DPP-4 inhibition improves function of endothelial progenitor cells from type 2 diabetic patients.

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RIASSUNTO

Presupposti e obiettivi.
Il diabete mellito (DM) è associato con una alterazione delle cellule pro angiogeniche (PACs). Le PACs sono cellule di origine midollare che contribuiscono all’omeostasi endoteliale. Il diabete altera le funzioni delle PACs favorendo quindi la disfunzione endoteliale. Gli inibitori dell’enzima DPP4, tra cui Saxagliptin (SAXA), sono una classe di farmaci usati nella terapia orale antidiabetica del diabete tipo 2. Poiché SDF-1α, chemochina coinvolta nella mobilizzazione delle PACs, è un substrato di DPP4, lo scopo dello studio è stato testare se l’inibizione di DPP4 possa modificare le capacità funzionali delle PACs.

Metodi. Le PACs sono state isolate da sangue periferico di soggetti sani o diabetici e dopo sette giorni di coltura, in terreno addizionato di SAXA e/o SDF-1α, sono stati eseguiti alcuni saggi funzionali: adesione ad un monostrato di HUVECs, migrazione, proliferazione, tubulizzazione e analisi di espressione genica.

Risultati. L’attività solubile di DPP-4 risulta maggiore rispetto all’attività della forma cellulare ed entrambe vengono equamente inibite da Saxagliptin. Al basale le colture di PACs da pazienti diabetici rispetto a quelle da soggetti sani contengono meno acLDL”Lectin” cells e mostrano un’espressione alterata dei geni legati all’adesione e alla regolazione del ciclo cellulare. Questo rispecchia l’alterata risposta delle PACs da diabetici nei saggi di adesione, proliferazione / clono genesi. Saxagliptin + SDF-1α migliora l’adesione e la formazione di tubuli da parte delle PACs da soggetti diabetici ma non in quelle da soggetti sani. Le cellule da pazienti diabetici che assumono Saxagliptin mostrano una maggiore attività angiogenica in vivo indotta da fattori di crescita rispetto a quelle da pazienti che non assumono Saxagliptin.

Conclusioni. Saxagliptin ripristina la funzione delle PACs nel diabete in vitro e migliora l’angiogenesi indotta in vivo. Questi dati suggeriscono che l’inibizione di DPP4 possa avere un effetto positivo sulle PACs di pazienti diabetici, che potrebbero stimolare la rigenerazione endoteliale e ridurre il rischio di malattia cardiovascolare.
ABSTRACT

Background and aims. Type 2 diabetes (T2D) is associated with reduction and dysfunction of circulating pro-angiogenic cells (PACs). PACs are a subset of BM-derived cells that contribute to endothelial homeostasis. Diabetes impairs EPC functions, thus prompting endothelial dysfunction and cardiovascular diseases. DPP-4 inhibitors, such as Saxagliptin, are a relatively new class of antidiabetic agents. Since SDF-1α, a chemokine involved in EPC trafficking, is a substrate of DPP-4, we aimed to test whether DPP-4 inhibition affects basal and SDF-1α-stimulated EPC function.

Methods. PACs were isolated from T2D (n=20) and healthy (n=20) subjects. Gene expression, clonogenesis, proliferation, adhesion, migration and tubulisation were assessed in vitro by incubating PACs with or without Saxagliptin and SDF-1α. Stimulation of angiogenesis by circulating cells from T2D patients treated with Saxagliptin or other non-incretinergic drugs was assessed in vivo using animal models. Soluble DPP-4 activity was predominant over cellular activity and was successfully inhibited by Saxagliptin. At baseline, PACs from T2D patients compared to healthy PACs contained less acLDL-Lectin” cells, and showed altered expression of genes related to adhesion and cell cycle regulation. This was reflected by impaired adhesion and clonogenesis / proliferative response of T2D PACs. Saxagliptin + SDF-1α improved adhesion and tube sustaining capacity of PACs from T2D patients, while it did not affect healthy PACs. While Saxagliptin modestly reduced angiogenesis by mature endothelial cells, circulating PAC-progeny cells from T2D patients on Saxagliptin treatment displayed higher growth factor-inducible in vivo angiogenetic activity, compared to cells from T2D patients on non-incretinergic regimen.

Conclusions. Saxagliptin reverses PAC dysfunction associated with T2D in vitro and improves inducible angiogenesis by circulating cells in vivo. These data add knowledge to the potential pleiotropic cardiovascular effects of DPP-4 inhibition.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CMTMR</td>
<td>5-(and-6)-(((4-chloromethyl)Benzoyl)Amino)tetramethylrhodamine</td>
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<tr>
<td>DIVAA</td>
<td>Directed In Vivo Angiogenesis Assay</td>
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<tr>
<td>DPP</td>
<td>Dipeptidyl peptidase</td>
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<tr>
<td>EBM</td>
<td>Endothelial basal medium</td>
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<tr>
<td>EGM</td>
<td>Endothelial growth medium</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>EPCs</td>
<td>Endothelial progenitor cells</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FSC</td>
<td>Forward scatter</td>
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<tr>
<td>GF</td>
<td>Growth factors</td>
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<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic peptide</td>
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<tr>
<td>GLP</td>
<td>Glucagon-like peptide</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
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<tr>
<td>PACs</td>
<td>Proangiogenic cells</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>SAVOR</td>
<td>Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus</td>
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<tr>
<td>SDF</td>
<td>Stromal cell derived factor</td>
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<td>SSC</td>
<td>Side scatter</td>
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T2D Type 2 diabetes

VEGF Vascular endothelial growth factor
INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders characterized by a chronic hyperglycemic condition resulting from defects in insulin secretion (the body cannot produce enough insulin), insulin action (the body cannot use insulin effectively) or both (1). It has become an ever-increasing problem throughout the world. International Diabetes Federation Diabetes’s most recent estimates indicate that 8.3% of adults – 382 million people – have diabetes, and the number of people with the disease is set to rise beyond 592 million in less than 25 years (2).

Diabetes mellitus (DM) is probably one of the oldest diseases known to man. It was first recognized around 1500 B.C.E. by the ancient Egyptians, who considered it a rare condition in which a person urinated excessively and lost weight. The term diabetes mellitus, reflecting that the urine of those affected had a sweet taste, was first used by the Greek physician Aretaeus, who lived from about 80 to 138 C.E. It was not until 1776, however, that Matthew Dobson actually measured the concentration of glucose in the urine of such patients and found it to be increased. Diabetes was a recognized clinical entity when the New England Journal of Medicine and Surgery was founded in 1812 (3).

In the intervening 200 years, major fundamental advances have been made in our understanding of the underlying causes of diabetes and the approach to its prevention and treatment. Although diabetes is still associated with a reduced life expectancy, the outlook for patients with this disease has improved dramatically, and patients usually lead active and productive lives for many decades after the diagnosis has been made. Many effective therapies are available for treating hyperglycemia and its complications.

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. Diabetes mellitus is subdivided into two groups: type 1 diabetes is immune-mediated and requires daily administration of insulin, whereas type 2 diabetes is the most common form and comprises of 90% of people with diabetes around the world (World Health Organization).
Both type 1 and type 2 cause hyperglycemia, which is the major source of morbidity and mortality in diabetes. Hyperglycemia results from the inability of the body to produce and/or to properly utilize insulin, the major hormone involved in glucose homeostasis. Type 2 diabetes is caused by a combination of insulin defect and insulin resistance, the latter being also responsible for endothelial dysfunction. Type 2 diabetes results from an imbalance between insulin sensitivity and insulin secretion. Under normal physiological conditions, plasma glucose concentrations are maintained within a narrow range, despite wide fluctuations in supply and demand, through a tightly regulated and dynamic interaction between tissue sensitivity to insulin (especially in liver) and insulin/glucagon secretion (4). In type 2 diabetes these mechanisms break down, with the consequence that the two main pathological defects in type 2 diabetes are impaired insulin secretion through a dysfunction of the pancreatic β-cell, and impaired insulin action through insulin resistance (5).

Insulin is released from pancreatic beta cells in response to a rapid increase in blood glucose concentration. In type 2 diabetic patients there is a decreased, and often absent, release of insulin than in healthy subjects. Furthermore, beta cell function deteriorates over the years following the diagnosis of type 2 diabetes (6). There are several potential causes of beta cell dysfunction in type 2 diabetes (7) and in particular one of these is the reduction of beta cell mass due to apoptosis.

Insulin resistance can be defined as the inability of target tissues to respond properly to normal circulating concentrations of insulin (8). The main insulin-sensitive tissues are skeletal muscle (accounting for 60-70% of whole-body glucose uptake), liver (30%) and adipose tissue (10%). To maintain euglycemia, the pancreas compensates by secreting increased amounts of insulin. The body's response to insulin resistance is to enhance the beta cell's secretion of insulin to maintain normal glucose tolerance. As long as the beta cell is able to compensate by increasing insulin production, normal glucose tolerance is maintained. The final sequence of events is a progressive deterioration in beta-cell function with subsequent decline in insulin-secreting ability.

Although both insulin resistance and impaired insulin secretion precede the development of postprandial hyperglycemia and the subsequent type 2 diabetic phenotype, insulin resistance is prominent in the prediabetic state and plays an important role in the pathogenesis of macrovascular disease. Insulin resistance is commonly the earliest manifestation in the development of type 2 diabetes, typically originating 5–10 years before postprandial glucose levels in the diabetic range. As long as the beta cell is able to compensate by increased insulin production, normal glucose tolerance is maintained.
Insulin resistance is characterized by impaired responses to the physiologic effects of this hormone on glucose, lipid, and protein metabolism, and by affecting vascular endothelial function. The endogenous insulin that is secreted is inefficiently capable of suppressing hepatic gluconeogenesis or stimulating glucose uptake and metabolism in the muscle and fat (9). Increases in plasma glucose concentrations by 50–100 mg/dL for as little as 24 hours can cause downregulation of the glucose transport system in the muscle (GLUT4), significantly increasing insulin resistance. Over time, insulin resistance peaks and then plateaus as increases in plasma insulin compensate to maintain the glycemic state.

Hyperglycaemia is a consequence of dysregulated glucose homeostasis. Hyperglycaemia becomes diagnostic of type 2 diabetes when secretion of insulin by pancreatic beta cells can no longer meet the demand of insulin-resistance for insulin, and undergo cell death as a result. In type 2 diabetes, residual insulin secretion may remain, but this is insufficient to overcome the tissue’s resistance to insulin. As a consequence of inappropriate or absent insulin secretion, glucose is no longer appropriately taken up into target and hepatic gluconeogenesis is not inhibited, causing a rise in blood sugar level (10),(11) (Figure 1).

**Figure 1.** The natural history of type 2 diabetes.
The vascular devastation of hyperglycemia can occur by several mechanisms, impacting the vessel wall, monocyte-derived macrophages, vascular smooth muscle cells, as well as many other cell types. For many years, glycemic control has been a determinate of the micro and macrovascular complications from diabetes. The United Kingdom Perspective Diabetes Study in type 2 diabetics and the DCCT trial (Diabetes Control and Complication Trial) in type 1 diabetics have shown reductions in microvascular and macrovascular complications with tighter glycemic control. From these and many other clinical trials the message is clear; effective diabetic management including glycemic control reduces complications (12).

The hallmark of macrovascular disease in the diabetic patient comprises the ugly triad of:

1. Coronary artery disease and its complications of myocardial infarction and congestive heart failure;
2. Cerebral and carotid arteriosclerotic vascular disease and its complications of stroke and cerebral ischemia;
3. Peripheral vascular disease and its complications of claudication, ischemia, and amputation (13).

The microvascular complications of diabetes include: Retinopathy, Nephropathy, Neuropathy. Most people with diabetes develop at least some form of eye disease (retinopathy), which can damage vision or provoke blindness. Persistently high levels of blood glucose, together with high blood pressure and high cholesterol, are the main causes of retinopathy. The network of blood vessels that supply the retina can become blocked and damaged in retinopathy, leading to permanent loss of vision. The most advanced stages of retinopathy can be managed through regular eye checks and by keeping blood glucose levels close to normal.

Kidney disease (nephropathy) is far more common in people with diabetes than in people without diabetes; and diabetes is one of the leading causes of chronic kidney disease. The disease is caused by damage to small blood vessels and epithelial cells, which can cause the kidneys to be less efficient, or to fail altogether. Maintaining near-normal levels of blood glucose and blood pressure can greatly reduce the risk of nephropathy.
When blood glucose and blood pressure are excessively high, diabetes can provoke damage to nerves throughout the body (neuropathy). This damage can lead to problems with digestion and urination, erectile dysfunction and a number of other functions. The most commonly affected areas are the extremities, particularly the feet. Nerve damage in these areas is called peripheral neuropathy, and can lead to pain, tingling, and loss of feeling. Loss of feeling is particularly dangerous because it can allow injuries to go unnoticed, leading to serious infections and ulceration, diabetic foot disease, and major amputations. Neuropathy includes mononeuropathy, diabetic amyotrophy, symmetric distal neuropathy, diabetic gastroparesis, diabetic diarrhea, neurogenic bladder, impaired cardiovascular reflexes, and sexual dysfunction (14). People with diabetes may develop a number of different foot problems as a result of damage to nerves and blood vessels. These problems can easily lead to infection and ulceration, which increase a person’s risk of amputation. People with diabetes face a risk of amputation that may be more than 25 times greater than that in people without diabetes (15). However, with good management, a large proportion of amputations can be prevented.

Hyperglycemia is the major causal factor in the development of endothelial dysfunction in patients with diabetes mellitus. Clinical trials have identified hyperglycemia as the key determinant in the development of chronic diabetic complications (16). Over the past 35 years, four seemingly independent major mechanisms of hyperglycemia-induced damage have been proposed to explain how hyperglycemia directly causes diabetic vascular complications. An increase in intracellular glucose will lead to an increase in the efflux of glucose to sorbitol via the polyol pathway and the activation of PKC C (protein kinase C) via de novo synthesis of DAG (diacylglycerol). In addition, glucose and glucose derived dicarbonyl compounds react non-enzymatically with the basic amino acids lysine and arginine in proteins to form AGEs (advanced glycosylation end-products) both extra- and intra-cellularly. These different pathways are interrelated and potentiate each other. Each of these mechanism has been studied independently of the others, and there has been no apparent common element linking them. Brownlee’s research has established that these biochemical pathways all arise from a single hyperglycemia-induced process: the overproduction of toxic free radicals produced by mitochondria. Brownlee’s unifying hypothesis considers that the oxidative stress launched by mitochondria is a common pathogenesis of chronic diabetic complications (17). He has also demonstrated that normalizing the levels of these excess free radicals inhibits the pathways through which cell damage occurs, and that all of these pathways can be activated, even in the presence of normal glucose levels, simply by using molecular genetic techniques to shut down the key enzyme that high glucose turns off. Figure 2 shows how, intracellularly, these four
biochemical mechanisms may all be the consequence of hyperglycaemia-induced overproduction of ROS in mitochondria (18).

Figure 2. Potential mechanisms by which hyperglycaemia and its immediate biochemical sequelae induce hyperglycaemic damage (source: Diabetes ©2003 American Diabetes Association, Inc.).

Endothelial dysfunction has received increasing attention as a potential contributor to the pathogenesis of vascular disease in diabetes mellitus; in fact it is a common finding in type 2 diabetic patients. Under physiological conditions, there is a balanced release of endothelial-derived relaxing and contracting factors, but this delicate balance is altered in diabetes and atherosclerosis, thereby contributing to further progression of vascular and end-organ damage (19). Over the last two decades it has become evident that the endothelium is not an inert, single-cell lining covering the internal surface of blood vessels, but in fact plays a crucial role in regulating vascular tone and structure. Importantly, a healthy endothelium inhibits platelet and leukocyte adhesion to the vascular surface and maintains a balance of profibrinolytic and prothrombotic activity (20).

Endothelial cell dysfunction is a broad term which refers to a condition in which the endothelium loses its physiological properties. Normally, the endothelium actively regulates vascular tone, limits leucocyte adhesion and thus inflammatory activity in the vessel wall, maintains vascular permeability to nutrients, hormones, other macromolecules and leucocytes, inhibits platelet adhesion and aggregation by producing prostacyclin and NO, limits activation of the coagulation cascade by the thrombomodulin/protein C, heparan sulphate/antithrombin and tissue factor/tissue factor pathway inhibitor interactions, and regulates fibrinolysis by producing t-PA and its inhibitor PAI-1 (18).
Several factors contribute to the dysregulation of endothelial cell functions, including smoking, high blood pressure, high cholesterol, obesity, hyperglycemia, advanced glycation end products (AGEs), and genetic factors (21). As such, abnormalities in endothelial function are detected early in the development of CVD, often before symptoms are clinically evident (22). Dysfunction of the vascular endothelium is regarded as an important factor in the pathogenesis of micro- and macroangiopathy (23) and endothelial function has gained increasing attention in the study of vascular disease. A considerable body of evidence in humans indicates that endothelial dysfunction is closely associated with the development of diabetic retinopathy, nephropathy and atherosclerosis in both type 1 and type 2 diabetes (24).

Endothelial progenitor cells

Diabetes is associated with defects in angiogenesis or in vessel repair (figure 3). Angiopathy is the term for vascular defects associated with angiogenic abnormalities. These include abnormally enhanced angiogenesis, defined as capillary vessel growth in the retina (25) leading to diabetic retinopathy (26) and insufficient angiogenesis implicated in abnormal wound healing, leading to diabetic skin ulcers (27).

Interestingly, EPC decrease is related to peripheral arterial disease severity, strengthening the pathogenetic role of EPC dysregulation in diabetic vasculopathy (28). The decrease in the number of angiogenic cells may also be because of the diabetes mellitus–induced abrogation of BM-derived cell mobilization. Hence, in control rats, EPCs showed a mobilization curve within 7 days, whereas diabetic rats are completely unable to mobilize EPCs after ischemic injury. As a consequence, diabetic rats show no compensatory increase in muscle capillary density. Defective EPC mobilization in diabetes mellitus is associated with altered release of CXCL12 and VEGF and inability to upregulate muscle HIF-1α (29).

Wound healing capacity in diabetic patients is decreased. The normal process of wound healing is characterized by five sequential processes: (a) hemostasis, (b) inflammation and debridement, (c) proliferation, (d) epithelialization, and (e) remodeling (30). The delayed wound healing in diabetic patients has been attributed to disturbances in the inflammation/debridement and proliferation phases (31). In the wound healing process, microangiopathy may also contribute to defects in the nutrient/oxygen supply, thus inhibiting normal healing processes (32). Both clinical research and animal models have confirmed such wound healing defects in diabetes (33).
Figure 3. Graphical representation of the proposed paradigm of neovascularization abnormalities in diabetes mellitus. Defective VEGF signaling results in impaired Flk-1 activation that affects a number of processes thought to be involved in arteriogenesis, including endothelial cell growth and migration, monocyte and endothelial progenitor cell (EPC) recruitment, and EPC release by the bone marrow. As a result, arteriogenesis is impaired. At the same time, decreased VEGF sensing, due to impaired Flk-1 activation, results in increased serum VEGF levels that lead to pathologic angiogenesis (retina, atheroma). AGE: advanced glycosylated end-products. (Journal of the American College of Cardiology Vol. 46, No. 5, 2005)

Since the 1930s repair of the damaged endothelium was thought to occur by the migration and proliferation of neighboring undamaged endothelial cells (ECs), and the regeneration of new blood vessels was believed to be mainly through the sprouting of ECs. In 1997, Asahara and colleagues reported (34) that some circulating blood cells possessed the potential to differentiate in vitro into adherent cells displaying cell surface antigens typical for endothelial cells and simultaneous loss of many typical hematopoietic markers. The cells displaying these proprieties were called circulating endothelial progenitor cells (EPCs). It is now known that EPCs originate in the bone marrow and contribute to the formation of new blood vessels in adults. The discovery of EPCs overturned the belief by which in the adult, vascular growth and remodelling was exclusively dependent on the activation of angiogenesis, being the process of vasculogenesis restricted to embryonic life (35). Their discovery led to the new concept that vasculogenesis and angiogenesis may occur simultaneously in the postnatal life because these cells are able to differentiate when needed into vascular endothelium, through a mechanism recapitulating embryonic vasculogenesis (36). They play important roles in vascular regulation at homeostasis and initiation of neoangiogenesis during wound healing, tissue ischemia, tissue remodelling, and tumorigenesis without becoming an integral part of the endothelial intima.

Angiogenesis is the process by which new blood vessels take shape from existing vasculature by sprouting of endothelial cells and before 1997 angiogenesis was considered responsible for most, if not all, blood vessel growth (37). Recent studies have shown that a
mechanism different from angiogenesis exists for formation of vessels in adults called postnatal vasculogenesis or neovascularisation. Vasculogenesis is the process of new blood vessel formation during embryonic development. This process involves the de novo formation of blood vessels by the differentiation of precursor cells into endothelial cells (EC), which eventually give rise to capillaries. This is the reason why the discovery of bone marrow (BM)-derived EPCs with angioblastic morphological and functional properties was a landmark in vascular biology that forever has changed the concept of neovascularization. Increasing evidence suggests that EPCs functionally contribute to the formation of new blood vessels in several models of tissue injury and remodelling, including wound healing, myocardial ischemia, retinopathy, stroke, peripheral vascular disease. The definition and characteristics of EPCs are a work in progress. EPCs are believed to differentiate from hemangioblasts along with other hematopoietic stem cells. Progenitor cells have been distinguished from stem cells due to the lack of self-renewing ability. However, EPCs are characterized as lineage-committed adult stem cells, as they have some degrees of stemness, including self-renewal, clonogenicity, and differentiation capacity. In order to isolate and quantify EPCs one can use two approaches: in vitro adhesion and growth and selection by cell surface phenotype using fluorescent-labelled antibodies and flow cytometry. In the human system, putative EPCs have been isolate and identified using three general approaches (Figure 4).
The first involves collecting low density mononuclear cells (MNCs) from human peripheral blood and preplating them for 48 hours on fibronectin coated dishes and then replating the nonadherent cells to quantify the emergence of the EPC colony forming units several days later. The putative EPCs have been referred to as colony forming unit-Hill (CFU-Hill). These CFU-Hill appearing colonies are composed of round hematopoietic cells that include myeloid progenitor cells, monocytes and T lymphocytes (38; 39).

A second method is based on adhesion of peripheral blood mononuclear cells to fibronectin-coated culture dishes was described by Vasa et al in 2001 (40; 41). Mononuclear cells from peripheral blood were plated on fibronectin and gelatine coated dishes in the presence of media supplemented with endothelial growth factors and fetal calf serum. After 4 days in culture, the non adherent cells where removed, and the adherent cells were assessed for the ability to ingest acetylated low density lipoprotein (LDL) or fluorescent labelled Ulex europaeus agglutinin 1 plant lectin. Many studies suggest that direct adhesion of peripheral blood mononuclear cells to fibronectin coated dishes may enrich for monocytes and is certainly not specific for EPCs. Whether or not the monocytes isolated in this manner, proceed to express endothelial specific markers and participate in angiogenesis depends on the culture conditions and growth factors used. This fact in particular has led to some confusion, since some investigators suggested that monocytes become EPCs. A more
plausible working hypothesis is that some monocytes-macrophage subsets are potent circulating regulators of the angiogenic response and have been called CAC, circulating angiogenic cells. They play important roles in vascular regulation at homeostasis and initiation of neoangiogenesis during wound healing, tissue ischemia, tissue remodelling, and tumorigenesis without becoming an integral part of the endothelial intima (42-45).

The clusters or colonies of cells that emerged from the previous methods were referred to as early outgrowth colonies.

Another different population of cells has been determined to emerge later in culture and at were referred to as late outgrowth colonies. The early outgrowth cells have largely been proved to be hematopoietic cells, the later outgrowth cells are clearly endothelial cells.

A different method of human EPC identification has relied on identification of a particular pattern of cell surface antigen expression on the cells. There are no unique or specific protein markers that can be used to prospectively isolate an EPC (46). In 1997, Asahara et al. reported the isolation of putative EPC from human peripheral blood, on the basis of cell-surface expression of CD34 and other endothelial markers (36).

In fact EPCs were initially described as a pool of circulating bone marrow derived CD34+ progenitor cells that display vasculogenic potential (47; 48). The choice of CD34 as a potential marker of the circulating angioblast was not surprising as it is known to be expressed on endothelial cells and is a marker used to isolate human hematopoietic stem and progenitor cells for clinical stem cell transplantation (49). Circulating EPCs originate primarily from the bone marrow and can be identified by differential expression of hematopoietic and endothelial cell markers. This is important because HSCs and EPCs probably share a common precursor, the hemangioblasts (50). Hemangioblasts reside mainly in the bone marrow and differentiate into HSCs and angioblasts. This process occurs mainly during early embryogenesis but was shown to exist in adults angioblasts will give rise to EPCs that upon stimulation with angiogenic factors are mobilized from bone marrow to peripheral blood (51; 52).

CD34 is a sialomucin expressed on a variety of mesoderm progeny including blood endothelial and fibroblasts and by numerous epithelial lineages and some cancer stem cell population (46). Thus, use of CD34 as an individual EPC marker is inadequate and would certainly require the search for additional potentially unique markers to discriminate all these different cellular lineages from the putative EPCs.

For this reason, EPC lineage was characterized via the expression of another hematopoietic marker, a 120 kDa glycosilated polypeptide, with 5 transmembrane-domains, CD133 (initially called AC133) (53). CD133 expression defines a very early population of progenitor cells, immature HSCs, in which the two sub-fractions of adult bone-marrow stem cells (the
hematopoietic and the mesenchymal subset) seem to be represented. CD133, is a highly conserved antigen with unknown biological activity, which is expressed on hematopoietic stem cells but is absent on mature endothelial cells and monocytic cells. EPCs have the capacity to proliferate, migrate and differentiate into endothelial lineage cells, but have not yet acquired characteristics of mature EC.

Since MNC are heterogeneous, other studies have tried to better define the EPC characteristics. EPCs should express CD34, CD133 and VEGF receptor-2 (VEGFR-2), also known as flk-1 or KDR. Recent studies have shown that also cells expressing CD14 antigen (monocytes/macrophages) (54) can effectively participate to neovasculogenesis, either CD34 negative or CD133 negative (55). Despite the different subpopulation characteristics, also these cells show analogue vasculogenic abilities (56) suggesting that EPCs represent a heterogeneous group at various differentiation stages.

Nowadays a unique definition of EPCs does not exist despite significant steps forward have been taken in order to reach a better definition and a detailed functional characterization of these cells. This may reflect that the endothelial progenitor is a dynamic phenotype in space and time. Indeed, the endothelial differentiation potential of circulating progenitors varies according to the local environment and changes over time, as can be recapitulated in the culture dish (57).
Functional Characteristics of EPCs.

Typical functions of EPCs in vitro are proliferation and clonogenesis, migration, adhesion, and tubulisation. An important functional characteristic is the capacity to form cord- and tubular-like structures in three-dimensional gels, like Matrigel (58), as mature endothelial cells do. Additionally, migratory capacity of EPCs is typically tested in vitro. A commonly used characterization of endothelial differentiation is based on EPC functions of uptaking acetylated low density lipoproteins (AcLDL) and binding lectin, Ulex europaeus agglutinin-1 (UEA-1). While ac-LDL uptake is dependent on scavenger receptors common to monocytes, UEA-1 binding is considered more specific for endothelial cells. Several functions of EPCs can also be established in vivo, such as formation of new blood vessels in relevant models in the laboratory animal.

Mobilization and homing of EPCs.

Several groups have demonstrated a significant restoration of blood flow and enhanced tissue regeneration after administering bone marrow-derived cells or EPCs after an ischemic event (59). The process whereby EPCs participate to postnatal vasculogenesis consists of four interrelated steps: mobilization, homing, invasion and differentiation into mature ECs and/or regulate preexisting ECs via paracrine or juxtacrine signals. The mobilization of EPCs from the bone marrow (BM), in order to re-establish an intact endothelial layer following denudation of endothelium, is a complex process. It is known that the majority of EPCs remain quiescent in the stem cell niche in the BM, which consists of fibroblasts, osteoblasts, mesenchymal cells, adipocytes, and endothelial cells. The BM niche is characterized by low oxygen tension and high levels of stromal cell derived factor-1α (SDF-1α) or CXC chemokine ligand 12 (CXCL12). Many studies have indicated that SDF-1α is a potent factor in mobilizing primitive progenitors from the BM niche (60; 61). SDF-1α, is a 7.97-kDa chemokine and is known to be an effective chemotactic factor for a variety of cell types that contains the G protein – linked receptor CXCR4. SDF-1 is produced constitutively in many organs, including BM, spleen, heart, liver and kidney; it plays a vital role in organ homeostasis, retention of HSCs and progenitors within the BM, and general development. In vivo stem cell homing to the BM, their retention, engraftment, and egress to the circulation, all involve SDF-1/CXCR4 interactions. In fact SDF-1α chemoattracts normal CD34+ CXC chemokine receptor-4 (CXCR4)- positive early hematopoietic cells (62). CXCR4 is the predominant receptor for SDF-1α and the cross–talk between CXCR4+ EPC and SDF-1α-expressing stromal cells may be partly responsible for their retention within the BM niche (63).
Under homeostatic conditions, most progenitors remain in the BM compartment, where they are retained by high SDF-1α expression induced by the hypoxic microenvironment. It is possible that low numbers of progenitor cells continuously recirculate under physiologic conditions (64). During alarm situations, stress, and injury, progenitor cell egress to the circulation is dramatically increased. This process is termed mobilization.

Tissue ischemia is considered the strongest stimulus for EPC mobilization, through the activation of hypoxia-sensing systems, such as hypoxia-inducible factor (HIF)-1. HIF-1 is a heterodimeric transcription factor composed of α (HIF-1α) and β (HIF-1β) subunits. While HIF-1β is constitutively expressed, HIF-1α expression is regulated by cellular oxygen concentrations. Under normoxic conditions, HIF-1α is rapidly degraded via the ubiquitin-proteasome pathway, while cellular hypoxia inhibits its ubiquitination and proteasomal degradation, allowing HIF-1α to dimerize with HIF-1β. The resulting active HIF-1 binds to enhancer DNA regions and promotes the transcription of oxygen-sensible genes that encode, among others, vascular endothelial growth factor (VEGF), SDF-1α, and erythropoietin (65).

There is a direct correlation between the plasma concentration and degree of mobilization, implying a concentration gradient determines response (66). After an ischemic insult, soluble factors, such as SDF-1α, are released by injured tissue and stimulate mobilization of progenitor cells from the BM. SDF-1α is selectively upregulated in transiently ischemic tissue guiding the recruitment of progenitor cells to a permissive or conditional niche, where they proliferate and assemble. Progenitor cells are recruited to ischemic tissue by high local levels of SDF-1α providing a permissive niche. During condition of ongoing local tissue hypoxia, progenitor cells proliferate and assemble into vascular structures, contributing to the remodeling of nascent vascular network, and eventual reoxygenation of the tissue. After neovascularization tissue oxygenation returns to normal levels, SDF-1α expression returns to baseline, and progenitor cell recruitment stops.

The dependence of progenitor cell recruitment and function on a hypoxic microenvironment suggests that targeting the hypoxic response mediated by the transcription factor HIF-1α may be a more rational strategy for achieving therapeutic angiogenesis in patients with congestive heart failure.

Recently, Rajagopalan and colleagues reported the first clinical results of HIF-1α gene therapy predicated on transcriptional activation of a patient's own gene. The high dose of HIF-1α gene therapy improved therapeutic outcomes in critical limb ischaemic patients. More importantly, it appears to be safe for clinical application (67). In fact, HIF-1α overexpression
on EPCs promotes hypoxia-induced EPC differentiation, proliferation and migration, establishing HIF-1α as a putative pharmacological target.

**Figure 5.** Schematic representation of mobilization of endothelial progenitor cells (EPC) from the bone marrow. Br J Clin Pharmacol. Oct 2009; 68(4): 484–492.

The figure 5 shows as the cells mobilization is a result of high levels of circulating SDF-1α, that appear to reverse the SDF-1α gradient across the BM barrier, forcing CXCR4⁺ cells to exit the BM (69). In other words EPCs are mobilized from the BM in response to peripheral tissue hypoxia and trauma, which cause the production and release of EPC-activation factors, such as HIF-1α (70), vascular endothelial growth factor (VEGF), erythropoietin (EPO), estrogens, or SDF-1α to a concentration greater than that in the BM (56). SDF-1α cleavage in the BM is postulated as a crucial process, breaking anchorage of stem cells to endosteal stroma and osteoblasts or vascular endothelium, shown to comprise the stem cell niche (71; 72),(73). Moreover, quiescence and immobility signals exerted by SDF-1α, which has been shown to maintain stem cells in their niche, are now disrupted. The mobilizing effect initiated by reversing the SDF-1α gradient was demonstrated by direct elevation of plasma SDF-1α (66).
**Figure 6.** Schematic model of organ-BM communication mediated by mutual CXCR4 SDF-1α interactions. (A) Throughout stress and inflammation conditions, the expression of SDF-1α is increased in the injured organs and consequently in the peripheral blood. Circulating SDF-1α is internalized by CXCR4 (1 and 2) across the physiologic blood vessel barrier (3) into the BM (4) where it binds CXCR4 in the stem cell niche (5). (B) Presentation of translocated SDF-1α by BM endothelial and other stromal cells recruits circulating CXCR4D hematopoietic stem and progenitor cells into the BM, which can proliferate and differentiate into immature and mature cells, mobilize into the damaged organ, and serve for host defense and organ repair(63).

**Figure 7.** Molecular mechanism regulating the multiple steps of endothelial progenitor cell biology during postnatal vasculogenesis. Recruitment and incorporation of EPCs into angiogenic sites requires a coordinated multistep process including mobilization, chemoattraction, adhesion, endothelial transmigration, migration, tissue invasion, in situ differentiation and paracrine and/or juxtacrine factor production. The major molecular mechanisms that have been implicated in the distinct steps of EPC biology are indicated. Figure adapted from Fusenig N, Marmé D (eds): Tumor Angiogenesis: Basic Mechanisms and Cancer Therapy. Springer; 2008. Chapter 17 with modifications.
The egress of stem cells from the BM is allowed by the activation of proteases such as elastase, cathepsin G, and matrix metalloproteinases (MMPs), which cleave adhesive bonds on stromal cells, and interact with integrins on hematopoietic stem cells (74) (Figure 7). 

Once mobilized, EPCs are released from the vascular zone of the BM into the peripheral circulation. To date, no clear definition exists as to when a circulating EPC turns into a mature, fully differentiated endothelial cell in vivo. An initiation of this differentiation may by the migration of EPCs from the BM into the peripheral circulation and after adhesion and insertion into the monolayer of surrounding mature endothelial cells this differentiation process may be completed. However, the exact differentiation cascade of EPCs in vivo is hardly comprehensible. Circulating EPCs can contribute to vascular repair not only by direct transdifferentiation into vascular endothelial cells but also and in particular via the secretion of proangiogenic cytokines.

Adhesion molecules of the selectin and integrin family are also essential for EPC arrest to endothelial cells. Chemokines have been shown to trigger integrin activation to mediate arrest of rolling leukocytes. Isolated human EPCs demonstrate significantly increased and constitutive adhesion on the \( \beta_2 \)-integrin ligands fibrinogen and ICAM-1 under physiological flow conditions in vitro (75). Functionally active \( \beta_2 \)-integrins further mediated arrest of EPCs to mature endothelial cells and their subsequent emigration into tissues, implying the importance of this integrin subclass not only after arterial injury but also during angiogenesis in ischemic regions, where sprouting of newly formed capillary structures is required. The association of \( \beta_2 \)-integrins with the homing of hematopoietic progenitor cells (including also EPC populations) during neovascularization was demonstrated in a mouse model of hind limb ischemia (75). In addition to \( \beta_2 \)-integrins, \( \beta_1 \)-integrins have also been involved in the homing of BM-derived progenitor cells to the remodeling vasculature. The \( \alpha_4 \beta_1 \) integrin (also known as very late antigen-4, VLA-4) plays an important role in EPC homing. Blockade of VLA-4 significantly reduces the incorporation of CD34\(^+\) cells to areas of active tumor vascularization, and, recent data revealed a critical involvement of VLA-4 in the homing of human EPCs to ischemic tissue (76). Vascular cell adhesion molecule (VCAM) and fibronectin are well known ligands for VLA-4. While VCAM is primarily upregulated in ischemic tissue, interaction of VLA-4 with fibronectin occurs mainly when denuded vascular ECM is exposed to the blood flow, e.g. after endothelial injury. It is conceivable that coating of biomaterials with EPC attracting compounds may be helpful for tissue engineering. Local delivery of VEGF-A within a region of endothelial denudation, e.g. after stent implantation, may be helpful to selectively attract EPCs and to achieve stent endothelialization, but has to be scrutinized with caution in the context of advanced atherosclerosis due to the risk of plaque destabilization. Finally, coating strategies with antibodies against progenitor cell
antigens (e.g. CD34) were reported to effectively accelerate endothelialization by capturing circulating EPCs after stent placement or vascular graft implantation.
EPC alterations in diabetes.

Traditionally, the development of diabetic complications has been attributed to the biochemical pathways driving hyperglycaemic cell damage, while reparatory mechanisms have been long overlooked. A more comprehensive view of the balance between damage and repair suggests that an impaired regenerative capacity of bone marrow (BM)-derived cells strongly contributes to defective re-endothelisation and neoangiogenesis in diabetes (77). It can therefore be predicted that defects in the mechanisms of repair can worsen, and hence vascular disease can be accelerated, in patients subjected to vascular noxae. Several lines of evidence indicate that diabetes should indeed be considered a disease of impaired damage control (78) with defects in the regenerative pathways that normally follow tissue injury.

Numerous studies have demonstrated an effect of hyperglycaemia on EPC biology. Culturing EPCs derived from healthy subjects under high glucose conditions impaires EPC number and function (79). If cultured in high glucose conditions, these cells present impaired function and decreased nitric oxide and MMP-9 production (80).

Both cytometric and culture methods have extensively demonstrated that type 1 and type 2 diabetic patients have less circulating EPCs than matched healthy subjects. Moreover, diabetic EPCs display functional impairment, such as reduced proliferation, adhesion, migration, and incorporation, into tubular structures (81). The reduction of the circulating CD34+ progenitor cell pool (the ancestor of all EPC phenotypes) occurs at an early stage in the natural history of type 2 diabetes and can be demonstrated in individuals with impaired glucose tolerance (82). The depletion of progenitor cells persists over time and is worse in patients with advanced complications (81),(83). Indeed, the levels of CD34+KDR+ EPCs are strongly negatively correlated to the degree of peripheral vascular complications in type 2 diabetes (28). The link between hyperglycaemia and altered EPCs is strongly supported by studies in type 1 diabetes. Type 1 diabetes causes early EPC dysfunction that is quite similar to that observed in type 2 diabetes (84).

These observations suggest that the burden of DM-related cardiovascular complications correlates with the number and function of EPCs: alterations in EPCs may contribute to the development and progression of atherosclerosis in patients with type 2 DM (85).The reduction in circulating EPCs in diabetic patients may recognize at least three pathophysiological explanations: impaired BM mobilization, defective proliferation, and enhanced apoptosis. Remarkably, in accordance with Brownlee's unifying hypothesis, oxidative stress appears as a major determinant of all of these mechanisms (86).
Experimental (87),(77) and human (88) studies also demonstrate that DM impairs the mobilization of EPCs in response to tissue ischemia or cytokines, such as granulocyte colony stimulating factor.

The migration of EPCs to sites of injury/ischemia is mediated by the gradient of SDF-1$\alpha$ and other chemoattractants, such as VEGF. In addition to the altered cytokine gradient, decreased NO, increased reactive oxygen species, and advanced glycation end products in DM impair this migration. These pathological processes induced by diabetes also increase apoptosis and decrease proliferation of EPCs (85).

Figure 8. Potential functional role of endothelial progenitor cells (EPCs) in vascular repair in normal subjects (top) and in patients with diabetes mellitus (DM; bottom). In subjects with DM, the number of bone marrow (BM) progenitor cells and their mobilization are reduced because of the alteration in the osteoblastic and vascular niches and the cytokines profiles, respectively, and the survival of circulating EPCs is reduced. As a result, the numbers of circulating EPCs are decreased in subjects with DM. Furthermore, the migratory, recruitment, and angiogenic functions of EPCs at ischemic tissue in subjects with DM are impaired and thus contribute to defective vascular repair and angiogenesis. (ATVB 2014;34:1136-1143).

In patients with DM, the high glucose environment reduces the level of VEGF and SDF-1$\alpha$ secretion from endothelial cells via the hypoxia-inducible factor/hypoxia-responsible element pathway and DPP-4 activity (87) (Figure 8).
Dipeptidyl peptidase-4

Because-impaired glucose metabolism and hyperglycemia are the primary initiating events that induces EPC alteration in DM, and EPC dysfunction is closely related to the degree of hyperglycemia, improved hyperglycemic control should be the initial therapeutic target. Currently, there are only limited data on the optimal antidiabetic therapy to reverse EPC dysfunction in DM. For type 2 diabetes the oral medication can be subdivided in these classes:

- Biguanide, metformin is the only member of the class and it is one of the oldest medications used in the treatment of type 2 diabetes. Metformin exerts its effects primarily by decreasing hepatic glucose output and has a comparatively lesser effect increasing insulin sensitivity.

- Sulfonylureas. This class include several medication that act on beta-cells to increase insulin release. They bind to the sulfonylurea receptor on the surface of the β-cell and inhibit potassium efflux, thus depolarizing the β-cells and facilitating insulin release. First-generation agents, such as acetohexamide, chlorpropamide, and tolbutamide have been replaced by second- and third-generation sulfonylureas, such as glyburide, glipizide, gliclazide and glimipiride.

- Glinides. The glinides, nateglinide and replaginide, exert their effects in a manner similar to sulfonylureas, by binding to the sulfonylurea receptor and inducing depolarization of the β-cells. However, they bind in a different manner to the sulfonylurea receptor and have a shorter half-life.

- Thiazolidinediones. The thiazolidinediones rosiglitazone and pioglitazone are insulin sensitizers. These drugs bind to peroxisome proliferator-activated receptors (PPARs) in cells, and this drug-PPAR complex (with one or more coactivators) acts on response elements in promoter regions to affect the transcription of as many as 100 genes.

- Alfa-glucosidase inhibitors. Acarbose and miglitol are the α-glucosidase inhibitors currently available in clinical practice. They act by inhibiting the intestinal enzyme that cleaves polysaccharides into monosaccharides, thereby limiting intestinal glucose absorption.

- GLP-1 receptor agonists, which promote glucose-induced insulin secretion.

- Dipeptidyl peptidase-4 (DPP4) inhibitors. DPP-4 inhibitors are a relatively new class of oral agents for the treatment of type 2 diabetes. They act by inhibiting the enzymatic degradation of glucagon-like peptide 1 (GLP-1), thereby potentiating glucose-induced insulin secretion after a meal.
Dipeptidyl peptidase-4 (DPP4) or adenosine deaminase complexing protein 2 (ADCP 2) or T-cell activation antigen CD26 (EC 3.4.14.5) is a serine exopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides (Figure 9), such as chemokines, neuropeptides, and peptide hormones. DPP4 is a type II transmembrane glycoprotein, expressed on the surface of many cell types, whose physiological functions are largely unknown. The enzyme exists as a membrane-anchored cell surface peptidase and as a second smaller soluble form present in the circulation. The soluble circulating form of DPP-4 (sDPP-4) that lacks the transmembrane and cytoplasmic domains of mDPP-4 is largely responsible for degradation of the majority of newly synthesized GLP-1 and GIP (89),(90),(91).

Evidence suggests that proteolytic cleavage of membrane membrane-associated DPP-4 (mDPP-4) is the major source of circulating sDPP-4 (92). DPP4 family consists of several members: DPP4, quiescent cell proline dipeptidase (QPP), fibroblast activation protein (FAP), DPP8 and DPP9. (93) DPP4 is widely expressed (lung, brain, pancreas, kidney, vessel, prostate, uterine, thymus, lymph nodes and spleen) on the cell surface of cells including epithelial, endothelial cells and immune cells such as natural killer (NK) cells, lymphocytes and monocytes. (93). The enzyme cleaves a diverse range of proteins including glucagon-like peptide (GLP)-1 and 2, glucose-dependent insulino tropic peptide (GIP), neuropeptide (NPY) and chemokines.

Both GLP-1 and GIP are gastrointestinal (Gut) hormones (incretins), secreted after ingestion of nutrients. Gut hormones are synthesized by specialized enteroendocrine cells located in the epithelium of the stomach, small bowel and large bowel and are secreted at low basal levels.
Plasma levels of most gut hormones rise briskly within minutes of nutrient intake and fall rapidly thereafter, mainly because they are cleared by the kidney and are enzymatically inactivated (94).

Incretins are involved in multiple functions and in particular they facilitate the disposal of glucose through the stimulation of insulin secretion from the endocrine pancreas. In 1932 La Barre proposed the name "incretin" for these hormones. The term incretin effect was used to describe the fact that oral glucose load produces a greater insulin response than that of an isoglycemic intravenous glucose infusion (95). The observation that GLP-1 is rapidly degraded by DPP-4 has fostered the development of specific protease inhibitors that prevent the rapid fall of GLP-1 in circulating plasma after eating (96). By 1995 DPP-4 inhibition was under consideration for the treatment of diabetes (97). Sitagliptin, from Merck and Co., was the first DPP-4 inhibitor to be approved by the U.S. Food and Drug Administration in October 2006. Nowadays DPP-4 inhibitors are a class of anti hyperglycemic agents indicated for improving glycemic control in patients with type 2 diabetes.

In addition to incretins, DPP-4 has several other physiologic substrates, including cytokines, chemokines, and neurohormones that can affect vascular function and metabolism. For example the figure 10 shows that DPP-4 cleaves multiple peptides which are capable of influencing cardiovascular function.

**Figure 10.** DPP-4 substrates that directly or indirectly regulate cardiovascular function. (Endocr Rev. Apr 2012; 33(2): 187–215. doi: 10.1210/er.2011-1052)
Interestingly, SDF-1α is a natural DPP-4 substrate and a major regulator of stem cell mobilization,(98) as well as EPCs / PACs function (67; 99) . Both GLP-1 and SDF-1α have been shown to increase the number of circulating EPCs and improve endothelial function (100; 101) which suggests that DPP-4 could be a possible candidate for pharmacotherapy in therapeutic vasculogenesis.

In patients with type 2 diabetes or chronic hyperglycaemia circulating levels of active DPP-4 have been shown to be higher than those in healthy controls (102; 103). The pleiotropic effects of DPP4 inhibitors, along with the control of glucose plasma concentrations, represent important mechanisms of action of these drugs, among which a potential cardiovascular protection activity appears to be of major importance, especially in T2DM. DPP4 inhibitors have a protective effect on the endothelium. This protection is particularly useful because endothelial cells exposed to high glucose exhibit enhanced DPP4 activity, independent of hyperosmolarity (104). DPP-4 inhibitors have anti-inflammatory and anti-oxidative effects (105),(106). The effects of DPP4-I on blood pressure appear more complex and less clear. DPP4-I administration can potentially decrease blood pressure by two distinct mechanisms: one at the renal level, by inhibiting sodium/hydrogen exchange, and the other at cardiac level, by inhibiting BNP degradation. Whether the effect of DPP4-I on BNP has any role on endothelial function in humans is presently unknown. However, it should be emphasized that very few clinical studies included reduction of blood pressure as a primary or prespecified end point (107).

Intensive glucose control has been shown to provide beneficial effects on retinopathy in both T1D (108) and T2D (109; 110) Few experimental studies have assessed the effect of DPP4-I on diabetic retinopathy. Only three experimental studies are available on the effect of DPP4-I on diabetic neuropathy (107) showing protective effects of DPP4-I.

Ishibashi et al. reported that linagliptin inhibited the generation of reactive oxygen species induced by advanced glycation end products (AGEs) in endothelial cells (105). Matsubara et al. reported that sitagliptin improves endothelial dysfunction in association with anti-inflammatory effects in patients with coronary artery disease and uncontrolled diabetes (111). Shiraki et al. reported that GLP-1 reduced TNF-α-induced oxidative stress in endothelial cells (106).

Many efforts, including preclinical studies, pooled analysis of phase III clinical trials, and randomized controlled trials, have been devoted to test the potential cardiovascular benefits of DPP4-I, while their impact on microangiopathy seems to be relatively neglected. So far, randomized controlled trials do not support cardiovascular protective effects of DPP4-I (112),(113) but studies with longer followup are likely needed to allow the effect of good
glycemic control achieved with DPP4-I to translate into improved cardiovascular outcomes (114).

Extensive experimental data and preliminary clinical studies indicate that DPP4-I may improve microvascular structure and function. Whether the effects of DPP4-I are mediated by improved glucose control or by pleiotropic off-target actions of DPP4-I on nonincretin substrates remains unclear. A few hints can help answer this question with available data. Preclinical findings obtained in vitro and using animal models of T1D (e.g., STZ-induced diabetes) suggest that favorable effects of DPP4-I are conveyed independently of glycemic effects (107).

![Figure 11](image)

**Figure 11.** DPP-4i facilitates endothelial repair, plaque stabilization, and neovascularization by preventing the degradation of its substrate, SDF-1α, which recruits BM derived EPCs to injured areas of the endothelium (115).

Treatment with DPP-4i blunts the DPP-4-mediated degradation of SDF-1α, thereby enhancing recruitment of EPCs to vascular beds in need of repair or expansion. Because SDF-1 is subject to inactivation via either DPP-4- or matrix metalloproteinase-mediated cleavage (67; 99), DPP-4 inhibitors have been used to enhance SDF-1 activity and increase stem cell number in both preclinical and clinical studies of cardiovascular injury (67).
METHODS

Patients. The study was approved by the Ethical Committee of the University Hospital of Padova and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2008. Informed consent was obtained from all patients. Type 2 diabetic patients were recruited at the outpatient clinic of the Metabolic Division, University Hospital of Padova. Healthy blood donor subjects were recruited anonymously from the local blood biobank, provided they were free from diabetes and cardiovascular disease. For diabetic patients, the following data were collected from the electronic outpatient clinic charts: age, sex, BMI, waist circumference, systolic and diastolic blood pressure, diabetes duration, HbA1c, lipid profile, concomitant risk factors, complications and medications. Coronary artery disease was defined as a past history of myocardial infarction or angina, confirmed by a coronary angiography showing stenosis >70% in at least one epicardial coronary vessel, or in the presence of a non-invasive stress test indicative of inducible myocardial ischemia. Peripheral arterial disease was defined as claudication, rest pain, or ischemic diabetic foot confirmed by an angiographic or ultrasound examination. Cerebrovascular disease was defined as a past history of stroke or evidence of carotid artery stenosis >30% at a ultrasound examination. Retinopathy was defined based on digital funduscopic photography scored remotely by expert ophthalmologists. Nephropathy was defined as overt macroalbuminuria (urinary albumin/creatinin ratio [ACR] >300 mg/g) or as chronic renal failure (estimated glomerular filtration rate [eGFR] <60 ml/min/1.73 m²). As the 2.5 mg renal dose-adjusted formulation of Saxagliptin was not yet commercially available in Italy when the study was performed, none of the patients in the in vivo study had chronic renal failure. Data on medications were also collected. For the in vivo angiogenesis assay, patients on Saxagliptin treatment (n=5) for >4 months and patients on other, non-incretinergic, regimen (n=5) were enrolled. As treatment had been previously decided on clinical ground and was not assigned by the investigators, this did not represent a clinical trial and was not registered as such.

Culture of pro-angiogenic cells (PACs) and functional assessment. PBMCs were isolated by density gradient centrifugation with Histopaque-1077. Cells were plated on human fibronectin-coated culture dishes and maintained in endothelial cell basal medium-2 (EBM-2). The medium was supplemented with EGM-2 MV SingleQuots containing FBS (5%), human VEGF-1, human fibroblast growth factor-2 (FGF-2), human epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and ascorbic acid. After 4 days in culture,
nonadherent cells were removed by washing with PBS, new medium was applied, and the cells were maintained through day 7 cultured with or without Saxagliptin (Saxa, DPP-4 inhibitor) and with or without SDF-1α.

PACs obtained after 7 days of culture without stimuli were immunophenotyped by fluorescence microscopy (Leica DM 6000B) for the ability to uptake AcLDL and bind FITC- \textit{Ulex europaeus} agglutinin Lectin. Adherent cells were first incubated with Dil-acLDL for 1h, then counterstained with FITC-Lectin and fixed in 2% paraformaldehyde. Images were acquired with the manufacturer’s software, and assembled using Adobe Photoshop.

In separate experiments, PACs were cultured from unselected PBMCs of healthy controls in the presence of Saxagliptin and/or SDF-1α and, at the end of the 7 day culture period, CD14⁺ PACs were separated from CD14⁻ PACs using the MS Column and MiniMACS Separator (Miltenyi Biotec). Then, functional assays and gene expression were analyzed separately for CD14⁺ and CD14⁻ PACs.

\textbf{Adhesion assay.} A monolayer of human umbilical vein endothelial cells (HUVECs) was prepared 48 hours before the assay by plating 2×10⁵ cells (passage 5 to 8) in each well of 24-well plate. PACs were labelled with CMTMR and 1×10⁵ cells were added to each well and incubated for 3 hours at 37°C. Non-attached cells were gently removed with PBS, and adherent PACs were fixed with 4% paraformaldehyde and counted in 10 randomly selected field.

\textbf{Matrigel Tubule Assay.} Matrigel (Sigma-Aldrich) was thawed and placed in 96-well plate at room temperature for 30 minutes to allow solidification. PACs (3×10³) were co-plated with 1.5×10⁴ human umbilical vein endothelial cells (HUVECs) and incubated at 37°C for 24 hours. The 5:1 HUVECs/PACs ration was chosen based on a preliminary dose-response experiments (not shown), which showed a suppressive effect of higher ratios on tube formation. Tubule formation was defined as a structure exhibiting a length 4 times its width. The length and the number of tubules was determined in 10 randomly selected fields.

In separate experiments performed to analyze the physical location of PACs co-cultured with HUVECs in the Matrigel tubule assay, PACs were red-labelled with the fluorescent dye Cell-Tracker Orange CMTMR (Life Technologies).

\textbf{Migration assay.} Cell migratory assays were performed using Transwell chambers with filter membranes of 3 mm pore size. Transwell chambers were inserted into the plate wells. PACs were seeded into the upper chamber (10⁴ cells per well in serum-free medium) in either the absence or the presence of SDF-1α at 37°C. At the end of the assay, 3 hours later, PACs
migrated in the lower compartment were collected and counted using the flow cytometer. Results are reported as ratio of the number of migrated cells and non migrated cells.

**Flow cytometry and immunomagnetic cell sorting.** For the characterization of PACs, cells were detached using EDTA and scraping. Cells were labelled with mouse anti-human PerCP-Cy5.5 CD45 (BD Pharmingen, cat# 552724), PE KDR (R&D Systems, cat# FAB357P), FITC CD68 (Dako Cytomation, cat#F7135), APC CD34 (BD Pharmingen, cat# 345804), PE CD14 (Beckman Coulter, cat# A07764), FITC CD26 (BD Pharmingen, cat# 555436), FITC CD31 (BD Pharmingen, cat# 555445). Events were acquired using a FACS Canto instrument (BD), after morphological gating in the SSC vs FSC plot. At least $10^5$ events were acquired for each analysis.

PACs treated with and without Saxagliptin and/or SDF-1α for 7 days were trypsinized into conical tubes, washed twice with PBS and fixed in 70% ice-cold ethanol. For DNA analysis, cells were centrifuged at 200 g for 10 min at 4°C and washed twice with PBS. For cell cycle analysis, after incubation at 37°C in the dark for 15 min, DNA content of the nuclei was determined by staining nuclear DNA with propidium iodide solution (50 μg/mL, sigma, USA) containing 50 μg/mL ribonuclease A. The DNA content was measured by a flow cytometry (FacsCanto) and scored.

For immunomagnetic selection of CD14$^{pos}$ and CD14$^{neg}$ cells from the initial PBMC population for PACs culture, we used the MS Column and MiniMACS Separator (Miltenyi Biotec). After isolation of PBMCs, cells were centrifugated at 300 g for 10 minutes. The cell pellets were resuspended in 80 μL of buffer (MACS buffer), 20 μl of CD14 MicroBeads per 10$^7$ total cells were added. After 30 minutes in the refrigerator, cells was washed by adding MACS buffer and resuspended in 500 μL of buffer. The magnetic separation was performed as described by the manufactures.

**Determination of DPP-4 activity.** DPP-4 activity was determined in conditioned medium or cell extracts from cell cultures using the DPP-4 drug discovery Kit (Enzo Life Sciences, Farmingdale, NY, USA) with the Gly-Pro-para-nitroaniline (pNA) chromogenic substrate, according to the manufacturer's instructions.

**Gene expression analysis.** After 7 days of culture total RNA was extracted from PACs using RNasey kit (Qiagen), following the manufacturer's protocol. RNA quantity was determined on a Nanodrop Spectrometer (termo Fisher scientific Inc) (using 1 OD260 = 40 μg RNA). A260/A280 ratios were also calculated for each sample. RNA was reverse
transcribed to generate cDNA using the First-Strand cDNA Synthesis Kit from Invitrogen following the manufacturer’s protocol. Samples were mixed by vortexing and briefly centrifuged and denaturated by incubation for 5 minutes at 70°C to prevent secondary structures of RNA. Samples were incubated on ice for 2 minutes to allow the primers to align. Gene-specific primer pairs were designed using Primer-BLAST (NCBI) and were each validated prior to use by gradient PCR and gel analysis to test for optimal annealing temperature, reaction efficiency and specificity (Table 1). Duplicates of sample cDNA were then amplified on the 7900HT Fast Real-Time PCR System (Applied Biosystems) using the Fast SYBR Green RT-PCR kit (Applied Biosystems) in 96-wells plates (micro amp optical, Applied Biosystems). Expression data were normalized to the mean of housekeeping gene to control the variability in expression levels and were analyzed using the 2^{-ΔCT} method.

Mouse colony forming assay. Hematopoietic colonies were grown from unfractioned bone marrow cells of C57Bl/6 mice and quantified using the Methocult system (Stem Cells inc. Vancouver, Canada). For all experiments using animals, the ‘Principles of laboratory animal care’ (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) as well as specific national laws were followed.

Spheroid assay. For preparation of methocell, 6 g of methyl-cellulose together with a magnetic stir bar were autoclaved in a 500 ml flask. Afterwards, 250 ml of 60°C basal EBM medium was added under sterile conditions and the suspension was stirred at 60°C for 40 min. Additional 250 ml of basal EBM medium were added and the solution was stirred at 4°C overnight. 50 ml portion of the solution were centrifuged for 2 h at 4000 rpm at room temperature. The highly viscose soluble fraction was separated from insoluble residue and was stored at 4°C. The following protocol was applied for the spheroid assay: 48,000 HUVEC were mixed with 6 ml of methocoel / HUVEC medium and seeded as 100 µl drops in a 96-well U-bottom dish using a multipette. The cells were incubated for 24 h in an incubator to form spheroids. The day after, spheroids were collected, centrifuged and the spheroid pellet was mixed with methocoel-mix The collagen gel was prepared on ice and 500 µl of collagen gel were added to the spheroid / methocoel solution, mixed by pipetting and seeded on a 24-well culture dish for 30 min in an incubator. The spheroids were cultured for 24 h at 37°C and 5% CO2. Spheroids were fixed with formaldehyde. For quantification, 10 spheroids were assessed for cumulative sprout length, number of sprouts and number of branch points.
In vivo angiogenesis assays. To gather information on the presence of functional circulating PACs and how they are modulated by Saxagliptin in type 2 diabetic subjects, we used the in vivo Matrigel plug angiogenesis assay with patients’ own PBMC. Briefly, PBMC were isolated with Histopaque (Sigma-Aldrich). Cell count and viability were assayed with an automated BioRad TC20 cell counter. Then, $3 \times 10^6$ PBMC were resuspended in 500 μL phenol-free Matrigel (BD, cat no. 356237) and implanted subcutaneously into the dorsum of immunodeficient RAG-2/gamma(c) double knock-out mice (in-house colony). Experiments involving animals were performed according to national guidelines and according to the ‘Principles of laboratory animal care’ (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm). The experiment was performed with PBMC of n=5 type 2 diabetic patients on Saxaglipthin therapy (>4 months) and n=5 type 2 diabetic patients on non-incretinergic therapy. Mice were anesthetized with 100 mg/ml Ketamine HCL and 20 mg/ml Xylazine. To minimize variability, the same mouse received Matrigel plugs from a Saxagliptin-treated and a control patient. Plugs were explanted 10 days later for macroscopic inspection, histology (H&E staining), and determination of the hemoglobin/protein content ratio (Drabkin’s solution and Bredford reagent respectively, Sigma-Aldrich), which is as a surrogate of perfusion.

In addition to the traditional Matrigel plug assay, we also used the Directed In Vivo Angiogenesis Assay (DIVAA, Amsbio, Abingdon, UK), which employs semi-closed small silicone cylinders called “angioreactors”, filled with Matrigel with or without a growth factor cocktail (fibroblast growth factor [FGF] + vascular endothelial growth factor [VEGF]) specifically design to stimulate vascular invasion. Angioreactors containing patients’ own PBMC were implanted subcutaneously in the dorsal flanks of RAG-2/gamma(c) double knock-out mice. Compared to the traditional plug assay, the sleek design of angioreactors provides a standardized platform for reproducible and quantifiable in vivo angiogenesis assays and prevents assay errors due to absorption of Matrigel by the mouse. In the present protocol, each anaesthetized mouse received implantation of 4 angioreactors, 2 containing cells from Saxagliptin-treated patients and 2 containing cells from control patients, each with or without adding growth factors. Angioreactor tubes were explanted 10 days later for gross inspection of vascular invasion and determination of perfusion by FITC Lectin detection, according to the manufacturer’s protocol. As fluorescence labelled *Griffonia simplicifolia* Lectin binds to alpha-D-galactosyl and N-acetyl galactosaminyl groups on the surface of endothelial cells, Lectin content is a measure of angioreactor tube vascular invasion.

Statistical analysis. Data are presented as mean ± standard error, or as percentage where appropriate. Normal distribution of the variables under investigation was verified using the
Kolmogorov-Smirnov test. Non normal variables were log transformed. Comparison between means was performed with the unpaired 2-tail Student’s t test. Comparison between more than 2 groups was performed with ANOVA. The Bonferroni correction was used to account for multiple testing, where appropriate. Frequencies were compared using the Chi square test. Statistical significance was accepted at p<0.05.
Characterization of cultured pro-angiogenic cells. Pro-angiogenic cells (PACs) cultured from human PBMCs constitute a heterogeneous population composed mostly of CD45+ lymphocytes and monocyte/macrophage lineage cells, plus a small population of stem/progenitor cells, possibly including EPCs (57). Accordingly, we found that PACs culture was comprised of i) small cells in the lymphocytic morphologic (FSC vs SSC) gate, some of which expressed KDR (21±3%) and CD31 (36±5%) and can correspond to angiogenic T cells present in PACs / early EPCs culture (116); ii) larger mononuclear cells expressing CD14, CD68, and CD31, likely belonging to the monocyte-macrophage population. The monocytic/lymphocytic cell ratio was 2.2±0.1 and fairly constant throughout experiments. As expected, expression of the stem/progenitor cell marker CD34 was low (Figure 12A). These data indicate that cells used in subsequent experiments correspond to the PAC phenotype already described in the literature. Cultured PACs were also characterized by the double positivity for the (non-unequivocal) endothelial markers acetylated LDL uptake and Ulex-Lectin binding (Figure 12B). Interestingly, the fraction of acLDL*Lectin+ cells in the PACs culture was significantly lower in T2D compared to healthy controls (30.8±0.8% vs 36.0±0.1%; p=0.003). There were no differences in the proportion of lymphocytic and monocytic cells in PACs cultures of T2D versus healthy subjects.

Figure 12. Phenotypic characterization of cultured PACs. A) PACs were characterized for surface marker expression by flow cytometry. In the side scattered (SSC) versus forward scatter (FSC) morphologic plot, lymphocytic cells (LYMPHs) and monocyte-macrophages (MONOs) were identified and gated separately. Histograms reporting the expression of relevant leukocyte (CD45, CD14, CD68) and endothelial markers (CD31, CD31, CD34).
KDR, CD34) are shown, together with mean percent expression from 3 replicates. The red line indicates negative control, while the blue line indicates the stained condition. B) Cells in the PACs culture were stained with the endothelial markers acLDL and Ulex Lectin. The fraction of cells that were positive for both markers were compared in cultures obtained from T2D or healthy control cells. *p<0.05 T2D vs Ctrl.

**In vitro DPP-4 activity sources and effective enzymatic inhibition.** We preliminarily tested the non-pharmacologic DPP-4 inhibitor Diprotin-A and several concentrations of the clinically available inhibitor Saxagliptin on DPP-4 activity in the conditioned medium obtained from HUVEC cultures. We found that 0.5 μM Saxagliptin was sufficient to reach maximal in vitro DPP-4 inhibition (~70%), which was equal to the degree of inhibition achieved using standard Diprotin-A concentrations (Figure 13A). The 0.5 μM Saxagliptin concentration, which has the same order of magnitude as the C_max obtained in vivo in humans after a 5 mg oral dose (0.1-0.3 μM), was chosen for subsequent experiments.

DPP-4 exists as a soluble secreted protein or a membrane-bound isoform, which also plays a role in signal transduction and adenosine metabolism (117). In cultured PACs, membrane DPP-4 (CD26) expression was restricted to a subpopulation of lymphocytes (~50% of the lymphocyte gate), whereas larger monocytic cells were CD26-negative (Figure 13B). By using PACs cultured from CD14^{pos} or CD14^{neg} cells, we compared soluble (cell-free conditioned medium) and cellular (cell extracts) DPP-4 activity and the effect of Saxagliptin. Soluble DPP-4 activity was 3-5 fold higher compared to cellular DPP-4 activity regardless of whether CD14^{pos} or CD14^{neg} cells were present in the culture, and was inhibited >50% by Saxagliptin. As expected, cellular DPP-4 activity was higher in CD14^{neg}(CD26^{+}) cells compared to CD14^{pos}(CD26^{+}) cells and it was inhibited >50% by Saxagliptin. The DPP-4 activity of CD14^{pos} cells, which do not express significant amounts of membrane CD26, can be explained by residual DPP activity provided by isoforms other than DPP-4 (e.g. DPP-8 and -9) (118), that are partly inhibited by Saxagliptin (Figure 13C). These data indicate that, in PAC cultures, soluble activity is higher than cellular activity of DPP-4, the latter being mostly attributed to lymphocytes than to monocytic cells, while both soluble and cellular DPP-4 are equally inhibited by Saxagliptin.
Figure 13. In vitro DPP-4 inhibition. A) Degrees of soluble DPP-4 inhibition in the conditioned medium by treatment of HUVECs with Diprotin-A (DPA) or different Saxagliptin concentrations, relative to the untreated control condition (CTRL). The experiment was performed in triplicate. * p<0.05 for ANOVA test. B) Expression of membrane DPP-4 (CD26) on monocytic (MONOs) and lymphocytic (LYMPHs) cells, identified by the side scattered (SSC) versus forward scatter (FSC) morphologic plot of PACs culture. C) Soluble and cellular DPP-4 activity and the extent of its inhibition of Saxagliptin were tested in PACs cultured from CD14\textsuperscript{pos} and CD14\textsuperscript{neg} preselected cells. *p<0.05 for CD14\textsuperscript{+} versus CD14\textsuperscript{−}; † p<0.05 for cellular versus soluble (n=3 replicates).
Functional effects of Saxagliptin on PACs from healthy controls and type 2 diabetic patients. We obtained PBMCs from n=20 healthy blood donors (50% males, aged 52±3) free of diabetes and cardiovascular disease, and n=20 patients with T2D (Table 2). We tested in vitro the ability of Saxagliptin 0.5 μM to modify PACs properties, such as clonogenesis and proliferation, adhesion, migration and tubulization. Given that DPP-4 inhibition is supposed to affect PACs by protecting SDF-1α (and other substrates) from enzymatic inactivation (100), we evaluated the effects of Saxagliptin with and without SDF-1α supplementation.

Gene expression. First, we determined the expression of genes related to PACs function and survival. In PACs isolated from T2D patients compared to healthy control PACs, we found significantly altered expression of BCL2, CDKN1A, VCAM1, ICAM1, ITGB2 and DPP4 (Figure 14A). Importantly, as previously noted (119), DPP4 gene expression was markedly increased in diabetic PACs, providing a rationale for pharmacologic DPP-4 inhibition. Expression of the selected genes in the entire PACs culture was unaffected by treatment with Saxagliptin and/or SDF-1α (not shown). In separated CD14⁺ monocytic PACs compared to CD14⁻ lymphocytic PACs, we found lower expression of BCL2 and higher expression of CKDN1A and VCAM1 at baseline (Figure 14B). In addition, Saxagliptin treatment increased BCL2, CDKN1A, ITGB2 and VCAM1 expression in CD14⁺ PACs, while the effect was modest or absent in CD14⁻ PACs.
Figure 14. Gene expression analysis of diabetic and healthy PACs. A) Gene expression was determined in the whole PACs population obtained from type 2 diabetic patients (T2D) and healthy subjects (Ctrl). Expression is reported relative to the housekeeping gene as $2^{\Delta \text{CT}}$. Note the logarithmic scale. *p<0.05 T2D versus Ctrl. B) Expression of a selected number of genes found to be differentially expressed in (A) was tested in CD14⁺ monocyctic and in CD14⁻ lymphocytic PACs separately. *p<0.05 in CD14⁺ vs CD14⁻ cells; †p<0.05 versus the control condition.

**Proliferation and clonogenesis.** While the culture of PACs from healthy controls yielded several rounded cell colonies in addition to interspersed cells, colonies were rarely seen during culture of PACs from T2D (Figure 15A). As expected from a PACs culture, the proliferation rate was low (3-5%). Treatment with Saxagliptin alone, but not in combination with SDF-1α, doubled the percentage of cells in the S+G2/M phases compared to the control.
condition only in healthy subjects’ PACs (Figure 15B). It has been previously reported that DPP-4 cleaves and inactivates several hematopoietic growth factors, suggesting that DPP-4 inhibition may sustain hematopoiesis (120). Saxagliptin and/or SDF-1α did not restore colony formation during PAC culture from T2D PBMCs, but tended to increase the number of colonies of PACs cultured from healthy controls. Furthermore, using the methylcellulose mouse bone marrow hematopoietic colony assay (Figure 15C), we report that treatment with Saxagliptin + SDF-1α was indeed able to increase the total number of hematopoietic colonies. A breakdown analysis showed that treatment with Saxagliptin + SDF-1α induced consistent trend increases of most colony types, especially granulocytes and macrophages (Figure 15D). These data indicate that, unlike in diabetic cells, in non diabetic cells Saxagliptin may favour proliferation, while the co-treatment with SDF-1α promotes hematopoietic clonogenesis. Differences in the proliferative response between healthy control and diabetic PACs may be related to the differential baseline expression of genes regulating cell cycle and survival, such as CDKN1A (encoding p21) and BCL2 (Figure 14). Saxagliptin and/or SDF-1α did not significantly affect the percentage of acLDL"Lectin" cells.

**Figure 15.** Effects of Saxagliptin on clonogenesis and proliferation. A) Representative microphotographs of PACs culture from type 2 diabetic patients and healthy controls exposed to Saxagliptin with or without SDF-1α supplementation. B) Analysis of cell cycle phases in cultured PACs of healthy subjects (left) and type 2 diabetic patients (right). *p<0.05 versus the unstimulated control condition (Ctrl). C) Representative microphotographs of murine hematopoietic colonies exposed to Saxagliptin with or without SDF-1α supplementation. D) Quantification of total (left) and types (right) of hematopoietic colonies in relation to treatment with Saxagliptin, with or without SDF-1α supplementation. *p<0.05 versus the unstimulated control condition (Ctrl).
Adhesion. Adhesion of PACs to mature endothelial cells represents an important step for the pro-angiogenic and vascular repairing activity of PACs. We thus tested adhesion of PACs to HUVECs in vitro and found that the number of adherent PACs was unaffected by treatment in healthy controls, while Saxagliptin significantly increased adhesion of T2D PACs in the presence of SDF-1α (Figure 16A, B). The significantly lower baseline adhesive capacity of diabetic PACs compare to control PACs and the differential response to Saxagliptin + SDF-1α may be related to the different baseline expression of adhesion molecule genes, such as ICAM1, VCAM1 and ITGB2 (Figure 14).

We used this assay to understand the relative contribution of membrane bound DPP-4 (CD26), which is expressed on CD14\textsuperscript{neg}, but not on CD14\textsuperscript{pos} PACs. Therefore, we cultured PACs from healthy controls starting from immunomagnetically purified populations of CD14\textsuperscript{pos} and CD14\textsuperscript{neg} cells. We found that Saxagliptin reduced adhesion by CD14\textsuperscript{pos}CD26\textsuperscript{neg} cells and increased adhesion by CD14\textsuperscript{neg}CD26\textsuperscript{pos} cells (Figure 16C). This indicates that inhibition of soluble DPP-4 only (as in cultures of CD14\textsuperscript{pos} cells) and inhibition of both isoforms (as in cultures of CD14\textsuperscript{neg} cells) can have opposing effects on PACs function and explains, at least in part, why Saxagliptin has modest or no overall net effect on the heterogeneous PACs culture. In addition, to better understand which is the saxagliptin-responsive cell type in the heterogeneous PACs culture, we separated CD14\textsuperscript{+} from CD14\textsuperscript{−} PACs cultured from unselected PBMCs in the presence of Saxagliptin and/or SDF-1α. We found that the adhesive capacity of CD14\textsuperscript{+} monocyteic PACs was significantly higher than that of CD14\textsuperscript{−} lymphocytic cells, and that it was enhanced by Saxagliptin with or without SDF-1α (Figure 16D).
Figure 16. Effects of Saxagliptin on adhesion of PACs. A) Representative microphotographs of red-labelled PACs adhering onto HUVEC monolayers (nuclei labelled in blue with Hoechst), using cells from type 2 diabetic patients and healthy controls exposed to Saxagliptin with or without SDF-1α supplementation. B) Quantification of adherent PACs from type 2 diabetic (T2D) and healthy (Ctrl) subjects in the untreated condition and according to the treatment with Saxagliptin, with or without SDF-1α supplementation. *p<0.05 versus the untreated control condition (Ctrl). C) Effects of Saxagliptin on cell adhesion was determined in cultures of healthy PACs obtained from pre-selected CD14+ and CD14− cells. *p<0.05 versus the untreated control condition (Ctrl). D) Baseline adhesion and effects of Saxagliptin +/- SDF-1α was tested in separated CD14+ and CD14− PACs, after cells were cultured from unselected PBMCs. *p<0.05 in CD14+ vs CD14− cells; †p<0.05 versus the control condition.

Migration. Migration of PACs was assessed using a transwell assay, with the chemokine SDF-1α being the prototypical positive control (98). We found that the percentage of migration was increased by SDF-1α using both healthy and diabetic PACs, whereas Saxagliptin alone or in combination with SDF-1α did not affect migration of both control and
diabetic PACs (Figure 17). It should be noted that the absolute numbers of migrating cells in this system was low, thus limiting the chance of reporting small effects as significant.

Figure 17. Migration of PACs cultured from type 2 diabetic patients (A) and healthy controls (B) in the presence and in the absence of Saxagliptin and SDF-1α was assessed using a transwell system. *p<0.05 versus the untreated control condition (Ctrl).

Tube formation. Tubulization by HUVECs co-cultured with PACs was assessed using the 2D Matrigel assay (Figure 18A). It is well known that PACs do not spontaneously form tubules in 2D Matrigel, but can sustain tubulogenesis by mature endothelial cells (57). We found that only T2D PACs treated with Saxagliptin + SDF-1α were able to increase tube length formation by HUVECs, while there was no effect on healthy PACs (Figure 18B-C). The number of branching points was not significantly different in T2D compared to controls and was unaffected by treatments (not shown). In addition, we evaluated the tube supportive capacity of CD14⁺ PACs versus CD14⁻ PACs and how they are affected by treatments. We found that HUVECs co-cultured with CD14⁺ monocytic PACs showed increased tube formation compared to co-culture with CD14⁻ lymphocytic PACs. Moreover, tube formation by HUVECs/CD14⁺ PACs was significantly increased by Saxagliptin treatment compared to the control condition (Figure 18D). Most of labelled PACs co-cultured with HUVECs remained located in cellular islands at tube intersections, while only some contributed physically to tube formation. No difference was seen in the percentages of tubes bearing integrated CD14⁺ (15±4%) versus CD14⁻ PACs (12±2%; p=0.41) (Figure 18E).

Finally, as the tube assay is strictly dependent on the cooperation between HUVECs and PACs, we explored the effect of Saxagliptin on in vitro angiogenesis assays of isolated HUVECs. We found that, using both the 2D Matrigel tubulisation assay and the spheroid
sprouting assay, Saxagliptin modestly but significantly reduced *in vitro* angiogenesis by mature endothelial cells (Figure 19A, B).

**Figure 18.** Effects of Saxagliptin on migration and tubulisation of PACs. A) Representative microphotographs of tube formation in 2D Matrigel by HUVECs co-cultured with PACs from type 2 diabetic patients and healthy controls exposed to Saxagliptin with or without SDF-1α supplementation. B-C) Quantification of total tube length formation by HUVECs alone and HUVECs co-incubated with PACs either untreated (Ctrl) or treated with Saxagliptin and/or SDF-1α. Panel B shows data obtained with PACs from type 2 diabetic patients, while panel C shows data obtained with healthy PACs. *p<0.05 versus HUVECs alone, set at 1.0. D) Quantification of total tube length formation by HUVECs alone and HUVECs co-incubated with CD14⁺ or CD14⁻ PACs from healthy controls. *p<0.05 in CD14⁺ vs CD14⁻ cells; †p<0.05 versus the control condition. E) Red-labelled PACs co-cultured with HUVECs in the tube forming assay were imaged under an inverted fluorescence microscioe to detect their spatial localization and quantify tubes with integrated CD14⁺ and CD14⁻ PACs.
In vivo effect of Saxagliptin on angiogenesis induced by circulating cells. To assess the relevance of the pro-angiogenic effects of Saxagliptin on circulating PACs compared to the possible anti-angiogenic effects on mature ECs, we used *in vivo* Matrigel assays. First, PBMCs from T2D treated with Saxagliptin (n=5) and PBMCs from T2D patients on non-incretinergic therapy (n=5; see Table 2) were embedded into Matrigel plugs and implanted subcutaneously into immunodeficient mice. Angiogenesis in this model is mediated by the cooperation between the intraplug cells (the circulating source of PACs) and mouse ECs invading the plug. We found a non-significant increase in vascular plug invasion and hemoglobin content (a surrogate of plug vascularization) in plugs containing PBMC from Saxagliptin-treated compared to those from untreated diabetic patients (Figure 19C). To further address this issue, we used the Directed In Vivo Angiogenesis Assay (DIVAA), which allows a more reproducible quantification of *in vivo* angiogenesis. DIVAA angioreactor tubes were implanted with cells obtained from saxagliptin-treated and non incretin drug-treated T2D patients, with or without growth factors (GF) that stimulate angiogenesis. During the course of the assay, implant grade silicone cylinders closed at one end, called angioreactors, are filled with 20 µl of Trevigen's basement membrane extract (BME) premixed with or without angiogenesis modulating factors. These angioreactors are then implanted subcutaneously in the dorsal flanks of nude mice. If filled with angiogenic factors, vascular endothelial cells migrate into, and proliferate in the BME to form vessels in the angioreactor. As early as nine days post-implantation, there are enough cells to determine an effective dose response to angiogenic factors. We did not find significant differences in the Lectin content (a proxy of perfusion in DIVAA experiments) in tubes containing cells from saxagliptin-treated patients versus controls in either the presence or absence of GF. However, the difference between Lectin content in tubes with and without GF, indicative of the ability of GF to increase PBMC-mediated angiogenesis in each patient, was significantly higher (p=0.03) for saxagliptin-treated patients compared to controls (Figure 19D).
Figure 19. Effects of Saxagliptin on angiogenesis. A) The effect of Saxagliptin on tube formation by HUVECs in 2D Matrigel was assessed. Total tube length as well as average tube length are reported. *p<0.05 (n=3 replicates) for Saxagliptin-treated versus the untreated control condition (Ctrl). B) The effect of Saxagliptin on sprouts formation by spheroids of HUVECs was assessed. Total sprouts length as well as the number of sprouts are reported. *p<0.05 (n=3 replicates) for Saxagliptin-treated versus the untreated control condition (Ctrl). C) In vivo angiogenesis in Matrigel plugs by PBMCs isolated from Saxagliptin-treated and non-incretinergic drug treated type 2 diabetic patients (n=5/group). Representative figures, as well as quantification of Hb content are shown. D) PBMC from Saxagliptin-treated and non-incretinergic drug treated type 2 diabetic patients (n=5/group) were also used for the Directed In Vivo Angiogenesis Assay (DIVAA) using angioreactor tubes with (+) and without (-) growth factors (GF). After visual inspection, Lectin content was determined as an indicator of vascular invasion of the tube. Lectin content quantification in tubes without and with GF, as well as the difference between the two are shown. *p<0.05 Saxa versus Ctrl.
In the present study, we show that DPP-4 inhibition with Saxagliptin reverses PACs dysfunction associated with T2D *in vitro* and improves inducible angiogenesis by patients’ cells *in vivo*. We have previously shown that DPP-4 inhibition increases circulating EPCs in a similar population of T2D subjects (100). Herein, we add to those findings showing that, not only the level, but also the function of vascular protective cells can be improved by DPP-4 inhibition.

PACs derived from PBMC cultures differ from EPCs quantified *ex vivo* by flow cytometry, as they are composed of a heterogenous population of angiogenic T cells and monocyte-macrophages, plus a small population of progenitor cells (57). The pro-angiogenic and vascular repair capacity of human PACs has been consistently demonstrated in pre-clinical studies (121; 122). In addition, autologous administration of bone marrow derived PACs was shown to improve left ventricular ejection fraction in patients with acute myocardial infarction (123), confirming the protective cardiovascular effects of these cells in humans. The mechanisms whereby PACs achieve vascular protection *in vivo* are not entirely clear, but data suggest that they are mainly derived from an intense paracrine activity, rather than definite endothelial differentiation and integration (77). Indeed, although a significant ontologic overlap between the endothelium and hematopoietic cells exists in the embryo (124), epigenetic brakes prevent blood-derived PACs to differentiate into mature endothelium in adulthood (125). The heterogeneous composition of PACs culture could be seen as a limit to the interpretation of the present findings. However, it should be noted that late EPCs and ECFC can be only stochastically isolated from peripheral blood of diseased subjects (57), thus limiting reproducibility of the findings. Whether PACs mainly represent an *in vitro* artefact or they also exist *in vivo* is a matter of debate, although the discovery of the so-called haemogenic endothelium suggest that endothelial-hematopoietic overlaps can occur also in adulthood (124). Hemogenic endothelial cells are rare, differentiated vascular endothelial cells that generate hematopoietic (blood) cells during embryogenesis. The main function of hemogenic endothelium is to generate definitive hematopoietic precursors and stem cells (126).

Several authors have reported that diabetes induces PACs dysfunction, through epigenetic changes (127), eNOS modulation (128), and humoral factors (129). We herein confirm that PACs isolated from T2D have impaired differentiation, clonogenesis and adhesion compared to PACs isolated from healthy controls. This was associated with changes in the expression of genes related to adhesion and regulation of cell cycle. Such differences, however, cannot be directly attributed to diabetes per se, because T2D patients were also older than controls
and had additional cardiovascular risk factors. The rationale for including healthy controls instead of matched non-diabetic patients was to assess the effects of Saxagliptin within each group and understand whether DPP-4 inhibition influences both diseased and healthy PACs. Interestingly, we found that, with the exception of proliferation, only PACs from T2D patients improved their function after treatment with Saxagliptin. This can be attributed to the marked upregulation of DPP4 gene expression in diabetic PACs. We have previously shown that DPP-4 activity is increased in serum/plasma of T2D compared to non-diabetic patients and is not directly related to glucose control (119). DPP-4 exists as either a soluble or membrane bound (cellular, CD26) isoform and the relative contribution of the two to the total DPP-4 activity and its biological effects were previously unknown. We show that soluble DPP-4 activity is higher than cellular DPP-4 activity, which is restricted to a lymphocyte subpopulation. This is particularly important in cultures of PACs, which are composed of angiogenic T cells and endothelial-like monocyte-macrophages. Indeed, we found that, though Saxagliptin did not significantly affect adhesion of healthy PACs, when PACs were isolated starting from CD14{sup}+{sub}(CD26/DPP-4{sup}-) cells or CD14{sup}- (CD26/DPP-4{sup}+) cells, the effects of Saxagliptin on adhesion were opposite. In addition, among the heterogeneous PACs population, CD14{sup}+ monocytic PACs compared to CD14{sup}- lymphocytic PACs showed higher functionality at baseline and were much more responsive to Saxagliptin-induced gene expression changes and stimulation of adhesion and tube supporting capacity. While such differences can be the result of cell-type specific responses to Saxagliptin, it is possible that cellular DPP-4 inhibition exerts different effects compared to soluble DPP-4 inhibition. While membrane bound DPP-4 may transduce intracellular signals and is a cofactor of adenosine deaminase (117), secreted DPP-4 is supposed to act mainly through cleavage of soluble mediators. Indeed, we found that Saxagliptin improved whole PACs function only in the presence of SDF-1α supplementation, whereas Saxagliptin alone or SDF-1α alone less effective. Based on the low SDF-1α concentrations in the medium (fg/ml) compared to the high DPP-4 expression / activity, it is rationale that only simultaneous SDF-1α supplementation and DPP-4 inhibition provided significant biological effects, a finding that supports the mechanistic theory whereby DPP-4i affects PACs by protecting SDF-1α (and possibly other factors) from enzymatic degradation. In addition to improving PACs function, DPP-4 inhibition also mobilizes EPCs via SDF-1α (100). This synergistic effect is expected to promote favourable outcomes in several diabetic complications (130), including wound healing (131; 132).

Fadini et al found [puoi dire “our group previously found”] a negative correlation between DPP-4 activity and plasma SDF-1α concentrations. Plasma DPP-4 activity was directly correlated to the BM/PB ratio of CD34{sup}+ cells, supporting the hypothesis that DPP-4 regulates CD34{sup}+ cell mobilization. They first analyzed DPP-4 activity in rats and found increased
activity in PB and decreased activity in BM of diabetic compared to non-diabetic rats. Then, they tested the bone marrow progenitor cell mobilization in wild type and DPP-4 deficient rats after either G-CSF stimulation or hind limb ischemia. This study, by combining a clinical data-driven discovery approach with animal models, shows that altered mobilization of stem/progenitor cells is associated with a high cardiovascular risk profile and, in the setting of diabetes, is driven by a tissue-specific DPP-4 dysregulation (118).

In contrast to the positive effects exerted by Saxagliptin + SDF-1α on T2D PACs, Saxagliptin +/- SDF-1α did not affect function of healthy control PACs when evaluated in the entire population, but differentially affected function of CD14⁺ monocyctic versus CD14⁻ lymphocytic cells. Saxagliptin also reduced angiogenesis by mature endothelial cells in vitro, suggesting cell-type specific effects. Therefore, to understand the overall net effects of Saxagliptin on angiogenesis in vivo, we used PBMCs isolated from Saxagliptin-treated and from control T2D patients treated with non-incretinergic drug. The Matrigel plug assay showed non-significantly higher perfusion obtained with Saxagliptin-treated compared to control cells. As this assay has wide variability depending on how the plug develops and is adsorbed in the mouse, we also used the more reliable and quantitative Directed In Vivo Angiogenesis Assay (DIVAA™). DIVAA is the first in vivo system for the study of angiogenesis that provides quantitative and reproducible results (133). The sleek design of the angioreactor provides a standardized platform for reproducible and quantifiable in vivo angiogenesis assays. Compared to the plug assay, the angioreactor prevents assay errors due to absorption of BME by the mouse. In addition, the angioreactor uses only a fraction of the materials conserving both BME and test compounds used, and up to four angioreactors may be implanted in each mouse, giving more data for analysis.

We found that the extent to which growth factors (VEGF + FGF) increased vascular invasion of the angioreactor was significantly higher for Saxagliptin-treated compared to control cells. This suggests that Saxagliptin increases the ability of circulating cells to respond to pro-angiogenic growth factors, possibly protecting them from enzymatic degradation.
CONCLUSIONS

Reversal of T2D PACs dysfunction and stimulation of inducible angiogenesis may translate in a microvascular (134) and cardiovascular protective activity of DPP-4i. While pooled data from short-term phase III randomized clinical trials in selected T2D patients showed potential cardiovascular benefit of Saxagliptin (135), the Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus (SAVOR) clinical trial, conducted on >16,000 T2D patients with a history or risk factors for cardiovascular events, showed a neutral effect of Saxagliptin on the rate of ischemic events (136). However, this event-driven study was terminated after just a median follow-up of 2.1 years, thus limiting the chance that protective effects of Saxagliptin translated into an event rate reduction. So far, experimental pre-clinical and clinical findings widely argue for potential cardiovascular protection by DPP-4 inhibition. The observation that Saxagliptin restores the function of PACs from T2D patients represents an additional step toward a better understanding of the pathobiology of DPP-4 in diabetes. PACs function can be restored by glucose normalization, as shown in islet-transplanted type 1 diabetic patients (137). Therefore, Saxagliptin may also impact PACs pro-angiogenic activity by improving glucose control (138). As HbA1c was only slightly and not significantly lower in Saxagliptin-treated patients compared to patients on non-incretinergic therapies, pleiotropic extraglycemic effects are likely implicated.
REFERENCES


67. !!! INVALID CITATION !!!


Table 1. Primer sequences for real time PCR analysis.

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Table 2. Characteristics of type 2 diabetic patients included in the study. Data are presented as mean ± standard error, or as percentage, where appropriate. There were no significant differences between the two groups of the in vivo study.

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<td>Number</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Age, years</td>
<td>65.2±2.3</td>
<td>67.4±2.6</td>
</tr>
<tr>
<td>Sex male, %</td>
<td>65.0</td>
<td>80.0</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>30.3±2.1</td>
<td>29.1±1.9</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>104.8±3.9</td>
<td>102.8±4.0</td>
</tr>
<tr>
<td>Diabetes duration, years</td>
<td>11.3±4.2</td>
<td>9.8±3.6</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl (mmol/l)</td>
<td>166.2±8.1</td>
<td>141.2±6.7</td>
</tr>
<tr>
<td></td>
<td>(9.2±0.5)</td>
<td>(7.8±0.4)</td>
</tr>
<tr>
<td>HbA1c, % (mmol/mol)</td>
<td>8.4±1.1</td>
<td>7.6±1.6</td>
</tr>
<tr>
<td></td>
<td>(68.3±9.0)</td>
<td>(59.6±12.5)</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>75.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>138.1±3.2</td>
<td>137.4±4.2</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>86.7±2.7</td>
<td>82.4±3.8</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl (mmol/l)</td>
<td>188.2±5.1</td>
<td>172.5±4.9</td>
</tr>
<tr>
<td></td>
<td>(4.9±0.1)</td>
<td>(4.4±0.1)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl (mmol/l)</td>
<td>46.6±2.2</td>
<td>48.2±1.1</td>
</tr>
<tr>
<td></td>
<td>(1.2±0.1)</td>
<td>(1.2±0.02)</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl (mmol/l)</td>
<td>98.1±4.9</td>
<td>81.2±3.2</td>
</tr>
<tr>
<td></td>
<td>(2.5±0.1)</td>
<td>(2.1±0.1)</td>
</tr>
<tr>
<td>Triglycerides, mg/dl (mmol/l)</td>
<td>149.3±12.4</td>
<td>162.1±14.8</td>
</tr>
<tr>
<td></td>
<td>(1.7±0.1)</td>
<td>(1.8±0.2)</td>
</tr>
<tr>
<td>Smoke, %</td>
<td>15.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Complications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease, %</td>
<td>25.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Peripheral vascular disease, %</td>
<td>20.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Cerebrovascular disease, %</td>
<td>40.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Retinopathy, %</td>
<td>20.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Nephropathy, %</td>
<td>25.0</td>
<td>0.0</td>
</tr>
<tr>
<td>eGFR&lt;60 ml/min/1.73 mq</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin therapy, %</td>
<td>50.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Oral therapy, %</td>
<td>45.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Diet alone, %</td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ACE inhibitors / ARBs, %</td>
<td>80.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Statin, %</td>
<td>65.0</td>
<td>60.0</td>
</tr>
</tbody>
</table>