelial cells.*

## EFFECTS OF GLP-1 PEPTIDES ON SIRT1 GENE EXPRESSION UNDER NORMOGLYCEMIA

HUVEC have been treated for 24h and 48 h with GLP-1 (7-37) and GLP-1 (9-37)NH2 in the presence and absence of DPP-4 inhibitor, in conditions of normoglycemia (glucose concentration 5.5 mM) (fig 13 and 14). Gene expression of SIRT1 was measured by means of real time PCR. As shown in figure 13 and figure 14 treatment with GLP-1

peptides did not modify SIRT1 expression either in the absence, or in the presence of DPP4 inhibitor.

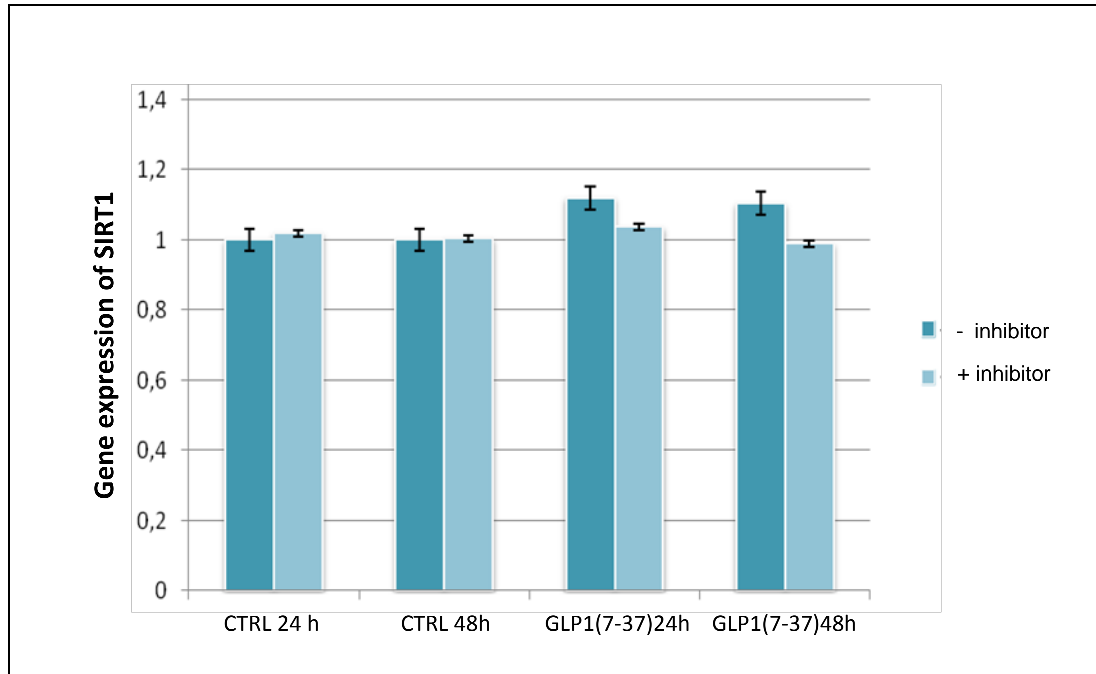


***Figure 13.*** *Effect of GLP-1 peptide (7-37), in the absence and presence of the DPP-4 inhibitor on gene expression of SIRT1 at 24 and 48 hours. The graph shows that there is no effect on the expression of SIRT. Values are expressed as mean ± SE, n = 3.*

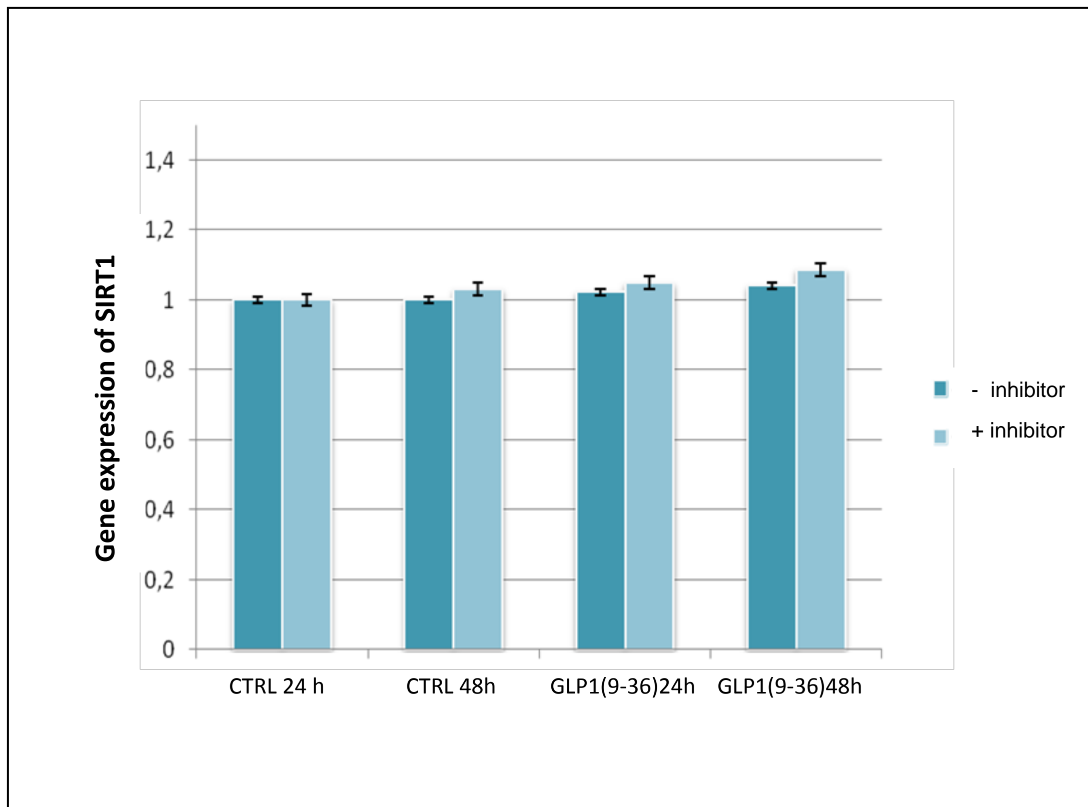


***Figure 14.*** *Effect of GLP-1 peptide (9-36) NH2, in the absence and presence of the DPP-4 inhibitor on gene expression of SIRT1 at 24 and 48 hours. The graph shows that there is no effect on the expression of SIRT. Values are expressed as mean ± SE, n = 3.*

## EFFECTS OF GLP-1 PEPTIDES ON SIRT1 GENE EXPRESSION UNDER HYPERGLICEMIA

Recently our group demonstrated that SIRT1 gene and protein expression was reduced in human linfomonocytes (THP-1) in the presence of high glucose levels (20 mM) for 48 h compared to what happens in normoglycemic conditions (210). This effect is due to an increase of oxidative stress and reactive oxygen species (ROS) production induced by hyperglycemia which inhibits SIRT1 expression. We investigated the effects of GLP-1 and DPP4 inhibitor on SIRT1 expression in condition of hyperglycemia (20mM) at 48 h, as hyperglycemia induced reduction of SIRT highest at 48 h (210).

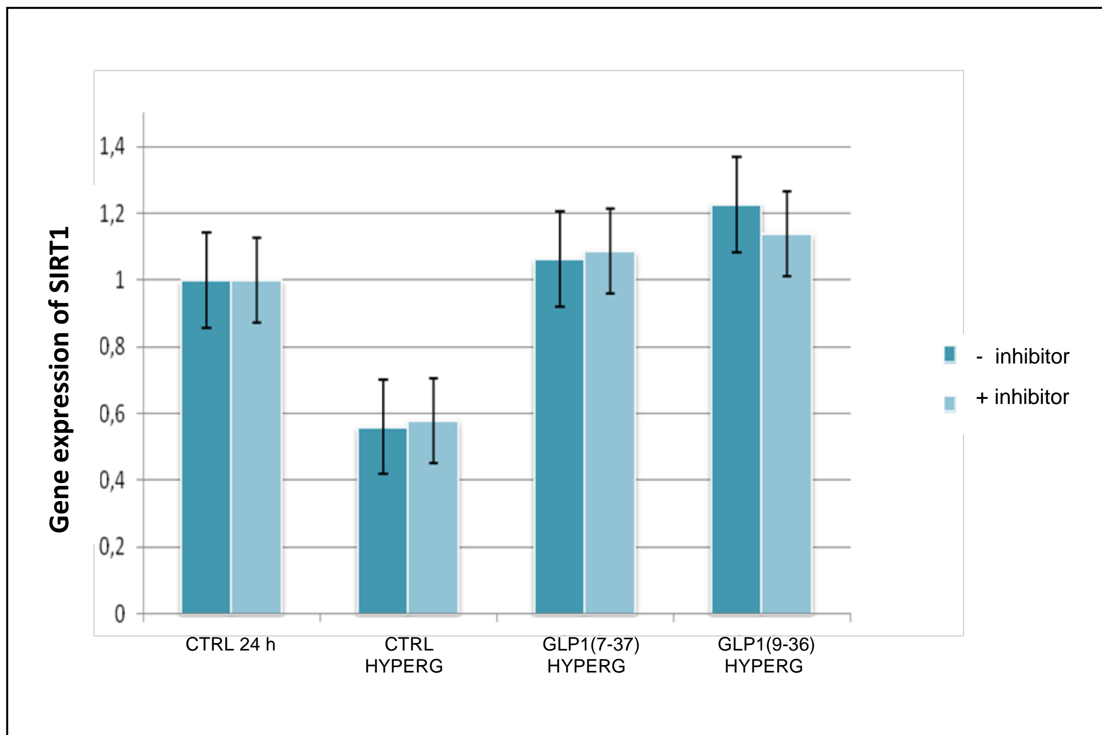


***Figure 15.*** *Gene expression of SIRT1 at 48 hours after treatment with the GLP-1 peptides and DPP-4 inhibitor under conditions of hyperglycemia (20 mM). Values are expressed as mean ± SE, n = 3*

Measurement of SIRT1 gene expression by real time PCR shows that in hyperglycemia SIRT expression is reduced compared to normoglicemia (5,5 mM) confirming previous observations (fig.15). Treatment of cells with GLP1 (7-37) and GLP1 (9-37) in conditions of hyperglycemia normalizes SIRT expression. The increase of SIRT expression levels induced by GLP-1 peptides was not affected by DPP-4 inhibitor treatment.

## EFFECTS OF GLP-1 PEPTIDES ON ACTIVATION OF p-AKT, p-AMPK AND p-ERK

GLP-1, by interacting with GLP-1 receptor coupled with G proteins or independently of the receptor activate different intracellular signal transducting pathways, in particular extracellular regulated enzymes (ERK1/2), IP3/Akt pathway, and AMPK, which induce different cellular and molecular effects, dependent on the cell type. To investigate wheter the GLP-1 induced increase of SIRT1 expression in condition of hyperglycemia is dependent on one or more of these intracellular pathways, we studied the effects of GLP-1 on Akt, AMPK and ERK activation.

EFFECTS OF GLP-1 PEPTIDES ON ACTIVATION OF p-AKT

HUVECs were treated for different times (5 min, 10 min, 30 min, 1 h, 3 h, 24h, 48h) with GLP-1 (7-37) and GLP-1 (9-37), in the presence or absence of DPP4 inhibitor. Fig.16 and Fig.17 show the effect of GLP-1 (7-37) on AKT measured by phosphorylation levels of AKT protein.

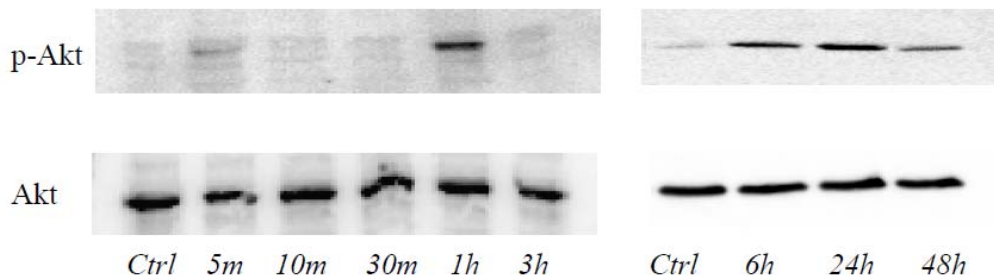


***Figure 16 .*** *Western blot of Akt and p-Akt after treatment of endothelial cells with GLP-1 (7-37).*

GLP-1 induced AKT phosphorylation is highest at 1 hour, it remains high up to 24 hours and it normalizes at 48 h. Treatment of HUVEC with GLP-1 (9-37) NH2 didn’t affect AKT phosphorilation . The presence of DPP4 inhibitor had no effect on AKT activation.

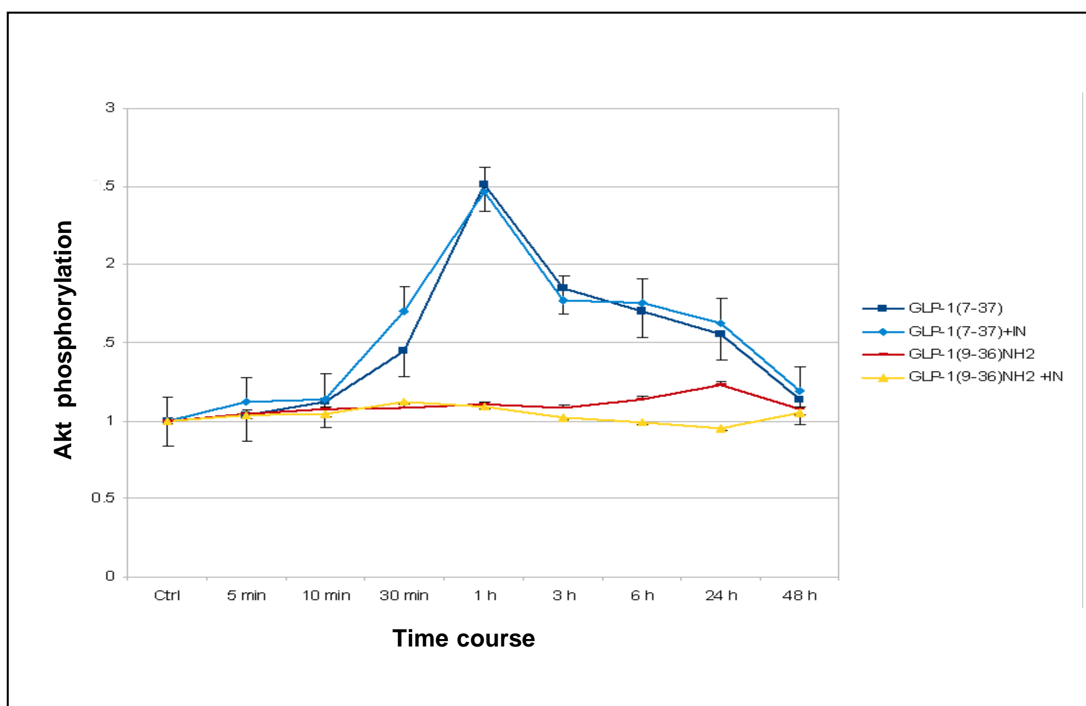


***Figure 17*** *. Akt phosphorylation over time after treatment with GLP-1 (7-37) and GLP-1 (9-36) NH2 in the presence and absence of DPP-4 inhibitor. Values are expressed as mean ± SE, n = 3.*

EFFECT OF GLP-1 PEPTIDES ON AMPK ACTIVATION

The effects of GLP-1(7-37) and GLP-1 (9-36) on AMPK activation were studied in conditions of normoglycemia (5.5mM). Figure 19 shows the effects of GLP-1(7-37) and GLP-1(9-36)NH2 on AMPK activation measured by AMPK phophorylation levels.

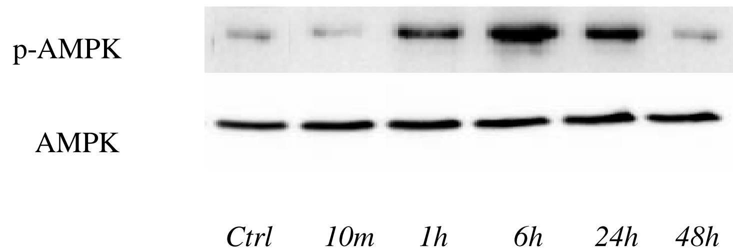


***Figure 18*** *. Western blot of AMPK and p-AMPK after exposure of endothelial cells with GLP-1 (9-36).*

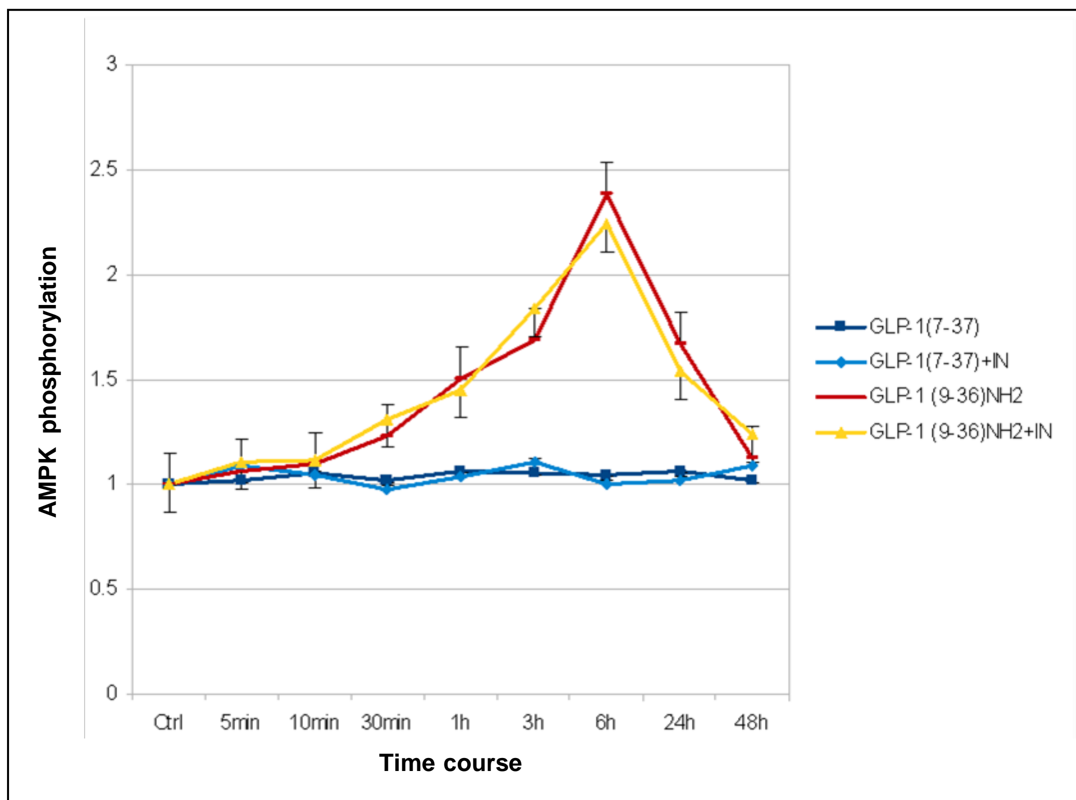


***Figure 19*** *. Phosphorylation of AMPK over time after treatment with GLP-1 (7-37) and GLP-1 (9-36) in the presence and absence of DPP-4 inhibitor. The values are expressed as mean ± SE, n = 3*

Figure 19 shows that treatment with GLP1 (9-36)NH2 induced a marked increase in AMPK phosphorylation achieving a peak after 6 hours, which is not affected by DPP4 inhibitor. GLP-1(7-37) had no effect on AMPK activation.

## EFFECTS OF GLP-1 PEPTIDES ON ERK ACTIVATION

HUVECs were treated for different times (5 min, 10 min, 30 min, 1 h, 3 h, 24h, 48h) with GLP-1 (7-37) and GLP-1 (9-37), in the presence or absence of DPP4 inhibitor. As it can be seen in figure 20 there is no effect of GLP-1 peptides and of DPP4 inhibitor on ERK activation.

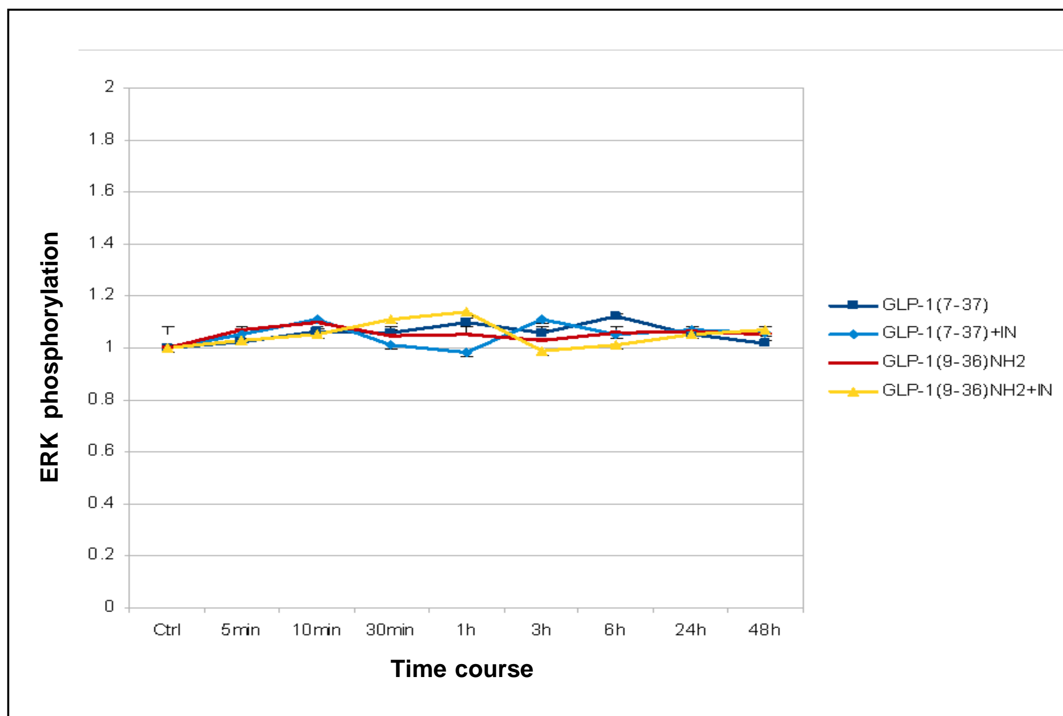


***Figure 20 .*** *ERK phosphorylation over time after treatment with GLP-1 peptides in the presence and absence of DPP-4 inhibitor.*

# DISCUSSION

Our study demonstrates that in human endothelial cells GLP-1 peptides, GLP-1 (7-37), and GLP-1 (9-36) NH2 antagonize the negative effects of hyperglycemia on the expression of SIRT1. In fact, both peptides have the ability to normalize SIRT1 gene expression, which is decreased in conditions of hyperglycemia. On the contrary, treatment with GLP-1 peptides, has no effect on the expression SIRT1 in normoglycemia.

The study also shows that the peptide GLP-1 (7-37) induces specifically the activation of phosphatidyl inositol-3 kinase (IP-3)/Akt pathway in HUVEC, while GLP-1 (9-36) leads to activation of the AMPK pathway. On the other hand we did not observe any effect of these peptides on the activation of ERK.

These data suggest a possible protective effect of GLP-1 peptides on vascular injury, manifested by increased expression of SIRT1 in conditions of hyperglycemia, that could involves two different intracellular signal transduction pathways for the two different peptides. The clinical importance of these aspects is evident if we analyze the results of a number of studies, which demonstrate the reduction of gene expression of SIRT1 in the endothelium in patients with diabetes, which plays a role in the onset and progression of atherosclerosis (196-209).

SIRT1 is a therapeutic target for the prevention and treatment of macrovascular complications, which are the leading cause of death in diabetes mellitus. The results of this study confirm a reduced expression of SIRT1 gene expression under conditions of hyperglycemia and demonstrate that GLP-1 peptides normalize the expression of SIRT1 reduced by hyperglycemia.

To our knowledge this is the first observation of the ability of GLP-1 to modulate the regulation of gene expression of SIRT1 in conditions of cellular stress, caused by the presence of high concentrations of glucose, and not in normoglycemic conditions, suggesting a specific protective effect against the vascular damage induced by hyperglycemia. Recent in vitro studies have shown that some effects of GLP-1, as the reduction of oxidative stress and reduced expression of adhesion molecules, occur only in pathological conditions (hyperglycemia or endothelial damage) and not in normal conditions (192). This finding is therefore of very important biological and clinical significance, since the normalization of the expression of SIRT1 in hyperglycemia may have a beneficial effect at endothelial level. In particular, it can regulate cell metabolism,

reduce inflammation, reduce production and increase disposal of reactive oxygen species, inhibit apoptosis and the development of atherosclerosis (196-209).

This study in addition to assessing the effects of GLP-1 on SIRT1 gene expression, investigated the possible molecular pathways through which GLP-1 peptides modulate the expression of SIRT, and in particular Akt, AMPK, ERK pathways were analyzed.

The majority of the actions of GLP-1 peptide (7-37) occurs as a result of the peptide interaction with the receptor GLP-1R, even if effects non mediated by GLP-1R have also been documented. The action of GLP-1 (9-36) is currently not well known. Probably the peptide does not bind to the GLP-1R, given the very low affinity for the receptor and non-receptorial mechanisms or the existence of a second receptor seem to be involved (59,84,140,141). The binding of GLP-1 to its receptor activates adenylate cyclase, resulting in increased cAMP and activation of various metabolic pathways including IP3/Akt and ERK pathways (143-147). Our results demonstrate also the activation of Akt in endothelial cells following treatment with the GLP-1 (7-37) peptide, while the other peptide studied, GLP-1 (9-36), did not stimulate IP3/Akt pathway. The activation of this pathway is very important because it regulates various cell processes such as increased transcription of eNOS, inhibition of the secretion of ET-1, inhibition of apoptosis and increased transcription of glucose transporters. In addition, some studies have shown the involvement of the IP3/Akt pathway also in the regulation of SIRT1gene expression. In particular, it has been demonstrated that, if activated, Akt pathway is able to reduce the synthesis of a specific microRNA (miR-199th), which is a regulation factor for SIRT1 expression, by binding to 3'-UTR (untranslated) sequence of SIRT1 mRNA, thus increasing the gene expression of SIRT1 (229). To reinforce the hypothesis of a link between Akt and SIRT1, a very recent study demonstrated the increased SIRT1 gene expression through IP3/Akt pathway activation induced by statins (230). Based on these experimental data it can therefore be hypothesized that increased expression of SIRT1, induced by GLP-1 (7-37), under conditions hyperglycaemia may involve the activation of IP3/Akt.

Activation of the AMPK pathway by GLP-1 active isotypes has also been described in different tissues, but whether this effect is mediated by the GLP1 receptor or by a non receptor mechanism is still unknown. In the liver this pathway mediates GLP1 inhibition of lypogenesis (242), in endothelial cells a GLP-1 analogue, liraglutide, exerts anti-inflammatory properties through activation of AMPK (71), in cardio-myocytes

AMPK pathway mediates GLP-1 protection from ischemia (243). On the other hand, it has been demonstrated that GLP-1 inhibits AMPK causing appetite loss through GLP-1 receptor binding mechanism.

Until now there was no evidence of any effect of GLP1(9-36) on the AMPK pathway. The data of our study show, for the first time, that the peptide GLP-1(9-36) induces activation of AMPK in endothelial cells possibly via a GLP1R independent mechanism while there is no effect by the other peptide, GLP-1 (7-37).

The identification of GLP-1R in endothelial cells implies a possible action with receptor mechanism by peptide GLP-1 (7-37) and an action with extra-receptorial mechanism by peptide GLP-1 (9-36), which has a low receptor affinity. The activation of the latter pathway is very important because it regulates various cellular processes such as control of cellular metabolism, inhibition of apoptosis and inhibition of inflammatory state.

Some studies have shown a possible link between AMPK pathway and SIRT1, and in particular evidence that activation of AMPK increases the expression of SIRT1 gene, resulting in inhibition of NF-kB and reduction in the inflammatory state, is emerging (234)

Activation of ERK following treatment of endothelial cells with GLP-1 peptides was also evaluated, but we could not find evidence for effects of ERK activation by GLP-1 peptides in our experimental conditions.

# CONCLUSIONS

The demonstration that GLP-1 is able to specifically normalize the expression of SIRT1 in hyperglycemia represents a new and promising therapeutic approach for type 2 diabetes mellitus and its complications, especially for the macrovascular complications. In addition to their metabolic effects GLP-1 agonists may improve endothelial function and reduce the risk of micro- and macrovascular complications. The finding of two different metabolic pathways activated by the two peptides studied suggests the existence of two independent mechanisms of action: GLP-1 (7- 37) by binding to the receptor activates the IP3/Akt pathway while GLP-1 (9-36) activates AMPK, probably through an extra-receptorial mechanism. This may stimulate research for new pharmacological pathways for the modulation of GLP-1 effects.

# REFERENCES


1. American Diabetes Association: *Standard of Medical Care in Diabetes*. Diabetes Care 2011; 34: 11-61
2. Harris M, Zimmet P: *Classification of Diabetes Mellitus and other categories of glucose intolerance*. In Alberti K, Zimmet P, Defronzo R, editors. International Textbook of Diabetes Mellitus. Second Edition. Chichester: John Wiley and Sons Ltd; 1997: 9-23
3. International Diabetes Federation: *Global Diabetes Plan 2011-2021*. 2011: 1- 26
4. Wild S, Roglic G, Green A, Sicree R, King H. *Global prevalence of diabetes: estimates for the years 2000 and projections for 2030*. Diabetes Care 2004; 27:1047-53.
5. Shaw JE et al. *Global estimates of the prevalence of diabetes for 2010 and 2030*. Diabetes Res Clin Pract. 2010; 87: 4-14.
6. Kannel WB, McGee DL: *Diabetes and cardiovascular risk factors: the Framingham study*. Circulation 1979; 59: 8-13
7. American Diabetes Association: *Clinical practice recommendations 2002*. Diabetes Care 2004; 27: 51-81
8. Haffner SM, Lehto S, Rönnemaa T, Pyörälä K, Laakso M. *Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction*. N Engl J Med 1998; 339: 229–234
9. Powers AC: *Diabete mellito morbilità e mortalità cardiovascolari.* In: Harrison Principi di Medicina Interna 16° edizione. Kasper DL, Fauci AS, Longo DL, Braunwald E, Hauser SL e Jameson LJ, Publ McGraw-Hill 2005; p 2427-2458
10. Masharani U: *Diabetes mellitus and hypoglycemia.* In: Current medical diagnosis and treatment 50° edition. McPhee SJ and Papadakis MA. Publ McGraw-Hill 2011; p 1140-1180
11. De Caterina R: *Endothelial dysfunction: common denominators in vascular disease*. Curr Opin Lipid 2000; 1: 9-23
12. Stehouwer CDA, Lambert J, Donker AJM, Van Hinsbergh VWM: *Endothelial dysfunction and pathogenesis of diabetic angiopathy.* Cardiovasc Res 1997; 1: 55-68
13. Schalkwijk GC, Stehouwer CDA: *Vascular complication in diabetes mellitus: the role of endothelial dysfunction*. Clin Sci 2005; 2:143-159.

14. Van den Oever IAM, Raterman HG, Nurmohamed MT, Simsek S: *Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus.* Mediator of Inflammation 2010; 1: 1-15
15. Hadi HAR, Al Suwaidi JA: *Endothelial dysfunction in diabetes mellitus.* Vascular and Risk Management 2007; 6: 853-876
16. Tan KCB, Chow W-S, Ai VHG, Lam KSL: *Effects of angiotensin II receptor antagonist on endothelial vasomotor function and urinary albumin excretion in type 2 diabetic patients with microalbuminuria.* Diabetes Metab Rev 2002; 18: 71–76.
17. Libby P. Inflammation in atherosclerosis. Nature 2002; 420 (6917) : 868–874
18. Tracy RP, Lemaitre RN, Psaty BM, et al: *Relationship of C-reactive protein to risk of cardiovascular disease in the elderly. Results from the Cardiovascular Health Study and the Rural Health Promotion Project.* Arterioscler Thromb Vasc Biol 1997; 17: 1121-1127
19. Biegelsen ES, Loscalzo J: *Endothelial function and atherosclerosis.* Coron Artery Dis 1999; 10: 241–256
20. Cowan DB, Langille BL: *Cellular and molecular biology of vascular remodeling*. Curr Opin Lipid 1996; **7**: 94–100.
21. Avogaro A, Albiero M, Menegazzo L, De Kreutzenberg S, Fadini GP: *Endothelial dysfunction in diabetes: the role of reparatory mechanism.* Diabetes Care 2011; 2: S285-S290
22. Verma S, Anderson TJ: *Fundamentals of endothelial function for the clinical cardiologist*. Circulation 2002; 105: 546–549
23. Flyvbjerg A: *Putative pathophysiological role of growth factors and cytokines in experimental diabetic kidney disease.* Diabetologia 2000; 43: 1205–1223.
24. Shamoon H, Duffy H, Fleischer N, et al. *The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus*. N Engl J Med 1993; 329: 977–986
25. Holman RR, Cull CA, Fox C, Turner RC: *United Kingdom prospective diabetes study (UKPDS) 13: relative efficacy of randomly allocated diet, sulphonylurea, insulin, or metformin in patients with newly diagnosed non-insulin dependent diabetes followed for three years.* Br Med J 1995; 10: 83–88
26. Dagher Z, Park YS, Asnaghi V, Hoehn T, Gerhardinger C, Lorenzi M: *Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy*. Diabetes 2004; 53: 2404–2411

27. Gabbay KH: *Hyperglycemia, polyol metabolism, and complications of diabetes mellitus*. Annu Rev Med 1975; 26: 521–536
28. Du X-L, Edelstein D, Rossetti L, et al.: *Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation.* Proc Natl Acad Sci USA 2000; 97: 12222–12226
29. Way KJ, Katai N, King GL: *Protein kinase C and the development of diabetic vascular complications.* Diabet Med 2001; 18: 945–959
30. Idris I, Gray S, Donnelly R: *Protein kinase C activation: isozyme-specific effects on metabolism and cardiovascular complications in diabetes.* Diabetologia 2001; 44: 659–673
31. Williams B, Gallacher B, Patel H, Orme C: *Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro.* Diabet Med 1997; 46: 1497–1503
32. Fowler MJ. *Microvascular and macrovascular complications of diabetes*. Clin Diabet 2008; 2: 77-82
33. Orsi E, Corbetta S, Peracchi M: *Disordini del ricambio glucidico In: Malattie del Sistema Endocrino e del Metabolismo 4°edizione*. Faglia G, Beck-Peccoz P Publ McGraw-Hill 2006; p 393-351
34. Kawashima S: *The two faces of endothelial nitric oxide synthase in the pathophysiology of atherosclerosis*. Endothelium 2004; 11: 99–107
35. Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM: *Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells.* Proc Natl Acad Sci USA 1996; 93: 9114–9119
36. Janssen-Heininger YMW, Poynter ME, Baeuerle PA: *Recent advances towards understanding redox mechanisms in the activation of nuclear factor κB*. Free Radic Bio Med 2000; 28: 1317–1327
37. Jansson P-A: *Endothelial dysfunction in insulin resistance and type 2 diabetes.* J Intern Med 2007; 262: 173–183
38. Kim JA, Montagnani M, Koh KK, Quon MJ: *Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms*. Circulation 2006; 113: 1888–1904

39. Muniyappa R, Montagnani M, Koh KK, Quon MJ: C*ardiovascular actions of insulin*. Endocr Rev 2007; 28: 463–491
40. Montagnani M, Chen H, Barr VA, Quon MJ: *Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179).* J Biol Chem 2001; 276: 30392–30398
41. Hartell NA, Archer HE, Bailey CJ: *Insulin-stimulated endothelial nitric oxide release is calcium independent and mediated via protein kinase B.* Biochem Pharmacol 2005; 69: 781–790
42. Wheatcroft SB, Williams IL, Shah AM, Kearney MT: *Pathophysiological implications of insulin resistance on vascular endothelial function.* Diabet Med 2003; 20: 255–268
43. Montagnani M, Golovchenko I, Kim I, Koh GY, Goalstone ML, Mundhekar AN, Johansen M, Kucik DF, Quon MJ, Draznin B. *Inhibition of phosphatidylinositol 3-kinase enhances mitogenic actions of insulin in endothelial cells.* J Biol Chem 2002; 277: 1794–1799
44. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD: *Insulin mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release.* J Clin Invest 1994; 94: 1172–1179
45. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD: *Obesity/insulin resistance is associated with endothelial dysfunction: implications for the syndrome of insulin resistance.* J Clin Invest 1996; 97: 2601–2610
46. Ido Y, Carling D, Ruderman N: *Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation*. Diabetes 2002; 51: 159–167
47. American Diabetes Association: *Scientific Session 2011*. June 24-28, 2011; San Diego California USA
48. ACCORD Study Group, Gerstein HC et al: *Effects of intensive glucose lowering in type 2 diabetes.* N Engl J Med 2008; 358: 2545-2559
49. ADVANCE Collaborative Group, Patel A. et al: *Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes.* N Engl J Med 2008; 358: 2560-2572
50. Duckworth W et al, VADT Investigators: *Glucose control and vascular complications in veterans with type 2 diabetes*. N Engl J Med 2009; 360: 129-139

51. DCCT Trial Research Group: *The Effect of Intensive Treatment of Diabetes on the Development and Progression of Long-Term Complications in Insulin-Dependent Diabetes Mellitus.* N Engl J Med 1993; 329: 977-986
52. Meier M et al. Cardiovascular disease and intensive glucose control in type 2 diabetes mellitus: moving practice toward evidence-based strategies. Vasc Health Risk Manag. 2009;5:859-71.
53. Hobb FD: *Type-2 diabetes mellitus related cardiovascular risk: new options for interventions to reduce risk and treatment goals*. Atherosclerosis 2006; 7: 29–32
54. Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ: *Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients*. Diabetes 2001; 50: 609-13
55. Nauck M, Stockmann F, Ebert R, Creutzfeldt W: *Reduced incretin effect in type 2 (non-insulin-dependent) diabetes.* Diabetologia 1986; 29: 46–52
56. Knudsen LB: *Glucagon like peptide-1: the basis of a new class of treatment for type 2 diabetes*. J Med Chem 2004; 47: 4128–4134
57. Drucker DJ, Nauck MA: *The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes.* Lancet 2006 Nov 11; 368: 1696-705 57.
58. Thorens B, Porret A, Bühler L, Deng SP, Morel P, Widmann C: *Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin-(9–39) an antagonist of the receptor*. Diabetes 1993; 42: 1678–1682
59. Knudsen LB, Kiel D, Teng M, Behrens C, Bhumralkar D, Kodra JT et al: *Small-molecule agonists for the glucagon-like peptide 1 receptor*. Proc Natl Acad Sci USA 2007; 104: 937–942
60. Buse BJ, Henry RR, Han J, Kim DD, Fineman MS, Baron AD: *Effects of exenatide (exendin-4) on glycemic control over 30 weeks in sulfonylurea treated patients with type 2 diabetes*. Diabetes Care 2004; 27: 2628–2635
61. Defronzo RA, Ratner RE, Han J, Kim DD, Fineman MS, Baron AD: *Effects of exenatide (exendin-4) on glycemic control and weight over 30 weeks in metformin-treated patients with type 2 diabetes*. Diabetes Care 2005; 28: 1092–1100
62. Kendall DM, Riddle MC, Rosenstock J, Zhuang D, Kim DD, Fineman MS, Baron AD: *Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients*

*with type 2 diabetes treated with metformin and a sulfonylurea.* Diabetes Care 2005; 28: 1083–1091

63. Drucker DJ, et al. DURATION-1 Study Group : *Exenatide once weekly versus twice daily for the treatment of type 2 diabetes: a randomised, open-label, non-inferiority study*. Lancet 2008; 372: 1240 – 1250
64. Chen D, Liao J, Li N, Zhou C, Liu Q, Wang G et al. (2007). *A nonpeptidic agonist of glucagon-like peptide 1 receptors with efficacy in diabetic db/db mice.* Proc Natl Acad Sci USA 104: 943–948.
65. Elbrønd B, Jakobsen G, Larsen S, Agersø H, Jensen LB, Rolan P, Sturis J, Hatorp V, Zdravkovic M. *Pharmacokinetics, pharmacodynamics, safety, and tolerability of a single-dose of NN2211, a long-acting glucagon like peptide 1 derivative, in healthy male subjects*. Diabetes Care 2002; 25: 1398–1404.
66. Flatt PR, Bailey CJ, Green BD: *Recent advances in antidiabetic drug therapies targeting the enteroinsular axis*. Curr Drug Metab 2009; 10:125–137
67. Courrèges JP, Vilsbøll T, Zdravkovic M, et al: *Beneficial effects of once daily liraglutide, a human glucagon-like peptide-1 analogue, on cardiovascular risk biomarkers in patients with Type 2 diabetes*. Diabet Med 2008; 25: 1129–1131
68. Vilsböll T, Zdravkovic M, Le-Thi T, et al: *Liraglutide, a long-acting human GLP-1 analog, given as monotherapy significantly improves glycemic control and lowers body weight without risk of hypoglycemia in patients with type 2 diabetes mellitus*. Diabetes Care 2007; 30: 1608–1610
69. Hirata K, Kume S, Araki S, Sakaguchi M, Chin-Kanasaki M, Isshiki K, Sugimoto T, Nishiyama A, Koya D, Haneda M, Kashiwagi A, Uzu T *Exendin-4 has an anti-hypertensive effect in salt-sensitive mice model.* Biochem Biophys Res Commun. 2009; 380 (1): 44-9
70. Davidson MH. *Cardiovascular effects of glucagonlike peptide-1 agonists.* Am J Cardiol. 2011 Aug 2;108 (3 Suppl):33B-41B.
71. Hattori Y, Jojima T, Tomizawa A, Satoh H, Hattori S, Kasai K, Hayashi T. *A glucagon-like peptide-1 (GLP-1) analogue, liraglutide, upregulates nitric oxide production and exerts anti-inflammatory action in endothelial cells.* Diabetologia. 2010; 53(10): 2256-63
72. Arakawa M, Mita T, Azuma K, Ebato C, Goto H, Nomiyama T, Fujitani Y, Hirose T, Kawamori R, Watada H. *Inhibition of monocyte adhesion to endothelial cells and*

*attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4*. Diabetes. 2010; 59(4): 1030-1037

73. Bayliss WM, Starling EH: *The mechanism of pancreatic secretion.* JPhysiol 1902; 28: 325-353
74. La Barre J, Still EU: *Studies on the physiology of secretin*. Am J Physiol 1930; 91: 649-653
75. La Barre J: *Sur le possibilités d'un traitment du diabète par l'incrétin*. Bull Acad R Med Belg 1932; 12: 620-634
76. Creutzfeldt W: *The pre-history of the incretin concept*. Regul Pept 2005;128: 87-91
77. McIntyre N, Holsworth DC, Turner DS: *New interpretation of oral glucose tolerance*. Lancet 1964; 2: 20-21
78. Creutzfeldt W. The incretin concept today. Diabetologia 1979. 16: 75–85
79. Mojsov S, Weir GC, Habener JF: *Insulinotropin: glucagon-like peptide-1(7-36) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas.* J Clin Invest 1987;79: 616-619
80. Kreymann B, Williams G, Ghatei MA, Bloom SR: *Glucagon-like-peptide-1 7-36: a physiological incretin in man*. Lancet 1987; 2: 1300-1304
81. Lauritsen KB, Moody AJ, Christensen KC, Lindkaer Jensen S: *Gastric inhibitory polypeptide (GIP) and insulin release after small-bowel resection in man.* Scand J Gastroenterol 1980; 15: 833–840
82. Mortensen K, Christensen LL, Holst JJ, Orskov C: *GLP-1 and GIP are colocalized in a subset of endocrine cells in the small intestine*. Regul Pept 2003; 114: 189–196
83. Theodorakis MJ, Carlson O, Michopoulos S, Doyle ME, Juhaszova M, Petraki K, Egan JM: *Human duodenal enteroendocrine cells: source of both incretin peptides, GLP-1 and GIP*. Am J Physiol 2006; 290: E550–E559
84. Drucker DJ, Baggio LL: *Biology of incretins: GLP-1 and GIP.* Gastroenterology 2007; 132: 2131-2157
85. Hermann C, Goke R, Richter G, Fehmann HC, Arnold R, Goke B: *Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients*. Digestion 1995; 56: 117–12
86. White JW, Saunders GF: *Structure of the human glucagon gene*. Nucl Acids Res 1986; 14: 4719–4730.

87. Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF: *Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing*. J Biol Chem 1986; 261: 11880–11889
88. Drucker DJ, Asa S*: Glucagon gene expression in vertebrate brain.* J Biol Chem 1988; 263: 13475–13478
89. Rouille Y, Martin S, Steiner DF: *Differential processing of proglucagon by the subtilisin-like prohormone convertases PC2 and PC3 to generate either glucagon or glucagon-like peptide*. J Biol Chem 1995; 270: 26488–26496
90. Dhanvantari S, Seidah NG, Brubaker PL: *Role of prohormone convertases in the tissue-specific processing of proglucagon*. Mol Endocrinol 1996; 10: 342–355
91. Myojo S, Tsujikawa T, Sasaki M, Fujiyama Y, Bamba T: *Trophic effects of glicentin on rat small-intestinal mucosa in vivo and in vitro*. J Gastroenterol 1997; 32: 300–305
92. Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ: *Oxyntomodulin from distal gut. Role in regulation of gastric and pancreatic functions*. Dig Dis Sci 1989; 34: 1411–1419
93. Dakin CL, Gunn I, Small CJ, Edwards CM, Hay DL, Smith DM, Ghatei MA, Bloom SR: *Oxyntomodulin inhibits food intake in the rat*. Endocrinology 2001; 142: 4244–4250
94. Baggio LL, Huang Q, Brown TJ, Drucker DJ: *Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure*. Gastroenterology 2004; 127: 546–558
95. Sowden GL, Drucker DJ, Weinshenker D, Swoap SJ: *Oxyntomodulin increases intrinsic heart rate in mice independent of the glucagon-like peptide-1 receptor*. Am J Physiol 2006; 292: R962–970
96. Drucker DJ, Erlich P, Asa SL, Brubaker PL: *Induction of intestinal epithelial proliferation by glucagon-like peptide 2*. Proc Natl Acad Sci USA 1996; 93: 7911–7916
97. Tang-Christensen M, Larsen PJ, Thulesen J, Romer J, Vrang N: *The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake*. Nat Med 2000; 6: 802–807
98. Lovshin J, Estall J, Yusta B, Brown TJ, Drucker DJ: *Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling*. J Biol Chem 2001; 276: 21489–21499

99. Lovshin JA, Huang Q, Seaberg R, Brubaker PL, Drucker DJ: *Extrahypothalamic expression of the glucagon-like peptide-2 receptor is coupled to reduction of glutamate-induced cell death in cultured hippocampal cells*. Endocrinology 2004; 145: 3495–3506

100. Irwin DM: *Molecular evolution of proglucagon*. Regul Pept 2001; 98: 1- 12

101. Estall JL, Drucker DJ: *Glucagon and glucagon-like peptide receptors as drug targets*. Curr Pharm Des 2006; 12: 1731–1750

102. Bell GI, Sanchez-Pescador R, Laybourn PJ, Najarian RC: *Exon duplication and divergence in the human preproglucagon gene*. Nature 1983; 304: 368–37197.

103. Wettergren A, Pridal L, Wojdemann M, Holst JJ: *Amidated and nonamidated glucagon-like peptide-1 (GLP-1): non-pancreatic effects (cephalic phase acid secretion) and stability in plasma in humans.* Regul Pept 1998; 77: 83–87

104. Orskov C, Rabenhoj L, Wettergren A, Kofod H, Holst, JJ: *Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans*. Diabetes 1994; 43: 535–539

105. Orskov C, Wettergren A, Holst JJ. *Biological effects and metabolic rates of glucagonlike peptide-1 7-36 amide and glucagonlike peptide-1 7-37 in healthy subjects are indistinguishable*. Diabetes 1993; 42: 658–661

106. Brubaker PL: *The glucagon-like peptides: pleiotropic regulators of nutrient homeostasis*. Ann N Y Acad Sci 2006; 1070: 10–26

107. Unger RH, Ohneda A, Valverde I, Eisentraut AM, Exton J: *Characterization of the responses of circulating glucagon-like immunoreactivity to intraduodenal and intravenous administration of glucose*. J Clin Invest 1968; 47: 48–65

108. Hermann C, Goke R, Richter G, Fehmann HC, Arnold R, Goke B: *Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients*. Digestion 1995; 56: 117–126

109. Schirra J, Katschinski M, Weidmann C, Schäfer T, Wank U, Arnold R et al: *Gastric emptying and release of incretin hormones after glucose ingestion in humans*. J Clin Invest 1996; 97: 92–103

110. Rocca AS, Brubaker PL: *Role of the vagus nerve in mediating proximal nutrient-induced glucagon- like peptide-1 secretion.* Endocrinology 1999; 140: 1687–1694

111. Balks HJ, Holst JJ, von zur Muhlen A, Brabant G: *Rapid oscillations in plasma glucagon-like peptide-1 (GLP-1) in humans: cholinergic control of GLP-1 secretion via muscarinic receptors.* J Clin Endocrinol Metab 1997; 82: 786–790

112. Anini Y, Hansotia T, Brubaker PL: *Muscarinic receptors control postprandial release of glucagon-like peptide-1: in vivo and in vitro studies in rats*. Endocrinology 2002; 143: 2420–2426
113. Anini Y, Brubaker PL: *Muscarinic receptors control glucagon-like peptide 1 secretion by human endocrine L cells*. Endocrinology 2003; 144: 3244–3250
114. Dubé PE, Brubaker PL. *Nutrient, neural and endocrine control of glucagon-like peptide secretion*. Horm Metab Res. 2004; 36 (11-12): 755-760
115. Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W: *Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus.* J Clin Invest 1993; 91: 301–307
116. Anini Y, Brubaker PL: *Role of leptin in the regulation of glucagonlike peptide-1 secretion*. Diabetes 2003; 52: 252–259
117. Brubaker PL: Minireview: Update on Incretin Biology: *Focus on Glucagon-Like Peptide-1.* Endocrinology 2010; 151: 1984–1989
118. Deacon CF, Nauck MA, Toft-Nielsen M, Pridal L, Willms B, Holst JJ: *Both subcutaneously and intravenously administered glucagon-like peptide 1 are rapidly degraded from the NH2-terminus in type II diabetic patients and in healthy subjects*. Diabetes 1995; 44: 1126–1131
119. Hansen L, Deacon CF, Orskov C, Holst JJ: *Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine*. Endocrinology 1999; 140: 5356–5363
120. Mentlein R: *Dipeptidyl-peptidase IV (CD26)–role in the inactivation of regulatory peptides*. Regul Pept 1999; 85: 9–24
121. Kieffer TJ, McIntosh CH, Pederson RA: *Degradation of glucosedependent insulinotropic polypeptide and truncated glucagonlike peptide 1 in vitro and in vivo by dipeptidyl peptidase IV*. Endocrinology 1995; 136: 3585–3596
122. Marguet D, Baggio L, Kobayashi T, Bernard AM, Pierres M, Nielsen PF, Ribel U, Watanabe T, Drucker DJ, Wagtmann N: *Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26.* Proc Natl Acad Sci USA 2000; 97: 6874–6879
123. Mentlein R, Gallwitz B, Schmidt WE: *Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine*

*methionine and is responsible for their degradation in human serum*. Eur J Biochem 1993; 214: 829–835

124. Deacon CF, Johnsen AH, Holst JJ: *Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo*. J Clin Endocrinol Metab 1995; 80: 952–957

125. Deacon CF, Plamboeck A, Moller S, Holst JJ: *GLP-1-(9-36) amide reduces blood glucose in anesthetized pigs by a mechanism that does not involve insulin secretion*. Am J Physiol Endocrinol Metab 2002; 282: E873-9

126. Green BD, Gault VA, Flatt PR, Harriott P, Greer B, O'Harte FPM: *Comparative effects of GLP-1 and GIP on cAMP production, insulin secretion, and in vivo antidiabetic actions following substitution of Ala8/Al2 with 2-aminobutyric acid.* Arc Biochem Biophys 2004; 428: 136–143

127. Ruiz-Grande C, Alarcon C, Alcantara A, Castilla C, Lopez Novoa JM, Villanueva-Penacarrillo ML, Valverde I: *Renal catabolism of truncated glucagon-like peptide 1*. Horm Metab Res 1993; 25: 612–616

128. Orskov C, Andreasen J, Holst JJ: *All products of proglucagon are elevated in plasma from uremic patients*. J Clin Endocrinol Metab 1992; 74: 379–384

129. Meier JJ, Nauck MA, Kranz D, Holst JJ, Deacon CF, Gaeckler D, Schmidt WE, Gallwitz B: *Secretion, degradation, and elimination of glucagon-like peptide 1 and gastric inhibitory polypeptide in patients with chronic renal insufficiency and healthy control subjects.* Diabetes 2004; 53: 654–662

130. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V: *Glucagon-like peptide-1(7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: Acute post-prandial and 24-h secretion patterns*. J Endocrinol 1993; 138: 159–166

131. Xiao Q, Boushey RP, Drucker DJ, Brubaker PL: *Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans*. Gastroenterology 1999; 117: 99–105

132. Ranganath LR, Beety JM, Morgan LM, Wright JW, Howland R, Marks V: *Attenuated GLP-1 secretion in obesity: cause or consequence?* Gut 1996; 38: 916–919

133. Ranganath L, Norris F, Morgan L, Wright J, Marks V: *Inhibition of carbohydrate-mediated glucagon-like peptide-1 (7-36)amide secretion by circulating non-esterified fatty acids*. Clin Sci (Lond) 1999; 96: 335–342

134. Vaag AA, Holst JJ, Volund A, Beck-Nielsen HB: *Gut incretin hormones in identical twins discordant for non-insulin-dependent diabetes mellitus (NIDDM) evidence for decreased glucagon-like peptide 1 secretion during oral glucose ingestion in NIDDM twins*. Eur J Endocrinol 1996; 135: 425–432
135. Vilsboll T, Agerso H, Krarup T, Holst JJ: *Similar elimination rates of glucagon-like peptide-1 in obese type 2 diabetic patients and healthy subjects*. J Clin Endocrinol Metab 2003; 88: 220–224
136. Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B, Drucker DJ, *International Union of Pharmacology XXXV: The glucagon receptor family*. Pharmacol Rev 2003; 55: 167–194
137. Montrose-Rafizadeh C, Avdonin P, Garant MJ, Rodgers BD, Kole S, Yang H, Levine MA, Schwindinger W, Bernier M: P*ancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells*. Endocrinology 1999; 140: 1132–1140
138. Hallbrink M, Holmqvist T, Olsson M, Ostenson CG, Efendic S, Langel U: *Different domains in the third intracellular loop of the GLP-1 receptor are responsible for Galpha(s) and Galpha(i)/Galpha(o) activation.* Biochim Biophys Acta 2001; 1546: 79–86
139. Stoffel M, Espinosa R III, Le Beau MM, Bell GI: *Human glucagon-like peptide-1 receptor gene. Localization to chromosome band 6p21 by fluorescence in situ hybridization and linkage of a highly polymorphic simple tandem repeat DNA polymorphism to other markers on chromosome 6*. Diabetes 1993; 42: 1215–1218
140. Kieffer TJ, Habener JF: *The glucagon-like peptides*. Endocr Rev. 1999; 20: 876–913
141. Ban K, Noyan-Ashraf MH, Hoefer J, Bolz SS, Drucker DJ, Husain M: *Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor dependent and -independent pathways*. Circulation 2008; 117: 2340–2350
142. Lin F, Wang R: *Molecular modeling of the three-dimensional structure of GLP-1R and its interactions with several agonists*. J Mol Model 2009; 15: 53–65
143. Holz GG: *Epac a new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic b-cell*. Diabetes 2004; 53: 5–13

144. Thorens B: *Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1*. Proc Natl Acad Sci USA 1992; 89: 8641–8645

145. Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF: *Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line.* Proc Natl Acad Sci USA 1987; 84: 3434–3438

146. Arnette D, Gibson TB, Lawrence MC, January B, Khoo S, McGlynn K. Vanderbilt CA, Cobb MH. Regulation of ERK1 and ERK2 by glucose and peptide hormones in pancreatic b cells. J Biol Chem 2003; 278: 32517–32525

147. Wheeler MB, Lu M, Dillon JS, Leng X-H, Chen C, Boyd AE III: *Functional expression of the rat glucagon-like peptide-I receptor, evidence for coupling to both adenylyl cyclase and phospholipase-C*. Endocrinology 1993; 133: 57–62

148. Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ, Prentki M. *Protein kinase Cz activation mediates glucagon-like peptide-1-induced pancreatic b-cell proliferation*. Diabetes 2001; 50: 2237–2243

149. Holst JJ, Orskov C, Nielsen OV, Schwartz TW: *Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut*. FEBS Lett 1987; 211: 169–174

150. Wang Y, Perfetti R, Greig NH, Holloway HW, DeOre KA, Montrose-Rafizadeh C, Elahi D, Egan JM: *Glucagon-like peptide-1 can reverse the age-related decline in glucose tolerance in rats*. J Clin Invest 1997; 99: 2883–2889

151. Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ: *Beta-cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1*. Diabetes 2005; 54: 482–491

152. Fehmann H-C, Habener JF: *Insulinotropic hormone glucagon-like peptide-I(7-37) stimulation of proinsulin gene expression and proinsulin biosynthesis in insulinoma FORMTEXT TC-1 cells*. Endocrinology 1992; 130: 159–166

153. Park S, Dong X, Fisher TL, Dunn S, Omer AK, Weir G, White MF: *Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function*. J Biol Chem 2006; 281: 1159–1168

154. Kodama S, Toyonaga T, Kondo T, Matsumoto K, Tsuruzoe K, Kawashima J, Goto H, Kume K, Kume S, Sakakida M, Araki E: *Enhanced expression of PDX-1 and Ngn3 by exendin-4 during beta cell regeneration in STZ-treated mice*. Biochem Biophys Res Commun 2005; 327: 1170–1178

155. Buteau J, Spatz ML, Accili D: *Transcription factor FoxO1mediates glucagon-like peptide-1 effects on pancreatic beta-cell mass*. Diabetes 2006; 55: 1190–1196
156. Heller RS, Kieffer TJ, Habener JF: *Insulinotropic glucagon-like peptide I receptor expression in glucagon-producing alpha-cells of the rat endocrine pancreas*. Diabetes 1997; 46: 785–791
157. Fehmann HC, Habener JF: *Functional receptors for the insulinotropic hormone glucagon-like peptide-I(7-37) on a somatostatin secreting cell line*. FEBS Lett 1991; 279: 335–340
158. Baggio LL, Huang Q, Brown TJ, Drucker DJ: *A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis*. Diabetes 2004; 53: 2492–2500
159. Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA: *Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients*. J Clin Endocrinol Metab 1996; 81: 327–332
160. Meier JJ, Gallwitz B, Salmen S, Goetze O, Holst JJ, Schmidt WE, Nauck MA: *Normalization of glucose concentrations and deceleration of gastric emptying after solid meals during intravenous glucagon-like peptide 1 in patients with type 2 diabetes*. J Clin Endocrinol Metab 2003; 88: 2719–2725
161. Wishart JM, Horowitz M, Morris HA, Jones KL, Nauck MA: *Relation between gastric emptying of glucose and plasma concentrationsof glucagon-like peptide-1*. Regul Pept 1998; 19: 1049–1053
162. Nauck MA, Niedereichholz U, Ettler R, Holst JJ, Orskov C, Ritzel R, Schmiegel WH: *Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans*. Am J Physiol 1997; 273: E981–E988
163. Bullock BP, Heller RS, Habener JF: *Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1receptor*. Endocrinology 1996 ; 137: 2968–78
164. Wei Y, Mojsov S. *Tissue-specific expression of the human receptor for glucagon-like peptide, I: brain, heart and pancreatic forms have the same deduced amino acid sequences.* FEBS Lett. 1995;358:219 –224.

165. Barragan JM, Rodriguez RE, Blazquez E: *Changes in arterial blood pressure and heart rate induced by glucagon-like peptide-1-(7-36)amide in rats.* Am J Physiol. 1994; 266: E459 –E466
166. Yamamoto H, Lee CE, Marcus JN, Williams TD, Overton JM, Lopez ME, Hollenberg AN, Baggio L, Saper CB, Drucker DJ, Elmquist JK: *Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons*. J Clin Invest. 2002; 110: 43–52
167. Yu M, Moreno C, Hoagland KM, Dahly A, Ditter K, Mistry M, Roman RJ. *Antihypertensive effect of glucagon-like peptide 1 in Dahl salts ensitive rats.* J Hypertens 2003; 21: 1125–1135.
168. Nikolaidis LA, Elahi D, Shen YT, Shannon RP. *Active metabolite of GLP-1 mediates myocardial glucose uptake and improves left ventricular performance in conscious dogs with dilated cardiomyopathy.* Am J Physiol Heart Circ Physiol. 2005; 289(6): H2401-8.
169. Sokos GG, Nikolaidis LA, Mankad S, Elahi D, Shannon RP: *Glucagon-like peptide-1 infusion improves left ventricular ejection fraction and functional status in patients with chronic heart failure*. J Card Fail. 2006; 12: 694–699
170. Nikolaidis LA, Mankad S, Sokos GG, Miske G, Shah A, Elahi D,Shannon RP: *Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion*. Circulation 2004; 109: 962–965
171. Nikolaidis LA, Doverspike A, Hentosz T, Zourelias L, Shen YT, Elahi D, Shannon RP: *Glucagon-like peptide-1 limits myocardial stunning following brief coronary occlusion and reperfusion in conscious canines*.J Pharmacol Exp Ther. 2005; 312: 303–308
172. Sokos GG, Bolukoglu H, German J, Hentosz T, Magovern GJ Jr, Maher TD, Dean DA, Bailey SH, Marrone G, Benckart DH, Elahi D, Shannon RP. *Effect of glucagon-like peptide-1 (GLP-1) on glycemic control and left ventricular function in patients undergoing coronary artery bypass grafting*. Am J Cardiol. 2007; 100(5): 824-9.
173. Bose AK, Mocanu MM, Carr RD, Brand CL, Yellon DM: *Glucagon-like peptide 1 can directly protect the heart against ischemia/reperfusion injury*. Diabetes. 2005;54:146 –151
174. Noyan-Ashraf MH, Momen MA, Ban K, Sadi AM, Zhou YQ, Riazi AM, Baggio LL, Henkelman RM, Husain M, Drucker DJ. *GLP-1R agonist liraglutide activates*

*cytoprotective pathways and improves outcomes after experimental myocardial infarction in mice*. Diabetes. 2009; 58(4): 975-83.

175. Golpon HA, Puechner A, Welte T, Wichert PV, Feddersen CO. *Vasorelaxant effect of glucagon-like peptide-(7-36) amide and amylin on the pulmonary circulation of the rat.* Regul Pept. 2001; 102: 81– 86.

176. Nystrom T, Gutniak MK, Zhang Q, Zhang F, Holst JJ, Ahrén B, Sjöholm A. *Effects of glucagon-like peptide-1 on endothelial function in type 2 diabetes patients with stable coronary artery disease.* Am J Physiol Endocrinol Metab 2004; 287: E1209–15.

177. Nystrom T, Gonon AT, Sjöholm A, Pernow J. *Glucagon-like peptide-1 relaxes rat conduit arteries via an endotheliumin-independent mechanism*. Regul Pept 2005; 125:173–177

178. Nystrom T. *The potential beneficial role of glucagon-like peptide-1 in endothelial dysfunction and heart failure associated with insulin resistance.* Horm Metab Res 2008; 40: 593–606.

179. Morales M, López-Delgado MI, Alcántara A, Luque MA, Clemente F, Márquez L, Puente J, Viñambres C, Malaisse WJ, Villanueva-Peñacarrillo ML, Valverde I. *Preserved GLP-I effects on glycogen synthase a activity and glucose metabolism in isolated hepatocytes and skeletal muscle from diabetic rats.* Diabetes. 1997; 46(8): 1264-9.

180. Perea A, Viñambres C, Clemente F, Villanueva-Peñacarrillo ML, Valverde I. *GLP-1 (7-36) amide: effects on glucose transport and metabolism in rat adipose tissue*. Horm Metab Res. 1997; 29(9): 417-421.

181. Ruiz-Grande C, Alarcn C, Mzrida E, Valverde I: *Lipolytic action of glucagon-like peptides in isolated rat adipocytes*. Regul Pept 1992; 13: 13–16

182. Villanueva-Penacarrillo ML, Marquez L, Gonzalez N, Diaz-Miguel M, Valverde I: *Effect of GLP-1 on lipid metabolism in human adipocytes*. Horm Metab Res 2001; 33: 73–77

183. Furnsinn C, Ebner K, Waldhausl W: *Failure of GLP-1 (7-36) amide to affect glycogenesis in rat skeletal muscle*. Diabetologia 1995; 38: 864–867

184. Nakagawa Y, Kawai K, Suzuki H, Ohashi S, Yamashita K:*Glucagon-like peptide-1(7-36)amide and glycogen synthesis in the liver*. Diabetologia 1996; 39: 1241–1242

185. Prigeon RL, Quddusi S, Paty B, D'Alessio DA: *Suppression of endogenous glucose production by glucagon-like peptide 1 independent of islet hormones: an extrapancreatic effect of an incretin hormone*. Am J Physiol 2003; 285: E701–E707
186. Green BD, Hand KV, Dougan JE, McDonnell BM, Cassidy RS, Grieve DJ. *GLP-1 and related peptides cause concentration-dependent relaxation of rat aorta through a pathway involving KATP and cAMP*. Arch Biochem Biophys 2008; 478: 136–42
187. Sonne DP, Engstrøm T, Treiman M. *Protective effects of GLP-1 analogues exendin-4 and GLP-1(9-36) amide against ischemia-reperfusion injury in rat heart.* Regul Pept. 2008; 146(1-3): 243-9.
188. Ban K, Kim KH, Cho CK, Sauvé M, Diamandis EP, Backx PH, Drucker DJ, Husain M. *Glucagon-like peptide (GLP)-1(9-36)amide-mediated cytoprotection is blocked by exendin(9-39) yet does not require the known GLP-1 receptor.* Endocrinology. 2010; 151(4): 1520-31.
189. Basu A, Charkoudian N, Schrage W, Rizza RA, Basu R, Joyner MJ. *Beneficial effects of GLP-1 on endothelial function in humans: dampening by glyburide but not by glimepiride*. Am J Physiol Endocrinol Metab. 2007; 293(5): E1289-95.
190. Richter G, Feddersen O, Wagner U, Barth P, Göke R, Göke B. *GLP-1 stimulates secretion of macromolecules from airways and relaxes pulmonary artery*. Am J Physiol. 1993; 265: L374-81.
191. Liu HB, Hu YS, Simpson RW and Dear AE. *Glucagon-like peptide-1 attenuates tumour necrosis factor-alpha-mediated induction of plasminogen activator inhibitor-1 expression.* J Endocrinol 2008; 196: 57–65.
192. Liu HB, Dear AE, Knudsen LB and Simpson RW. *A long-acting glucagon-like peptide-1 analogue attenuates induction of plasminogen activator inhibitor type-1 and vascular adhesion molecules*. J Endocrinol 2009; 201: 59–66.
193. Ishibashi Y, Matsui T, Takeuchi M, Yamagishi S. *Glucagon-like peptide-1 (GLP-1) inhibits advanced glycation end product (AGE)-induced up-regulation of VCAM-1 mRNA levels in endothelial cells by suppressing AGE receptor (RAGE) expression.* Biochem Biophys Res Commun. 2010; 391(3): 1405-8.
194. Oeseburg H, de Boer RA, Buikema H, van der Harst P, van Gilst WH, Silljé HH. *Glucagon-like peptide 1 prevents reactive oxygen species-induced endothelial cell senescence through the activation of protein kinase A*. Arterioscler Thromb Vasc Biol. 2010; 30(7): 1407-14.

195. Erdogdu O, Nathanson D, Sjöholm A, Nyström T, Zhang Q. *Exendin-4 stimulates proliferation of human coronary artery endothelial cells through eNOS-, PKA- and PI3K/Akt-dependent pathways and requires GLP-1 receptor*. Mol Cell Endocrinol. 2010; 325(1-2): 26-35.
196. Potente M, Ghaeni L, Baldasseri D, Mostoslavsky R, Rossig L, Dequiedt F, Haendeler J, Mione M, Dejana E, Alt FW, Zeiher AM, Dimmeler S: *SIRT1 control endothelial angiogenic function during vascular growth*. Genes Dev. 2007; 21: 2644-2658
197. Ota H, Akishita M, Eto M, Iijima K, Kaneki M, Ouchi Y: *Sirt1 modulates premature senescence-like phenotype in human endothelial cells*. J Mol Cell Cardiol 2007; 43: 571-579
198. Menghini R, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, Vasa-Nicotera M, Ippoliti A, Novelli G, Melino G, Lauro R, Federici M: *MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1*. Circulation 2009; 120: 1524-153215.
199. Mattagajasingh I, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, DeRicco J, Kasuno K, Irani K. *SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase*. Proc Natl Acad Sci U S A. 2007; 104(37): 14855-60.
200. Salminen A, Kauppinen A, Suuronen T, Kaarniranta K. *SIRT1 longevity factor suppresses NF-kappaB -driven immune responses: regulation of aging via NF-kappaB acetylation*. Bioessays. 2008; 30: 939-42
201. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. *Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase*. EMBO J. 2004; 12: 2369-80
202. Baker RG, Hayden MS, Ghosh S. *NF-κB, inflammation, and metabolic disease.* Cell Metab. 2011; 13: 11-22
203. Manna SK, Mukhopadhyay A, Aggarwal BB. *Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation.* J Immunol 2000; 164: 6509-19.
204. Stein S, Matter CM. *Protective roles of SIRT1 in atherosclerosis*. Cell Cycle. 2011;10(4): 640-7.

205. Balestrieri ML, Rienzo M, Felice F, et al. *High glucose downregulates endothelial progenitor cell number via SIRT1.* Biochim Biophys Acta 2008; 1784: 936-945

206. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE, Xie R, Disch JS, Ng PY, Nunes JJ, Lynch AV, Yang H, Galonek H, Israelian K, Choy W, Iffland A, Lavu S, Medvedik O, Sinclair DA, Olefsky JM, Jirousek MR, Elliott PJ, Westphal CH. *Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes.* Nature 2007; 450:712–716

207. Gracia-Sancho J, Villarreal G Jr, Zhang Y,García-Cardeña G. *Activation of SIRT1 by resveratrol induces KLF2 expression conferring an endothelial vasoprotective phenotype*. Cardiovasc Res 2010; 85: 514-519

208. Yang J, Wang N, Feng P: *Roles of SIRT1 in High Glucose induced Endothelial Impairment: Association with Diabetic Atherosclerosis*. ArchMed Res. 2011; 42: 354-60.

209. Orimo M, Minamino T, Miyauchi H, Tateno K, Okada S, Moriya J, Komuro I: *Protective role of SIRT1 in diabetic vascular dysfunction*. Arterioscler Thromb Vasc Biol 2009; 29: 889-94

210. de Kreutzenberg SV, Ceolotto G, Papparella I, Bortoluzzi A, Semplicini A, Dalla Man C, Cobelli C, Fadini GP, Avogaro A. *Downregulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome:potential biochemical mechanisms*. Diabetes. 2010; 59: 1006-

211. Zhou S, Chen HZ, Wan YZ, Zhang QJ, Wei YS, Huang S, Liu JJ, Lu YB, Zhang ZQ, Yang RF, Zhang R, Cai H, Liu DP, Liang CC. *Repression of P66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction.* Circ Res. 2011; 109(6): 639-648

212. Bordone L, Cohen D, Robinson A, Motta MC, van Veen E, Czopik A, Steele AD, Crowe H, Marmor S, Luo J, Gu W, Guarente L. *SIRT1 transgenic mice show phenotypes resembling calorie restriction.* Aging Cell. 2007;6(6): 759-767

213. Taylor D. M., Maxwell M. M., Luthi-Carter R., Kazantsev A. G. B*iological and Potential Therapeutic Roles of Sirtuin Deacetylases*. Cell. Mol. Life Sci. 2008; 65: 4000 – 4018

214. Michan S, Sinclair D. *Sirtuins in mammals: insights into their biological function.* Biochem. J. 2007; 404: 1–13

215. Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, Valenzuela DM, Yancopoulos GD, Karow M, Blander G, et al: *SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic β cells*. Cell 2006; 126: 941–954
216. Tanno M, Sakamoto J, Miura T, Shimamoto K, Horio Y. *Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1*. J. Biol.Chem. 2007; 282: 6823–32
217. Valter D. Longo, Kennedy B.K. *Sirtuins in aging and age-related disease*. Cell 2006;126: 257-268
218. Haigis MC, Guarante L: *Mammalian Sirtuins-emerging roles in physiology, aging and calorie restriction*. Genes Dev 2006; 20: 2913-2921
219. Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W: *Negative control of p53 by Sir2α promotes cell survival under stress*. Cell 2001; 107: 137–148
220. Deng CX: *SIRT1, is it a tumor promoter or tumor suppressor?*. Int. J. Biol. Sci. 2009; 5: 147–52
221. Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, Bultsma Y, McBurney M, Guarente L: *Mammalian SIRT1 represses forkhead transcription factors*. Cell 2004; 116: 551–563
222. Tadahiro Kitamura, Jun Nakae, Yukari Kitamura, Yoshiaki Kido, William H. Biggs, Christopher V.E. Wright, Morris F. White, Karen C. Arden, Accili D: *The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic β cell growth.* J Clin Invest 2002; 110:1839–1847
223. Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, et al: *Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ*. Nature 2004: 429: 771–76
224. Jeong J, Juhn K, Lee H, Kim SH, Min BH, Lee KM, Cho MH, Park GH, Lee KH . *SIRT1 promotes DNA repair activity and deacetylation of Ku70*. Exp Mol Med. 2007; 39(2): 8-13
225. Westerheide SD, Anckar J, Stevens SM Jr, Sistonen L, Morimoto RI. *Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1*. Science 2009; 323(5917): 1063-6.
226. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, et al: *Resveratrol improves mitochondrial*

*function and protects against metabolic disease by activating SIRT1 and PGC-1α.* Cell 2006; 127: 1109–1122

227. Chen J, Zhou Y, Mueller-Steiner S, Chen LF, Kwon H, Yi S, Mucke L, Gan L. *SIRT1 protects against microglia-dependent amyloid-β toxicity through inhibiting NF-kB signaling*. J. Biol. Chem. 2005; 280: 40364–40374
228. Fayard E, Tintignac LA, Baudry A, Hemmings BA: *Protein kinase B/Akt a glance*. J Cel Sci 2005; 118: 5675-5678
229. Sayed D, Abdellatif M: *AKT-ing via microRNA*. Cell Cycle 2010; 9: 3213-3217
230. Ota H, Eto M, Kano MR, Kahyo T, Setou M, Ogawa S, Iijima K, Akishita M, Ouchi Y: *Induction of endothelial nitric oxide synthase, SIRT1, and catalase by statins inhibits endothelial senescence through the Akt pathway*. Arterioscler Thromb Vasc Biol 2010; 30: 2205-2211
231. Canto C, Auwerx J: *AMP-activated protein kinase and its downstream transcriptional pathways*. Cell Mol Life Sci. 2010; 67: 3407–3423
232. Steinberg GR, Kemp BE: *AMPK in health and disease.* Physiol Rev. 2009; 89: 1025–1078
233. Green MF, Anderson KA, Means AR. *Characterization of the CaMKKβ-AMPK signaling complex*. Cell Signal. 2011;23(12):2005-12
234. Salminen A, Juha MT, Hyttinen J, Kaarniranta K: *AMP-activated protein kinase inhibits NF-κB signaling and inflammation: impact on healthspan and lifespan*. J Mol Med 2011; 89: 667–676
235. Lage R, Dieguez C, Vidal-Puig A, Lopez M: *AMPK: a metabolic gauge regulating whole-body energy homeostasis*. Trends Mol Med 2008; 14: 539–549
236. Ruderman NB, Cacicedo JM, Itani S, Yagihashi N, Saha AK, Ye JM, Chen K, Zou M, Carling D, Boden G, Cohen RA, Keany J, Kraegen EW, Ido Y: *Malonyl-CoA and AMPK-activated protein kinase (AMPK): possible links between insulin resistance in muscle and early endothelial cell damage in diabetes*. Biochem Soc Trans. 2003; 31: 202–206
237. Hummasti S, Hotamisligil GS: *Endoplasmic reticulum stress and inflammation in obesity and diabetes*. Crc Res 2010; 107: 579–591
238. Gustafson B. *Adipose tissue, inflammation and atherosclerosis*. J Atheroscler Thromb 2009; 17: 332–341

239. Sturgill TW, Ray LB, Erikson E, Maller JL: *Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II*. Nature. 1988; 334: 715-8
240. Boulton TG, Nye SH, Robbins DJ, et al: *ERKs: a family of protein serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF*. Cell 1991; 65: 663-75
241. Seger R, Krebs EG: *The MAPK signaling cascade*. *FASEB J*. 1995; 9: 726-35
242. Ben-Shlomo S, Zvibel I, Shnell M, Shlomai A, Chepurko E, Halpern Z, Barzilai N, Oren R, Fishman S. *Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase.* J Hepatol. 2011; 54(6): 1214-23..
243. Huisamen B, Genade S, Lochner A. *Signalling pathways activated by glucagon-like peptide-1 (7-36) amide in the rat heart and their role in protection against ischaemia*. Cardiovasc J Afr. 2008; 19(2):77-83

.