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$CD34^+$ stem cells and epigenetic memory: key players and pharmacological targets in diabetic cardiovascular complication.

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Summary

Abstract

Diabetes mellitus (DM) is a chronic multifactorial disease characterized by accelerated rates of inflammation and multiple vascular complications, whose associated cardiovascular (CV) complications represents the leading cause of morbidity and mortality in type 1 or type 2 (T2DM) patients. Bone marrow (BM) derived-$CD34^+$ stem cell population, which identifies both hematopoietic precursors and putative endothelial progenitor cells, plays a key role in maintaining vascular endothelium homeostasis and regeneration. To date, $CD34^+$ stem cells depletion is considered to be involved in the impairment of CV homeostasis in DM. Besides the reduction in number and function of $CD34^+$ stem cells with pro-angiogenic properties, hyperglycemia promotes alterations in the innate immune system elements including abnormal elevation of inflammatory monocyte subpopulation and alteration in macrophage polarization in DM patients. Since inflammation is leading cause in atherosclerosis, this unbalance could be at the basis of the increased risk of developing cardiovascular disease (CVD) in T2DM patients. Emerging evidence suggests that this alteration could be the result of hyperglycemia-induced pre-programming at BM level. Epigenetic modifications may induce long-term changes in gene function and metabolism. The involvement of these mechanisms in the diabetic pre-programming of BM stem cells would explain the perpetuation of cell dysfunction in their progeny despite a good glycemic control, a phenomenon known as “hyperglycemic memory”.

Recently, a new class of glucose lowering drugs, the agonists of glucagon-like peptide 1 receptor (GLP-1R), which includes liraglutide (LIRA), have displayed the ability to reduce the risk of major CV events and mortality as well as atheroprotective and anti-inflammatory effects. This pleiotropic CV benefit appears to be above and beyond the effects on glycaemia and the mechanisms whereby LIRA exerts such striking CV protective effects are still largely unknown.

In the light of these data, we hypothesized that hyperglycemia could epigenetically induce, at BM level, $CD34^+$ stem cell alterations resulting in blood stream release of pro-angiogenic cells with defective functionality and myeloid cell populations characterized by more aggressive phenotype. Moreover, we propose that LIRA may exert CV protective effects and reduce the systemic and chronic pro-inflammatory status of the patients through improvement of $CD34^+$ stem cell function and rebalance of circulating inflammatory progenitor cells.

Herein, by *in vitro* model of DM based on umbilical cord blood (UCB)-derived $CD34^+$ stem cells, we demonstrated that the CXCR4/SDF-1α axis impairment induced by HG exposure was related to epigenetic changes at level of CXCR4 promoter that persisted despite the recovery into normal glucose (NG). Notably, these data were confirmed in BM-derived $CD34^+$ stem cells

of T2DM patients undergoing cardiac surgery for coronary artery disease (CAD). In addition, we showed that HG-$CD34^+$ stem cells displayed a senescent-associated secretory phenotype (SASP) characterized by epigenetic upregulation and activation of NFkB-p65 transcription factor and release of inflammatory cytokines (TNFα, IL6). Interestingly, these cells, once differentiated into myeloid lineage, generated higher level of pro-inflammatory intermediate ($CD14^{++}CD16^+$) monocytes when compared to NG cells. Importantly, BM-$CD34^+$ stem cells from CAD-DM patients also displayed a senescent/inflammatory phenotype and increased intermediate monocytes generation when compared with cells from CAD patients without T2DM. Finally, LIRA treatment showed to prevent the functional impairment of HG-$CD34^+$ stem cells as well as the upregulation and nuclear translocation of NFkB-p65 and abnormal myeloid differentiation.

In conclusion, our *in vitro* and *vivo* data show that $CD34^+$ stem cells can "memorize" the hyperglycemic environment in the form of epigenetic modifications that collude to alter their functionality and their myeloid differentiation towards inflammatory monocyte subpopulations. Finally, the results suggest that part of favorable effect of LIRA on CV system are exerted by improvement $CD34^+$ stem cell function and its immunomodulatory properties.

Introduction

1. Diabetes Mellitus

1.1 - Definition and Epidemiology

Ancient Egyptian firstly described the symptoms of diabetes mellitus 3000 years ago, but the term "diabetes" was then coined by Araetus of Cappodocia (81-133AD) and derives from the Greek verb *diabaino* (διά-βαίνω) that means "to flow through", referring to the frequent polydipsia and polyuria observed in diabetic patients [1]. The adjective "mellitus", from the Latin *mel* "honey, sweet", was added later by Thomas Willis indicating the sweet taste of urine typical of this disease [1]. To date, as defined by the World Health Organization (WHO), "diabetes mellitus" (DM) describes heterogeneous disturbances of metabolism with multiple aetiology characterized mainly by chronic hyperglycemia, with alteration of carbohydrates, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [2].

Numerous pathogenic processes are involved in the development of DM, including abnormalities that develop in resistance to insulin action and processes that destroy the β-cells of patients with consequent insulin deficiency. Reduced insulin tissue response and/or reduced secretion derive from the impairment of one or more points in the complex pathways of hormone action and these processes can contribute, alone or together, to the establishment of hyperglycemia in diabetic patients [2, 3].

Chronic hyperglycemia is associated with long-term damage, dysfunction and failure of different organs resulting in reduced life expectancy [3]. Specifically, hyperglycemia gives rise to a risk of both microvascular damage, which leads to retinopathy, nephropathy, neuropathy, and macrovascular complications (i.e. ischemic heart disease, stroke and peripheral vascular disease) [4].

Characteristic symptoms of DM are thirst, polynuria, polydipsia, polyphagia, blurring of vision and weight loss; however, their severity is due to the type and duration of the disease. In most severe forms, uncontrolled DM can establish ketoacidosis or a non-ketotic hyperosmolar state, which may lead to stupor, coma and death in absence of treatment [5]. Otherwise, patients with type 2 DM may be asymptomatic in the early years of the disease and, consequently, hyperglycemia may be present for a long time before the diagnosis causing pathological and functional changes.

According to WHO study, the global prevalence of DM was estimated to be 2,8% (171 million) in 2000 and was projected to reach 4,4% in 2030, meaning 366 millions of all-age group people worldwide [3]. However, these numbers are increasing due to population growth, aging, urbanization, increasing prevalence of obesity and physical inactivity. Consistently, the

International Diabetes Federation reported that approximately 463 million adults (20-79 years) worldwide were affected by DM in 2019 and expects the number to rise to 700 million in 2045 [6]. Moreover, health care costs for DM were at least $ 760 billion in 2019 and DM is one of the main causes of death in developed countries, remaining one of the most challenging health problems of the 21st century [6].

1.2 - Classification

From the first widely accepted classification of DM published by WHO in 1980, the classification criteria have undergone changes and redefinition over time [2]. Patients' classification has important implications for the treatment strategies. However, it is not always an easy issue to classify patients, since they often do not fit into a single class. To date, the most accepted classification has been proposed by the American Diabetes Association (ADA) in 1997 and provides four classes: type 1, type 2, other types and gestational diabetes mellitus (GDM) [7].

Type 1 Diabetes Mellitus (T1DM)

This type of diabetes constitutes 5%-10% of the subjects diagnosed with diabetes, 80%-90% of whom are children and adolescents. Previously indicated as insulin-dependent diabetes or juvenile onset diabetes, T1DM is due to the autoimmune destruction of β-cells of the pancreas, usually leading to absolute insulin deficiency [5]. Autoantibodies against the pancreatic islet cells are the hallmark of T1DM and can be detected in the serum even months or years before the onset of the disease. Other autoantibodies that can be present in T1DM patients are autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD, GAD65), protein tyrosine phosphatase (IA2 and IA2β) and zinc transporter protein (ZnT8A) [3, 5]. Although immune-mediated DM commonly occurs in childhood and adolescence, it can rise up at any age. Latent autoimmune diabetes in adults (LADA) is typically diagnosed after 35 years of age and accounts for 2%-12% of all cases of diabetes. These patients, presenting type 2 Diabetes Mellitus (T2DM) phenotype, are often misdiagnosed as T2DM and initially treated with oral hypoglycemic agents, but they eventually become insulin-dependent more rapidly than T2DM patients and have circulating β-cell autoantibodies [8].

The genetic predisposition to T1DM has been long investigated and the first genetic association that contribute to T1DM risk was observed for the human leukocyte antigen (HLA) region. The HLA genes, which encoded for classical HLA class I (A, B and C) and class II (DR, DQ and DO) antigens, are involved in the immune response and, to date, the primary HLA association

has been linked with class II genes, in particular with those encoding for the DR and DQ molecules [9]. Cornerwise, many DR-DQ haplotypes have a protective role, being negatively associated with T1DM risk. However, the exact biological mechanism of HLA-conferred susceptibility is still a matter of debate.

Some forms of T1DM have no known etiologies and are so called *idiopathic diabetes*. These rare forms are not due to autoimmunity, are not HLA-associated and display almost complete insulin deficiency [3, 5].

Type 2 Diabetes Mellitus (T2DM)

This form of DM is the prevalent one and accounts for 90–95%. Most of these patients are adults, despite the recent increased incidence in youth mainly due to the change in the lifestyle with more sedentary life and less healthy food [10]. Albeit the specific etiologies are not known and there are probably many different causes of this form of DM, it is note that obesity is the major reason behind insulin resistance that is mainly responsible for T2DM. Specifically, T2DM can range from predominant insulin resistance state with relative insulin deficiency (rather than absolute) to prevailing defective secretion with insulin resistance [3, 5]. Insulin resistance occurs when cells in insulin-target tissues no longer respond properly to the hormone insulin. This condition leads to an increased demand for insulin in those tissues; however, defects in β-cells function could prevent their response [11]. Moreover, insulin secretion may decrease with the increased demand for insulin in the course of time due to the gradual destruction of β-cells that could transform some of T2DM patients from being independent to become dependent on insulin [5]. However, at least initially, managing hyperglycemia through body-weight reduction and/or pharmacological treatment could improve insulin resistance [3]. Many comorbidities have been associated to insulin resistance and DM, including obesity, nephropathy, essential hypertension, dyslipidemia and systemic inflammation, defining a condition that have been termed metabolic syndrome [5].

Despite T2DM presents familial predisposition suggesting the involvement of genetic predisposition, the genetics of this form are complex and not yet clearly defined [3].

Especially since the beginning T2DM symptoms are mild, the diagnosis can be delayed for years so to increase a risk of developing long-term macrovascular and microvascular complications.

Other Types of Diabetes Mellitus

These types of DM have different etiologies and include:

- *Genetic Defects of the β-Cell:* characterized by monogenetic defects in β-cell function. Often the hyperglycemia starts at early age classifying it as neonatal diabetes, if it occurs before the age of six months, or Maturity Onset Diabetes of the Young (MODY), before the age of 25 years. They are inherited in an autosomal dominant pattern and, to date, they have been associated to abnormalities at six genetic loci on different chromosomes.
- *Genetic Defects in Insulin Action:* determined by mutations of the insulin receptor leading to metabolic abnormalities, this syndrome was termed type A insulin resistance.
- *Other Genetic Syndromes Sometimes Associated With Diabetes:* genetic syndromes such as Down syndrome, Klinefelter syndrome and Turner syndrome.
- *Diseases of the Exocrine Pancreas:* induced by trauma, infection, pancreatitis, pancreatectomy and pancreatic carcinoma which injures the pancreas causing diabetes.
- *Endocrinopathies:* diseases which present excess amounts of hormones that antagonize insulin action (e.g. acromegaly, Cushing syndrome and glucagonoma) can cause diabetes.
- *Drug- or Chemical-Induced Diabetes:* some drugs can impair insulin secretion, destroy pancreatic β-cells or impair insulin action [3].

Gestational Diabetes Mellitus (GDM)

This type of DM has been defined as an altered glucose tolerance with onset or first recognition during pregnancy. Since the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy, GDM is defined to appear in the II-III trimester of pregnancy, whereas high-risk women found to have DM at their initial pre-natal visit do not receive a diagnosis of GDM according to International Association of the Diabetes and Pregnancy Study Groups deliberations. Although most cases resolve with delivery, it represents an important risk factor for the pregnant woman and the fetus [3].

1.3- Glucose metabolism

Glucose is the major source of energy required for organs to function and can be made available in circulation following intestinal adsorption after food intake, glycogenolysis (the breakdown of glycogen into glucose) or gluconeogenesis (the glucose formation from non-carbohydrate substrate). In normal physiological conditions, the regulation of plasma glucose concentration results from the fine balance between glucose entering and removal from the circulation, maintaining blood concentration in a relatively controlled range during fasting and feeding states to adequately supply the metabolic demands. These processes are driven by the main glucose regulatory hormones, including insulin, glucagon, amylin, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) [12].

Insulin is a small protein composed of two polypeptide chains, A- and B-chain, linked by disulfide bonds. It is synthesized by pancreatic β-cells as preproinsulin, a continuous single polypeptide consisting of an N-terminal signal peptide plus A- and B-chain linked by a C-peptide. The signal peptide allows the translocation of the preproinsulin to the rough endoplasmic reticulum, where it is cleaved and the protein is folded to form proinsulin. The final active conformation is then obtained after the cleavage of the C-peptide and insulin is finally stored into secretory vesicles. Insulin is secreted in response to the increased glucose-blood concentration after meals. Glucose through the insulin-independent transporter GLUT2 enters the β-cells, where it is phosphorylated to G6P by the low-affinity hexokinase glucokinase leading to the increase of the intracellular ATP/ADP ratio and to the closure of the ATP-sensitive K^+ channel. This results in depolarization of the β-cell membrane, which opens voltage-gated L-type Ca^{++} channels. Increased intracellular Ca^{++} concentration activates microtubule-mediated exocytosis of insulin-containing secretory granules. In the postprandial phase, insulin is, at first, rapidly released and, subsequently, its synthesis is increased in response to blood glucose. Insulin exerts its biological action by binding to a specific receptor on the surface of its target cells and activating a complex downstream pathway. The insulin receptor consists of two extracellular α subunits and two transmembrane β subunits that are disulfide linked into a $\alpha_2\beta_2$ heterotetrameric complex. After insulin binding to the extracellular α subunits, the receptor dimerizes and undergoes autophosphorylation, thereby triggering a series of intracellular transphosphorylation reactions on different proximal substrates, such as members of the insulin receptor substrate family (IRS1/2/3/4) [13]. Tyrosine phosphorylation of the IRS proteins interacts with effector molecules containing the Src homology2 (SH2) domains (i.e. phosphatidylinositol 3-kinase). These mechanisms lead to several important effects, including the membrane translocation of insulin-sensitive glucose transporters (GLUT-

4) [13] that increase glucose uptake by insulin-sensitive tissue (i.e. skeletal muscle, adipose tissue and liver). Simultaneously, insulin promotes glycogenesis by the liver and inhibits glucagon secretion from the pancreatic α-cells and, together, these actions lead to the reduction of blood glucose concentration following the fed state. Besides glucose, other factors that can stimulate insulin secretion are the incretin hormones (GLP-1 and GIP), the increased plasma concentration of some amino acids and the parasympathetic stimulation. Normal fasting blood glucose concentration in humans is 80 to 90 mg per 100 ml and fasting states are associated with very low levels of insulin secretion [12, 14].

Amylin, or islet amyloid polypeptide (IAPP), is a neuroendocrine hormone consisting in a 37-amino acids peptide that is stored and secreted by β-cells together with insulin. Indeed, it has a secretory and concentration profile similar to insulin. Amylin suppresses post-prandial glucagon secretion and slows gastric emptying exerting its action mainly through the central nervous system [15].

Glucagon is a main catabolic hormone secreted by α-cells to sustain plasma glucose during the fasting state. It acts primarily on the liver stimulating hepatic glucose production through glycogenolysis and gluconeogenesis while inhibiting glycogenesis and glycolysis. In the postprandial phase, glucagon secretion is suppressed. In DM patients the reduced suppression of glucagon secretion leads to an elevated hepatic glucose production playing a primary role in the establishment of hyperglycemia [16].

Incretin hormones include several peptides released from the gut in response to food ingestion. The two main hormones in the glucose regulation homeostasis are GLP-1 and GIP and both of them stimulate insulin secretion in a glucose-dependent manner. GIP is synthesized by enteroendocrine K-cells and, besides its insulinotropic activity, it exerts several actions, including promotion of growth and survival of the pancreatic β-cell and stimulation of adipogenesis [17]. GLP-1 is produced by enteroendocrine L-cells as a part of the proglucagon polypeptide and released, following tissue specific post-translational processing, in two forms: GLP-1-(7-37) and GLP-1-(7-36)NH(2), both binding to a specific receptor (GLP-1R) on pancreatic α-cells. Besides stimulating insulin secretion, GLP-1 inhibits pancreatic glucagon secretion and slows gastric emptying. It has a plasma life of 2 minutes being rapidly degraded by dipeptidyl peptidase-4 (DPP-4) [18].

1.4- Diagnosis and Therapies

Diagnostic criteria have been reviewed over time and, to date, the diagnosis of DM is based on the measurement of random plasma glucose, fasting plasma glucose (FPG), oral glucose tolerance test (OGTT) and on the evaluation of hemoglobin A1c (Hb1Ac) [5].

According to "Italian standards for the treatment of diabetes mellitus 2018" approved by Associazione Medici Diabetologi and Società Italiana di Diabetologia, the diagnosis of DM, in the presence of typical symptoms of the disease (polyuria, polydipsia and weight loss), is made with the finding of random blood glucose ≥ 200 mg / dl (regardless of food intake), even on one occasion [19]. Conversely, in the absence of the typical symptoms of the disease, the diagnosis must be made with the confirmation, on at least two occasions, of:

- FPG ≥ 126 mg/dL (the blood sample should be taken at 8.00 in the morning with at least 8 hours of fasting) [20];
- Glycemia ≥ 200 mg/dl 2 hours after oral glucose loading, performed with 75 g of anhydrous glucose (OGTT);
- HbA1c ≥ 6.5% (48 mmol/mol). Hemoglobin A1c represents a small percentage of total hemoglobin able to bind glucose depending on its plasma concentration. The levels of HbA1c are not influenced by daily fluctuations in the blood glucose concentration but reflect the average glucose levels over the prior 6 to 8 weeks. However, IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) standardization of results is required for this test and it is important to consider that values can be influenced by various factors.

Moreover, there are values of these glycemic parameters that identify subjects at risk of DM and CVD:

- FPG 100-125 mg/dL (Impaired Fasting Glucose, IFG);
- Blood glucose 2 hours after oral glucose loading 140-199 mg / dL (reduced glucose tolerance or Impaired Glucose Tolerance, IGT);
- HbA1c 6.00-6.49% (42-48 mmol/mol) (only with IFCC aligned dosage).

In subjects with IFG and / or IGT or Hb1A with values of 42-48 mmol / mol (6.00-6.49%), the presence of other risk factors for diabetes (e.g. obesity, familiarity for diabetes) must be assessed in order to program an intervention to reduce the risk of DM onset. Furthermore, in these subjects it is also appropriate to look for the presence of any other CV risk factors (e.g. dyslipidaemia, hypertension) to define the overall CV risk and establish the appropriate therapeutic measures [19].

The major goal of DM treatment is to control blood glucose levels, preventing complications of the disease. An interprofessional approach, involving both lifestyle modifications with diet and exercise and pharmacologic therapies, is needed to achieve optimal glycemic control [21]. T1DM is typically managed with insulin as well as dietary changes and exercise, whereas T2DM may require at first dietary changes and weight reduction. When these measures fail to control the elevated blood sugar, oral or injected hypoglycemic medications are prescribed and finally, if those medications become ineffective, treatment with insulin may be required. Overall, good lifestyle modification needs always to be combined to pharmacological therapies. Pharmacological treatments for T2DM have multiplied in line with the new understanding of the pathophysiologic defects underlying the disease and they are designed to target multiple defects, such as decreased insulin secretion, decreased incretin effect, increased lipolysis, increased glucose reabsorption, decreased glucose uptake, neurotransmitter dysfunction, increased hepatic glucose production and increased glucagon secretion [21, 22]. Therapeutic noninsulin glucose lowering agents include:

- *Biguanides* (Metformin): increase hepatic adenosine monophosphate-activated protein kinase activity, thus reducing hepatic gluconeogenesis and lipogenesis as well as increasing insulin-mediated uptake of glucose in muscles [23];
- *Sulfonylureas* (glipizide, glyburide, gliclazide, glimepiride): bind to ATP-sensitive K^+ channel in the β-cells of the pancreas leading to the inhibition of those channels and depolarizing the resting membrane potential of the cell, thereby causing an influx of Ca^{++} and the stimulation of insulin secretion [24];
- *Meglitinides* (Repaglinide and nateglinide): exert their effect via different pancreatic β-cell receptors, but they mimic sulfonylureas by regulating ATP-sensitive K^+ channel in pancreatic β-cells, thereby causing an increase in insulin secretion [25];
- *Thiazolidinediones* (rosiglitazone, pioglitazone): bind to peroxisome proliferator-activated receptor gamma to increase peripheral uptake of glucose and decrease hepatic glucose production [26];
- *α-Glucosidase inhibitors* (acarbose, miglitol, voglibose): competitively inhibit alpha-glucosidase enzymes in the intestine that digest the dietary starch thus inhibiting the polysaccharide reabsorption as well as the metabolism of sucrose to glucose and fructose [27];

and three new classes comprehending:

- *Dipeptidyl peptidase-4 (DPP-4) inhibitors* (sitagliptin, saxagliptin, vildagliptin, linagliptin, alogliptin): inhibit the enzyme DPP- 4 and prolong the action of glucagon-

like peptide. This inhibits glucagon release, increases insulin secretion and decreases gastric emptying thus decreasing blood glucose levels [28];

- *Sodium-glucose co-transporter 2 (SGLT2) inhibitors* (dapagliflozin and canagliflozin): inhibit SGLT-2 in proximal tubules of renal glomeruli, causing inhibition of 90% glucose reabsorption and resulting in glycosuria in people with diabetes which in turn lowers the plasma glucose levels [29];
- *Glucagon-like peptide-1 (GLP-1) receptor agonists* (liraglutide, dulaglutide, semaglutide): synthetic analogues of the native human incretin hormone GLP-1, which stimulates GLP-1 receptors to enhance insulin release and decrease glucagon secretion from the pancreas, they also delay gastric emptying leading to appetite suppression [30].

The therapy choices are based on the targeting of disease defects following a patient-centered approach, which considers factors beyond glycemic control including CV risk reduction. Specifically, two important clinical trials on GLP-1 receptor agonists demonstrated their ability to reduce the risk of major cardiovascular events (MACE) in T2DM patients at high CV risk [31, 32]. Moreover, also SGLT2 inhibitors, in the same type of patients, demonstrated to have positive effects on CV outcomes by reducing the risks of primary composite CV outcome and of death from any cause [33]. Those results have prompted an update to the 2017 American Diabetes Association standards of care, which now recommend consideration of empagliflozin or liraglutide for patients with suboptimally controlled longstanding T2DM and established atherosclerotic CVD [22].

1.4- Diabetes Complication

Patients with DM have an increased risk of developing acute and/or chronic complications. Acute complications, such as severe hypoglycemia or ketoacidosis, being related to the total insulin deficiency, are more common in T1DM. Whereas chronic complications are more frequent in T2DM and can affect multiple organ systems, including eyes, kidneys, nerves, blood vessels and heart [34]. Overall, vascular damage underlies these chronic complications that can be divided in micro- and macrovascular complications [35, 36]. Microvascular complications include:

- *Retinopathy*: induced by the damage of small blood vessels that spray the retina, which can lead to blindness;
- *Nephropathy:* which is a progressive reduction of the kidney filter function caused by damage to small blood vessels in the kidneys and leading to renal failure if untreated;
- *Neuropathy:* which consists in the nerve damage through different mechanisms, including direct damage by the hyperglycemia and decreased blood flow to nerves by damaging small blood vessels. It can cause loss of sensitivity, damage to the limbs, dysfunctions of the heart, eyes, stomach and impotence in males. It represents one of the most common complications of DM.

Macrovascular complications include cardiovascular diseases (CVD), such as ischemic heart disease and peripheral artery disease, which are the most prevalent cause of morbidity and mortality in diabetic patients [37]. In these patients the risk of CVD is 2 to 4 times higher if compared to the rest of the population; indeed, hyperglycemia, by inducing progressive endothelial dysfunction, represents the primary driver of chronic vascular disease and leads to long-term vascular damage [38]. Notably, an important issue is that vascular complications in DM patients may persist and progress despite the strict glycemic control, raising the idea that hyperglycemia has long-lasting deleterious effects and that glycemic control is often not enough to completely reduce the onset and development of DM complications. The concept that early glycemic environment is remembered in the target organs is been named hyperglycemic or "metabolic memory" and clinically emerged following the results of important clinical trials on both T1DM and T2DM [39]. This phenomenon suggests that hyperglycemia-dependent mechanisms induce stable cell changes that are responsible for their persistent dysfunction, even after glycemic normalization. Interestingly, emerging evidence report the existence of the hyperglycemic memory not only in terminally differentiated cells but also in stem and progenitor cells, which are the fundamental players of tissue homeostasis and repair [40].

Indeed, hyperglycemia has been observed to induce stem/progenitor cells dysfunction that is only partially reversible after the normalization of glucose levels [41]. This latest finding raises a crucial issue: the inability of the current therapeutic strategies to preserve progenitor capacity precludes the intrinsic auto-regenerative potential of the tissues and consequently, the prevention of DM complications.

The study of the mechanisms underlying metabolic memory is therefore mandatory to understand and to prevent the onset and development of persistent vascular complications.

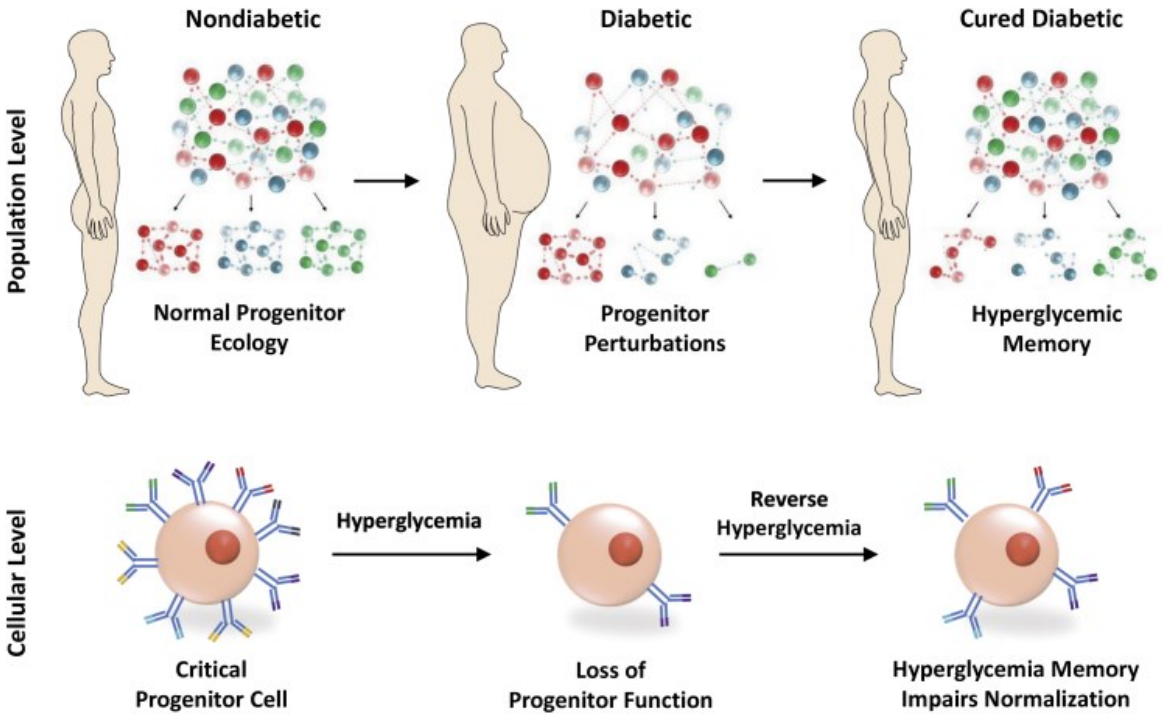

Figure 1. "Diabetes causes population-wide and single-cell alterations within progenitor cells. Exposure to sustained hyperglycemia causes depletion of critical progenitor cell subsets and alterations within individual progenitor cells. Hyperglycemic memory prevents the normalization of progenitor function on curing of diabetes" [40].

1.5- Oxidative Stress, Inflammation and Diabetic Complications

Several studies have revealed a direct link between oxidative stress and DM. Indeed, increased reactive oxygen species (ROS) production seems to be the main cause of endothelial dysfunction and pro-inflammatory state, which characterize DM and are at the basis of its micro- and macrovascular complications [42]. ROS, including superoxide anion (O_2^-) and hydrogen peroxidase (H_2O_2), are highly unstable molecules consisting of radical and non-radical oxygen species formed by the partial reduction of oxygen mainly during mitochondrial oxidative phosphorylation, but also in cellular response to xenobiotics, cytokines and bacterial invasion. Mitochondrial ROS production is generated by the respiratory chain in which the release of electrons reduces O_2 into O_2^-, to form ATP, in a process known as oxidative phosphorylation. Several enzyme systems catalyze the reactions to neutralize free radicals and reactive oxygen species. For instance, O_2^- is converted by the enzyme superoxide dismutase (SOD) into H_2O_2, which can be converted to H_2O and O_2 by catalase (CAT) [43]. Low or moderate ROS levels are physiological. However, an unbalance due to increase in ROS levels and/or a decrease in the cellular antioxidant capacity generates oxidative stress condition [44]. ROS can react with various substrates such as DNA, lipid and protein, and damage them. Moreover, ROS are able to directly inactivate antioxidant enzymes and important anti-atherosclerotic enzymes such as endothelial nitric oxide synthase (eNOS) and prostacyclin synthase.

Hyperglycaemia increases ROS production through various pathways including polyol pathway, increased formation of advanced-glycation end products (AGEs) and protein kinase C (PKC) activation. These pathways, fostered in turn by ROS, are known to be involved in the pathogenesis of DM-complications because associated with defective angiogenesis, activation of pro-inflammatory pathways and induction of pro-inflammatory genes expression through long-lasting epigenetic changes [45].

Oxidative stress has been always strictly related to inflammation that has a pivotal role in atherosclerosis. Inflammation is a natural defence mechanism driven by immune cells that are known to produce free radicals against pathogens or inflammatory stimuli [42]. Moreover, inflammatory stimuli activate numerus intracellular pathways including protein kinases (i.e. Mitogen-activated protein kinase [MAPK] and Phosphoinositide 3 [PI3] kinase), nuclear factor kappa-B (NFkB) and signal transducer and activator of transcription (STAT) pathways, leading to the expression of cytokines, enzymes and growth factors involved in tissue repair and inflammation resolution. Notably, several evidences indicated that oxidative stress plays a pathogenic role in chronic inflammation. Indeed, high levels of ROS have been reported to

stimulate the activation of inflammatory processes leading to the synthesis and secretion of pro-inflammatory cytokines, contributing to the establishment of chronic inflammatory state [46]. In particular, NFkB is a pleiotropic transcription factor, involved in multiple biological processes, which has been found to be a master regulator of genes implicated in atherosclerosis and diabetes mellitus [47, 48]. The NFkB or Rel family consists of five subunits: p50, p52, p65 (also known as RelA), c-Rel and RelB [49] that can form homodimers or heterodimers. In the cytoplasm, IkB inhibitory proteins block the ability of NFkB to bind to DNA resulting in the inactivation the NFkB complex. IkB kinase complex phosphorylates IkB thus leading to its ubiquitination and degradation. This process reveals the NFkB DNA-binding domain and nuclear localization sequence allowing NFkB nuclear translocation and the consequent regulation of target genes [49, 50]. Numerous studies reported hyperglycemia-induced increase NFkB activity in different cell types both *in vitro* and *in vivo*. Of note, Hoffman et al. observed a significant increase in NFkB activity in peripheral mononuclear cells of T2DM and T1DM patients when compared to non-DM patients [51]. Moreover, NFkB activity was found increased in monocytes from T1DM patients with microvascular complications when compared to those from T1DM patients without microvascular complications [52]. This difference correlated with an increased release of monocyte cytokines, such as IL-6 and TNFα, suggesting the contribution of NFkB in the DM associated inflammation. Interestingly, high levels of IL-6 and TNFα, two pro-inflammatory cytokines, have been also detected in T2DM patients [49, 53]. Furthermore, studies describe that the systemic pro-inflammatory state in DM patient includes also alteration in the cell populations of the innate immune system. Monocytes and macrophages are cellular components of the innate immune system [54]. Human monocytes originate from a common myeloid precursor in the BM and give rise to tissue macrophages and dendritic cells. The different expression of the lipopolysaccharide receptor CD14 and the FcγIIIR CD16 distinguishes the three major monocytes populations: classical ($CD14^{+}CD16^{-}$), non-classical ($CD14^{+}CD16^{++}$) and intermediate ($CD14^{++}CD16^{+}$). Classical monocytes are about the 80-95% of the total circulating monocytes. They are highly phagocytic and important scavenger cells involved in wound healing, angiogenesis and coagulation processes. Non-classical monocytes constitute the 2-11% of total population with patrolling behavior *in vivo*, pro-inflammatory action, inflammatory cytokines secretion in response to infection. The intermediate monocytes are the smaller subpopulation, ranging from 2 to 8%. They are characterized by ROS production and have been described as the major source of pro-inflammatory cytokines upon stimulation [55]. Once monocytes reach the tissue are named macrophages. Here, they can be activated by different stimuli and polarize towards type 1 (M1)

or type 2 (M2) macrophages. M1 macrophage polarization is involved in the initial phase of the inflammatory response. These, once activated, increase the expression and production of pro-inflammatory cytokines/chemokines and enhance their ability to kill microbial pathogens through ROS and NO production. Inflammatory resolution involves M2 macrophages that, characterized by reduced pro-inflammatory activity, promote angiogenesis and help tissue healing and remodeling. Interestingly, emerging evidence reported that DM patients display abnormal elevation of intermediate ($CD14^{++}CD16^{+}$) inflammatory monocytes [56, 57] and alteration in macrophage polarization towards M1 phenotype [58, 59]. Cumulatively, these alterations have been reported to drive the development of atherosclerosis and increase the risk of CVD in DM patients [60].

2. $CD34^+$ Stem Cells

2.1- Characterization and Regenerative Potential

$CD34^+$ stem cells are bone marrow (BM)-derived cells characterized by the expression of the transmembrane phosphoglycoprotein CD34, which was first identified in 1984 on hematopoietic stem and progenitor cells (HSPCs) [61, 62]. The structure of CD34 has been well investigated, whereas little is known about its function. Studies suggest the involvement of this protein in cytoadhesion, in particular by linking HSPCs to BM extracellular matrix or to stromal cells, and in the regulation of cell differentiation and proliferation [63, 64]. HSPCs were originally discovered in the BM, however, they can also be isolated from umbilical cord blood (UCB) or "mobilized" in the peripheral blood by several physiological, pharmacological or pathological stimuli [65, 66].

Predominantly regarded as a marker of HSPCs, CD34 marker identifies several cell types of BM origin with great regenerative potential, including endothelial progenitors cells (EPCs) [61]. Since their discovery, HSPCs have been the focus of intensive research and their clinical value to promote repair/regeneration has been proven in both hematopoietic and non-hematopoietic tissues, such as liver, brain, and heart [67].

In 1997, Ashara et al. described for the first time EPCs as $CD34^+$ KDR^+ mononuclear blood cells, which were able to differentiate in into endothelial-like phenotype cells *in vitro* and may contribute to adult neoangiogenesis [68], starting years of research about the regenerative potential of these cells. To date, despite the debate on EPC definition is still ongoing, two major EPCs populations have been isolated from peripheral blood using cell culture methodologies: the myeloid angiogenic cells (MACs, also known as circulating angiogenic cells (CACs), pro-angiogenic cells (PACs), or "early EPCs") and the endothelial colony forming cells (ECFCs, also called "outgrowth endothelial cells" (OECs) or "late EPCs") [69]. Evidence suggests that these two populations both contribute to vascular repair through different mechanisms [69]. MACs, despite not having the ability to become endothelial cells, can promote angiogenesis through paracrine actions by releasing soluble factors that stimulate endothelial tube formation *in vitro* and vascular repair *in vivo* [70]. Moreover, MACs can interact directly with ECFCs by recruiting them at the site of injury. This interaction is also able to activate ECFCs and enhance their angiogenic properties *in vitro* [71], [72]. ECFCs, indeed, display a potent intrinsic vasculogenic capacity, with a significant proliferative potential and the capacity to self-assemble into functional blood vessels *in vivo* [69, 73]. Although these two populations differ in functions and surface markers characterization, they cooperate in neovascularization.

Despite the EPC definition remains controversial, cells expressing the common progenitor cell marker CD34 have attracted the attention of the scientific community due to their potential in regenerative medicine. Besides their clinical relevance in the outcome of BM transplants [74, 75], $CD34^+$ cells have been investigated for their ability to promote angiogenesis and CV repair/regeneration [76]. Increasing evidence suggests that BM-derived circulating progenitor cells, including $CD34^+$ cells, contribute to vascular homeostasis in adults [68, 77] not only as a pool of EPCs, but also as source of growth/angiogenesis factors [78]. In 2003, Kawamoto et al. showed for the first time the therapeutic neovascularization potential of autologous $CD34^+$ mononuclear cells in myocardial ischemia [79]. Afterwards, several clinical trials have been designed to evaluate the ability of $CD34^+$ cells to induce cardiac regeneration [76] and to verify the safety and efficacy of $CD34^+$ cells treatment for the recovery of cardiac functionality in patients affected by different cardiac pathologies (e.g. acute myocardial infarction, AMI; dilated cardiomyopathy, DCM; ST-elevation myocardial infarction, STEMI; Refractory angina). Overall, the results showed significant benefits in the cardiac function recovery in terms of left ventricular ejection fraction improvement [80-82].

2.2- Disease Correlation

Evidence in literature suggests that $CD34^+$ cells have an important protective role in different organs and their reduced levels have been associated with different pathologies. Specifically, low levels of circulating $CD34^+$ cells have been correlated with poor prognosis in chronic hemodialysis patients, in which vascular risks and all-cause mortality were significantly increased [83]. Circulating $CD34^+$ cell levels have been also related to vascular risk associated with cognitive impairment [84]. Furthermore, a decreased $CD34^+$ cell number significantly correlated with age in early Alzheimer's disease, suggesting a deficient regenerative hematopoietic support for the central nervous system in the early stage of this pathology [85]. Moreover, numerous studies demonstrated the correlation between circulating $CD34^+$ cell levels and the risk of CVD. Indeed, these cells were found to inversely correlate with coronary artery disease and the number of atherosclerotic risk factors [86]. In DM Fadini et al. also showed a close negative correlation between $CD34^+$ cells and CV risk, demonstrating that the number of circulating $CD34^+$ cells can predict CV outcomes in diabetic patients [87]. Few years later, the same author unequivocally demonstrated that circulating $CD34^+$ cell number is a clinical-grade biomarker able to improve long-term CV event prediction in T2DM patients [88], further supporting the relevant role of these cells in the maintenance of CV system homeostasis.

2.3- Diabetic Environment

In physiological conditions, once mobilized and released in the blood circulation, $CD34^+$ stem cells adhere to the endothelium and migrate to the target site where they contribute to the repair or generation of new blood vessels. [89]. To date, it is well known that all these processes are compromised by hyperglycemia [38]. Moreover, emerging studies describe that DM induces defects in the BM microenvironment, including microvascular disease (microangiopathy), damage of the nerve terminals (neuropathy), $CD34^+$ stem cells rarefaction and impaired stem cells mobilization (mobilopathy) [90, 91]. The BM niche has an important role in hematopoietic stem/progenitor cell and mesenchymal stem cell homeostasis [92]. Therefore, diabetes-induced alteration of niche microenvironment may have a negative outcome on its resident populations, leading to the generation of cells with harmful atherosclerotic properties [93]. To this regard, it has been observed that DM environment can promote BM-derived progenitor cells differentiation into pro-calcific cells [94] or smooth muscle progenitor cells [95]. Furthermore, Nguyen et al. reported that circulating progenitor cells from T1DM generated higher number of myofibroblast progenitor cells [96].

Besides the impairment of innate CV regeneration ability, T2DM patients also display a chronic low-inflammatory status that plays a central role in the development of diabetic complications [53]. There is a strong evidence that chronic hyperglycemia promotes alterations in the innate immune system elements and chronic low-grade systemic inflammation in the absence of overt infection [57]. Specifically, DM has been shown to promote a pro-inflammatory milieu consisting in peripheral pro-inflammatory monocytes increase [56, 57], alteration of macrophage polarization [97] and high levels of circulating inflammatory cytokines [98, 99]. Interestingly, Loomans and colleagues demonstrated, for the first time, that hyperglycemia was able to alter the differentiation fate of BM precursor cells, reducing the potential to generate vascular regenerative cells and favoring the development of pro-inflammatory cells [100]. In agreement with these findings, it has been recently reported that T2DM patients display a significant decreased in provascular progenitor content along with an increased circulating inflammatory cell burden [101]. All together, these data suggest that the DM environment, besides leading to the reduction in number and function of vasoprotective and regenerative cells, might preprogram BM-derived progenitor cells, such as $CD34^+$ stem cells, favoring the generation of cells with anti-angiogenic and pro-inflammatory properties that could actively participate to the development of diabetic vascular complications.

3. Epigenetics

3.1- Epigenetics Mechanisms

The term “epigenetics” literally means “outside conventional genetics” and is now used to describe the study of stable alterations in genes that arise during development and cell proliferation [102]. Epigenetics allows the cells of a multicellular organism to be genetically homogeneous but structurally and functionally heterogeneous owing to the differential expression of genes. Specifically, approximately 147 base pairs of DNA are wrapped around histone protein (H2A, H2B, H3 and H4) in the repeating units of chromatin, i.e. the nucleosomes. Modifications to both DNA and histone tails can regulate gene accessibility to the transcriptional machinery, conferring to the chromatin an active or inactive structure. Epigenetic changes can be transient and/or stable and heritable; this intrinsic characteristic allows the cell to promptly respond to environmental stimuli as well as to normally develop and differentiate toward distinct cell lineages without involving DNA mutations. This implies that disease-induced environmental cues may contribute to the aberrant epigenetic makeup and consequent pathophenotype [103]. The major mechanisms in epigenetic regulation involve DNA methylation, which are stable and long-term modifications, histone modifications that are more flexible (short-term), such as methylation/acetylation, and post-transcriptional RNA regulation.

- *DNA methylation* at CpG sites, in which a cytosine nucleotide is located next to a guanidine nucleotide, occurs by the covalent addition of a methyl (CH3) group to DNA; these methyl groups project into the major groove of DNA and inhibit transcription. The enzyme families that catalyze the DNA methylation and demethylation process are DNA methyl transferases (DNMTs) and the ten-eleven translocation (TET) family of 5-mC hydroxylases respectively [103-105].
- *Histone modification:* DNA is wrapped around a histone octamer, formed by two copies each of histone H2A, H2B, H3 and H4. The transcriptional activation or repression process depends on the recruitment of protein complexes that modify the histone tails through acetylation, methylation and phosphorylation and ubiquitination, making it more or less accessible for transcription. Multiple histone modifications concur in the regulation of gene transcription at promoter or enhancer level. The acetylation involves the addition of an acetyl group on a lysine or an arginine leading to DNA distension and gene transcription. The Histone AcetylTransferase (HAT) enzymes are responsible for the acetylation process, whereas the Histone DeACetylases (HDAC) enzymes carry out

the removal of the acetyl group. Histone acetylations frequently occur in promoters (H3K4ac, H3K9ac, H3K14ac) and in enhancers (H3K27ac). Histone methylation is achieved by Hstone MethylTransferase (HMT) enzymes, which can add from one to three methyl groups to the same amino acid. Depending on the degree and site of methylation, this modification can be associated with transcription activation or repression.

Phosphorylation occurs on the amino acids serine, threonine and tyrosine by action of specific kinases and plays a key role in chromatin remodeling during several processes, such as DNA-damage repair, transcription regulation, mitosis, meiosis and apoptosis [106]. At last, Histone Ubiquitination promotes the addition of an ubiquitin molecule on the C-terminal lysine of the H2A and H2B histones and is also involved in several processes such as DNA-damage repair and transcription regulation, besides playing a crucial role in stem cell maintenance and differentiation [103, 107].

- *Non-coding RNAs* stand out in long non-coding RNAs (lncRNAs) and small noncoding RNAs (sncRNAs), which include microRNAs (miRNAs) and small inhibitory RNAs (siRNAs). Non-coding RNAs participate in the modulation of gene transcriptional silencing through the recruitment of remodeling complexes as well as by the binds to their target mRNAs. All in all these mechanisms lead to the reduction of mRNA stability and/or translation [103, 108].

3.2- Epigenetic Changes associated with DM Complications

Emerging studies suggest that epigenetic mechanisms may regulate the complex interaction between genes and the environment, playing a key role in several diseases including DM and its complications [109]. The Diabetes Control and Complications Trial (DCCT) (1983-1989) and the subsequent follow-up study Epidemiology of Diabetes Interventions and Complications (EDIC) showed that T1DM patients who had received long-term intensive control had improved kidney function and decreased risk of retinopathy progression and CVD compared with those who had been in the conventional treatment group [110, 111]. Furthermore, a 10-year follow-up of the UK Prospective Diabetes Study (UKPDS) demonstrated that intensive treatment also reduced the risk of developing micro- and macrovascular complications in T2DM patients [112]. Overall, these findings demonstrated the importance of early metabolic control to reduce long-term complications but also confirmed that hyperglycemia can have long-lasting deleterious consequences resulting in the development of complications despite

glycemic normalization [113]. This phenomenon has been termed hyperglycemic memory by clinicians and the emerging concept is that prolonged exposure to altered glycemic conditions may epigenetically imprint human cells permitting vertical or horizontal transfer of the defect to cell progenies [114, 115]. Several studies demonstrated that DM conditions are able to elicit epigenetic changes in a variety of cell types, inducing alteration that can contribute to CVD onset and progression. Epigenome-wide analysis on primary human vascular cells revealed that hyperglycemia is able to induce histone H3K9/K14 acetylation and DNA methylation alterations, leading to the induction of genes and pathways that are associated to the endothelial dysfunction [116]. El-Osta and coworkers reported that transient hyperglycemia induced, in a ROS-dependent manner, epigenetic histone modifications in aortic endothelial cells, both *in vitro* and *in vivo*. These epigenetic changes were responsible for NFkB-p65 gene upregulation and persisted despite the return to normal glucose levels [117]. Another study demonstrated that the histone methyltransferase Set7 epigenetically regulated the increased expression of NFkB in peripheral mononuclear cells from T2DM patients contributing to inflammation and endothelial cells dysfunction [118]. Moreover, Zhong et al showed that the persistent NFkB activation and pro-inflammatory gene expression after transient high glucose exposure was associated with miRNAs expression changes in human aortic endothelial cells *in vitro* [119].

We also recently demonstrated that CXCR4/SDF-1α axis impairment in $CD34^+$ cells exposed to high glucose was associated with epigenetic changes that persisted despite the recovery into normal glucose conditions. In particular, we found that repressive epigenetic modifications at level of CXCR4 promoter reduced the expression of CXCR4 with cell migration impairment toward SDF-1α. Notably, these data were also confirmed in $CD34^+$ stem cells isolated from BM of T2DM patients undergoing coronary artery by-pass surgery [120]. Taken together, these data suggest that hyperglycemic environment can be "memorized" by the cells in the form of epigenetic changes that, by contributing to the self-perpetuating alteration of gene expression, may be potentially responsible for the onset and progression of micro- and macrovascular complications in DM, despite glycaemia correction.

Interestingly, Gallagher and colleagues observed the decrease of H3K27me3, a repressive histone methylation mark, at *IL-12* gene promoter level in BM progenitors isolated from a murine model of glucose intolerance. This epigenetic signature, passed down to wound macrophages, programmed their polarization toward a pro-inflammatory phenotype [121]. Other studies described similar findings although in different genes and experimental settings [122]. For example, Yan et al recently demonstrated that DNMT1 dysregulation skewed macrophage polarization towards M1 phenotype in the wounds of T2D mice [123].

These observations entail important concepts. First, they underline that epigenetic mechanisms are involved in the establishment of hyperglycemic memory and contribute to hyperglycemia-induced cells dysfunction, taking part in the onset and perpetuation of DM-CV complication. Moreover, they reveal that epigenetic changes induced by hyperglycemia affect not only terminally differentiating cells but also stem/progenitor cells, with direct implication on tissue homeostasis and repair. Finally, they suggest that hyperglycemia might "epigenetically preprogram" cells at BM level, inducing modifications that can be transmitted during the differentiation process to the cell progeny favoring the generation of abnormal cell types potentially involved in DM-CV complication and DM-increased inflammatory status.

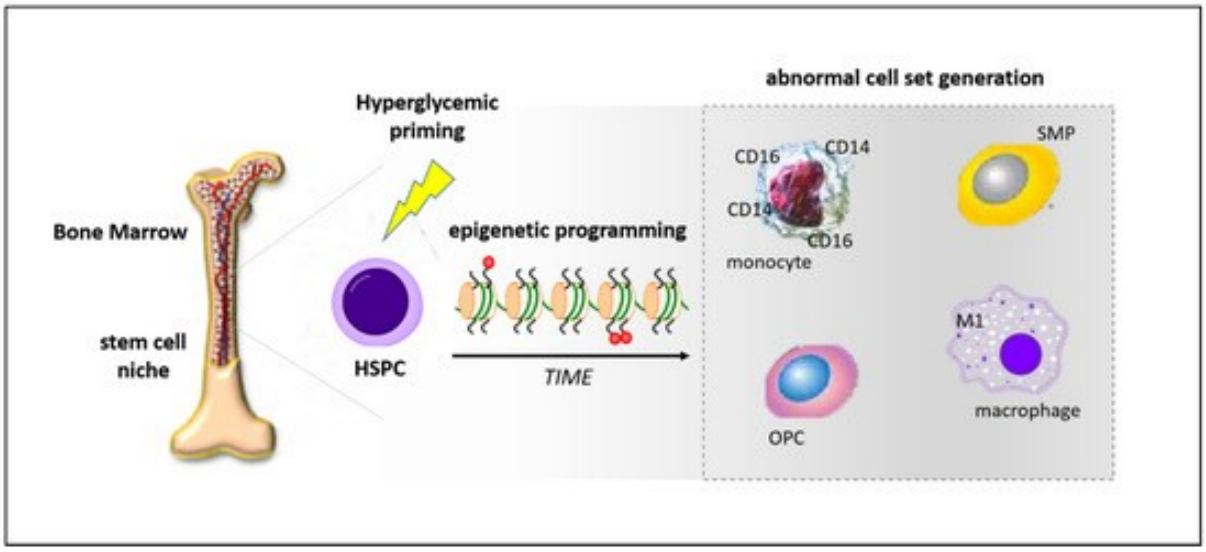

Figure 2. Schematic representation of the epigenetic programming induced by hyperglycemia on hematopoietic stem progenitor cells (HSPCs) at the bone marrow level [93].

4. Epigenetic Reversibility and DM Cardiovascular Complication Improvement

4.1- Therapeutic Tools

Cardiovascular complications are the major contributors to morbidity and mortality in patients with T2DM [124, 125]. Current clinical guidelines suggest metformin as the first drug to use at the time of T2DM diagnosis, in addition to lifestyle modifications, unless patient contraindications. The progression of T2DM might require a combination therapy to maintain HbA1c at target. The choice of medication to add is based on clinical characteristics of the patient and their preferences. Current recommendation indicates a stepwise approach and oral agents have been the standard of care. For patients with an indicator of high risk or established atherosclerotic cardiovascular disease (ASCVD), the addition of drugs that have been proven to reduce major adverse CV events (MACE) and/or CV mortality is now recommended. On the contrary, for patients without high risk or established ASCVD, the choice of a second agent to add to metformin is not yet guided by empiric evidence [126, 127].

Recently, a new class of antidiabetic drugs, the agonists of glucagon-like peptide-1 receptor (GLP-1R), displayed pleiotropic CV effects in T2DM patients. Alongside glucose-dependent insulinotropic peptide (GIP), GLP-1 is an incretin; thus, its biological functions include stimulating insulin secretion in a glucose-concentration-dependent fashion, suppressing glucagon release, promoting satiety and increasing peripheral glucose disposal [128]. Although it has been reported to couple to other G proteins, GLP-1R signal primarily involves G_s proteins. Its activation on β-cells also leads to rapid increases in levels of cAMP and intracellular calcium followed by insulin exocytosis in a glucose-dependent manner [129]. Besides the pancreas and gastrointestinal tract, GLP-1R has been identified also in the nervous system, heart, vascular smooth muscle cells, monocyte and macrophage [130] . GLP-1 displays beneficial effects on the vascular system [130]. Some studies showed that GLP-1 upregulates the activity and protein expression of endothelial nitric oxide synthase (eNOS) and prevent ROS-induced cell senescence in human umbilical vein endothelial cell (HUVEC) in normal glucose conditions [131, 132]. Moreover, GLP-1 also improved proliferation and differentiation of EPCs [133] and decreased high-glucose-induced ROS production in cardiac microvascular endothelial cells [134]. Consistently, GLP-1R agonists have shown cardioprotective action as well as a protective role on vascular endothelium [93]. For instance, Wei and colleagues demonstrated an improved coronary artery endothelial function, reflected by increased coronary flow velocity reserve and reduced circulating levels of inflammatory markers, in newly diagnosed patients

with T2DM [135]. Whereas, interestingly, other studies reported similar results, even in a pre-diabetic setting [136, 137].

4.2- Liraglutide

While human GLP-1 has a short half-life, liraglutide (LIRA), which shares with it 97% sequence identity, is a long-acting GLP-1R agonists and has a plasma half-life of 13 hours after subcutaneous administration [128].

Results from the LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) clinical trial demonstrated that LIRA is able to reduce the risk of death from CV disease in T2DM patients [138]. It was a randomized trial versus placebo involving 9340 patients with T2DM and high CV risk. Specifically, data analysis of the trial demonstrated a significant reduction of the 3-point of MACE, i.e. the occurrence of CV death, nonfatal myocardial infarction or nonfatal stroke, in both T2DM patients with poly-vascular and single-vascular disease. Similar results were obtained for the secondary expanded outcome also including hospitalization for unstable angina, coronary revascularization, or hospitalization for heart failure and CV death. These clinical outcomes raised the possibility that GLP-1R agonist treatment could reverse hyperglycemic memory because these patients already had a high risk of CV disease. However, available data on the mechanisms underlying the impact of LIRA on CV protection in T2DM patients are still limited. *In vitro* studies demonstrated that LIRA, aside from reducing oxidative stress, is able to prevent the onset of high-glucose induced endoplasmic reticulum stress, inhibits tumor necrosis factor-alpha (TNF-α) and vascular adhesion molecule expression induced by high glucose in HUVEC [139-141]. Moreover, *in vivo* LIRA treatment also inhibited the progression of atherosclerotic plaque formation and enhanced plaque stability in ApoE-/- mice suggesting a potential role for LIRA in the prevention and stabilization of atherosclerotic vascular disease [142]. Interestingly, experimental evidence has demonstrated the atheroprotective and anti-inflammatory effect of LIRA at different tissue levels. Specifically, LIRA has been observed to exert a strong anti-inflammatory effect on human aortic endothelial cells [143]. Moreover, Bruen et al observed that LIRA decreased atherosclerotic lesion formation in ApoE-/- coincident with a reduction in pro-inflammatory and increased anti-inflammatory monocyte/macrophage populations *in vivo* supporting a therapeutic role for LIRA as an atheroprotective agent via modulating macrophage cell fate [144]. Further investigation of the mechanisms by which LIRA exerts its protective

CV action would provide evidences to support the benefits of using LIRA in patients without high risk or established ASCVD, preventing their onset.

LIRA has shown to exert effects on a variety of stem/progenitor cells, including mesenchymal stem cells and adipocyte precursors [145, 146]. However, so far there are no studies in humans describing on epigenetic basis the cellular and molecular effects of LIRA on dysfunctional $CD34^+$ stem cells and their peripheral pro-angiogenic/inflammatory progeny in T2DM patients.

Aims

In the light of the data reported in literature, we aimed at investigating, by using an *in vitro* model of diabetes, whether epigenetic mechanisms contributed in the reduction of intrinsic vascular homeostatic capacity and in differentiation drift of cord blood-derived $CD34^+$ stem cells, alterations known to drive the development of atherosclerosis and increase the risk of CVD in T2DM patients. Our findings were then confirmed in BM-$CD34^+$ stem cells isolated from sternal biopsies from diabetic patients undergoing coronary bypass surgery. Finally, since we hypothesized that at least part the ancillary CV protective effect of LIRA may be mediated by the ability of the drug to modulate BM stem/progenitor cell function and differentiation, we sought to evaluate whether the drug was able to prevent hyperglycemia-induced dysfunction of cord blood-derived $CD34^+$ stem cells.

Materials and Methods

Experimental Design

We first evaluated the effects of high glucose (HG) on the epigenetic makeup of naïve umbilical cord blood (UCB) -derived $CD34^+$ stem cells. This allowed us to avoid readout misinterpretations derived from aging or other risk factors, discriminating between epigenetic modifications established as a direct consequence of hyperglycaemia exposure, rather than those related to confounding variables. As depicted in Figure 1, we expanded $CD34^+$ stem cells in HG conditions until $CD34^+$ stem cells lost glucose tolerance. To assess LIRA effects on $CD34^+$ stem cells exposed to HG, two additional experimental points were set up with HG+LIRA (MedChemExpress®) 50nM and HG+LIRA100nM. Afterwards, in order to reproduce metabolic memory *in vitro*, cells expanded in HG condition were recovered in NG for 3 days (exHG), as previously described [147]. We then performed molecular and epigenetic analyses of the gene(s) involved in $CD34^+$ stem cell dysfunction upon HG, exHG and LIRA treatments. Following the metabolic memory experiment, $CD34^+$ stem cells of the different treatments were induced to differentiate toward myeloid lineage and characterized. The results have been validated in BM-derived $CD34^+$ stem cells isolated from patients with coronary artery disease (CAD) with or without DM.

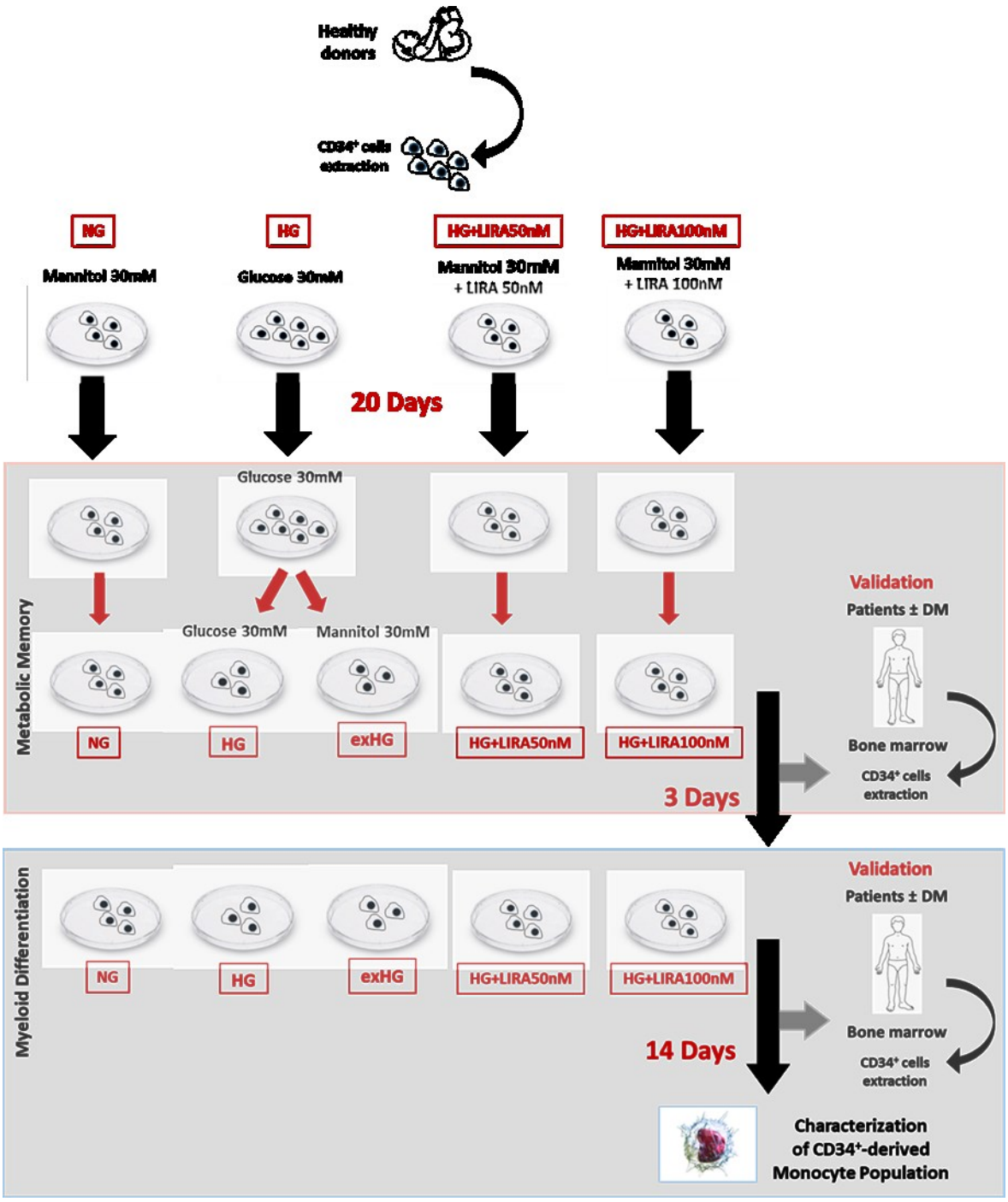

Figure 1. Experimental design. Schematic representation of the study. UCB-derived $CD34^+$ stem cells were expanded in NG, HG, HG+LIRA50nM and HG+LIRA100nM conditions for up to 20 days. Afterward, part of the HG-$CD34^+$ stem cell population was returned to NG conditions for 3 days, after which cells form all the treatment were induced to differentiate toward myeloid lineage. At the end of the experiment, cells were used for functional and molecular characterization. CAD±DM indicates coronary artery disease patients with or without diabetes mellitus; exHG, ex-high-glucose conditions; HG, high-glucose; NG, normal-glucose; HG+LIRA50nM/100nM, high-glucose plus liragluutide 50nM or 100nM;UCB, umbilical cord blood.

Study Participants

All experiments have been carried out upon approval of local ethic committees (No. 2015/ST/232 and R196/14-CCM 205). Informed written consent was obtained from all patients before BM harvesting. CAD and CAD-DM subjects have been selected by stringent matching of age, pharmacological treatments and major risk factors. At admission, CAD-DM patients were treated with sulfonylureas or metformin±insulin. Clinical characteristics of patients included in the different analyses are shown in supplementary Table S1 and S2.

UCB-CD34$^+$ Stem Cell Isolation and Expansion

UCB was collected from the umbilical cord of healthy full-term deliveries without gestational DM. CD34$^+$ stem cells were isolated and expanded as previously described, with some slight modification [148]. Briefly, mononuclear cell fraction was isolated from UCB by density gradient centrifugation using Ficoll-Paque (Lymphoprep; Sentinel Diagnostics). CD34$^+$ stem cells were then magnetically sorted from the mononuclear cell fraction using the MidiMACS system (CD34 Microbead Kit; Miltenyi Biotec GmbH). Isolated cells were seeded $2*10^5$ cells/wells in 300 µL in SFEM medium (Voden Medical Instruments) containing 20 ng/mL of interleukin-6 (IL-6), 20 ng/mL of interleukin-3 (IL-3), 50 ng/mL of fms-like tyrosine kinase 3 (Flt-3) and 50 ng/mL of stem cell factor (SCF) (PeproTech EC Ltd) in 48 multi-well plate and expanded up to 30 days at 37 °C in a humidified atmosphere with a concentration of 5% CO_2. At the beginning of the study five experimental conditions were set: hyperglycemic (30 mmol/L of glucose; HG), normoglycemic (30 mmol/L of mannitol; NG) and hyperglycemic condition plus LIRA (50nM and 100nM).

Metabolic Memory Experiment

To reproduce the metabolic memory phenomenon *in vitro*, HG-CD34$^+$ stem cells were returned to physiological glucose conditions for 3 days (exHG-CD34$^+$), as previously described [147] while the other cells were replaced each in their respective treatment. All the cells were plated at $3*10^5$ cells/wells in 300 µL in SFEM medium plus 20 ng/mL of IL-6, 20 ng/mL of IL-3, 50 ng/mL of Flt-3 and 50 ng/mL of SCF (PeproTech EC Ltd) in 48 multi-well plate. Metabolic memory experiment was started after 20 days of expansion, when HG-CD34$^+$ stem cells started to lose glucose tolerance.

Sternal BM Biopsy and CD34$^+$ Stem Cell Isolation

Sternal blood from the BM of patients with CAD±DM was obtained by needle aspiration. Aspirates were suspended in saline buffer. Mononuclear cell fraction as well as CD34$^+$ stem

cells were isolated as aforementioned and then expanded up to 20 days in in SFEM medium plus 20 ng/mL of IL-6, 20 ng/mL of IL-3, 50 ng/mL of Flt-3 and 50 ng/mL of SCF (PeproTech EC Ltd).

$CD34^+$ Stem Cells Differentiation toward Myeloid Lineage

Myeloid differentiation was induced following the protocol by Stec et al. [149]. At the end of the metabolic memory experiment, UCB-$CD34^+$ stem cell were replaced each of which maintained their respective treatment, at $1*10^5$ cells/wells in 300 µL in IMDM medium (Gibco™, Thermo Fisher) containing 20% of fetal calf serum (Hyclone™, Euroclone), 25ng/mL of SCF, 30ng/mL of IL-3, 30ng/mL of Flt-3 and 30ng/mL of macrophage colony-stimulating factor (M-CSF) (PeproTech EC Ltd) in 48 multi-well plate and expanded up to 14 days. After 20 days of expansion, BM-$CD34^+$ stem cells from CAD±DM patients were induced to differentiate in the same manner.

$CD34^+$ Stem Cell Growth Curves

$CD34^+$ stem cells were seeded at an initial density of $2*10^5$ cells/well and cultured for up to 20 days in NG, HG, HG+LIRA50nM and HG+LIRA100nM conditions. Cells were counted on days 5, 10, 15 and 20.

Migration Assays

Cell migration was determined by the use of Transwell culture inserts (5-µm pore membrane; Corning Incorporated), according to the manufacturer's instructions. In brief, 1×10^5 cells/well $CD34^+$ stem cells were seeded onto the upper chamber and allowed to migrate toward the lower chamber containing, or not, SDF-1α (50 ng/mL; PeproTech EC Ltd). Transwells were incubated at 37°C, 5% CO_2, for 4 hours. Migrated cells were counted and results are expressed as a migration index.

Flow Cytometric Assays

$CD34^+$ stem cells were incubated for 30 minutes with allophycocyanin-conjugated monoclonal antihuman CXCR4 antibody (BD Biosciences), Annexin V (BD Biosciences) and a CellROX green fluorescent assay kit (Life Technologies Italia) for detection of the CXCR4 receptor, early apoptosis and reactive oxygen species (ROS), respectively. To characterize the monocyte subpopulation cells were incubated with allophycocyanin-conjugated monoclonal antihuman CD14 antibody (BD Biosciences) and with PE- conjugated monoclonal antihuman CD16 antibody (BD Biosciences). The membrane expression of GLP-1 receptor on $CD34^+$ stem cells was investigated using the allophycocyanin-conjugated monoclonal antihuman GLP-1R

antibody (R&Dsystems), incubated for 30 minutes. The Beckman Coulter FACS Gallios platform was used to analyze samples by use of appropriate physical gating. At least 10^4 events in the indicated gates were acquired.

Western Blot Analyses

AKT phosphorylation was evaluated by stimulating for 15 minutes $CD34^+$ stem cells ($1.2*10^6$/sample) with 50 ng/mL of SDF-1α (Results Part I, Figure 3B). AKT and ERK1/2 phosphorylation was evaluated by stimulating for 5, 10 and 15 minutes $CD34^+$ stem cells (10^6/sample) with 100nM of LIRA (Results Part III, Figure 4A). AKT and ERK1/2 phosphorylation was also evaluated by stimulating for 10 minutes $CD34^+$ stem cells (10^6/sample) with 100nM of LIRA following 10 minutes of Exendin-3 (9-39) (EXE; TORCIS) 150nM pre-incubation (Results Part III, Figure 4B).

Cells were lysed in Laemmli buffer and 30 uL of proteins resolved on 10% SDS- PAGE. Whole-cell lysate (80 ug) was used to evaluate proteins expression. Proteins were transferred to PVDF membranes (Millipore, Merck SpA) and then incubated with the primary antibodies reported in Table S3. After washing, membranes were incubated with secondary antibody, which was linked to horseradish peroxidase (Pierce Antibody, Pierce Biotechnology) and revealed by ECL detection (Pierce). Results were quantified using the Alliance 9.7 Western Blot Imaging System 8 (UVItec Ltd).

DNA Extraction and Bisulfite Modification

Genomic DNA was isolated by the PureLink Genomic DNA kit (Invitrogen), following the manufacturer's protocols. Nucleic acid samples were quantified by NanoDrop and integrity was analyzed by 1% agarose gel electrophoresis. Bisulfite conversion and subsequent purification was performed with the MethylCode Bisulfite Conversion Kit (Invitrogen), starting from 300 ng of total DNA.

RNA Extraction, cDNA Preparation and Quantitative Polymerase Chain Reaction Reactions

Total RNA from $CD34^+$ stem cells was isolated by use of the Direct-zol RNA Kit (Zymo Research), following the manufacturer's protocols. Total RNA (500 ng) was converted to cDNA with the Superscript III kit (Life Technologies), according to the manufacturer's protocol. Retrotrascribed RNA was used to quantify gene expression. Data, expressed as fold-change (2DDCT) over NG after normalization to each housekeeping gene, were log2-trasformed before analysis. Primers are reported in Table S5 and S6. All reactions were performed with SYBR Green Supermix 2X (Bio-Rad Laboratories) on CFX96 Real–Time

System PCR (Bio-Rad). GLP-1R gene amplification products were resolved by 1% agarose gel electrophoresis to verify the expected band size (~300bp).

Quantitative Polymerase Chain Reaction Analysis of CXCR4 Promoter Methylation

Methylation of the CXCR4 promoter was evaluated on bisulfite-treated DNA by "2-step SYBR Green–based polymerase chain reaction (PCR)", a new technique devised by our laboratory and described in detail by Bianchessi et al. [150]. Briefly, our two-step method requires two sequential rounds of PCR reactions summarized in Figure 2.

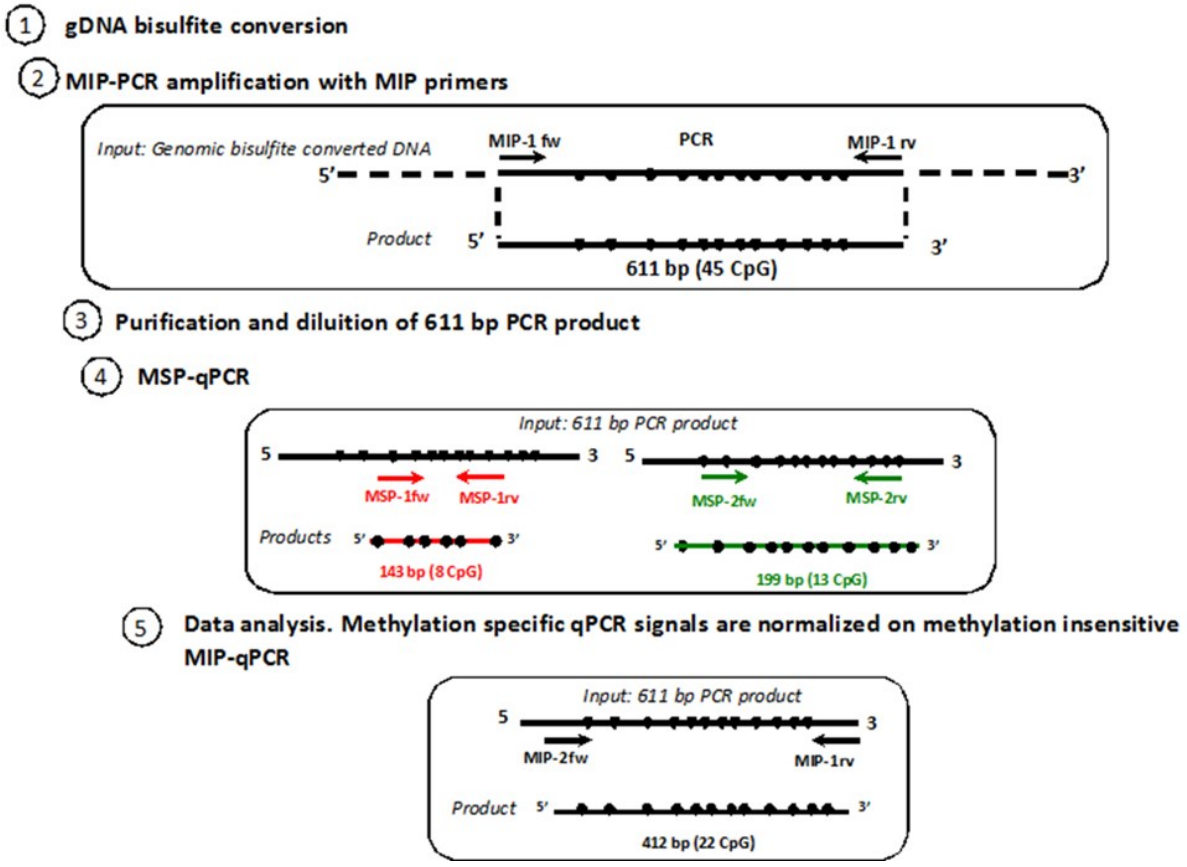

Figure 2. Two-step PCR method outline. Schematic diagram of technique based on two steps PCR. Bisulfite-converted genomic DNA is used as template in the first amplification of 611 bp region corresponding to the entire CpG-island methylation independent primers (MIP-1fw and MIP-1rv). Nested qPCR consists of three independent reactions performed with diluted input PCR product from the first PCR round. Two nested methylation specific primers reactions (MSP-qPCR1, in red and MSP-qPCR2, in green) evaluate the methylation status of target template. A nested methylation insensitive qPCR (MIP-qPCR) is used as reference control to normalize the amount of methylated target detected by the MSP-qPCRs with the DCt method. Black circles (•) are a schematic representation of methylated CpG sites and do not reflect the real number of CpG which are indicated in parentheses.

CXCR4 Promoter Sequencing

The CXCR4 promoter was amplified from bisulfite-treated DNA as previously described.24 Converted DNA was cloned into the pCR4-TOPO-TA cloning vector (Invitrogen) and transformed in Escherichia coli strain DH5a. Ten colonies for each sample were randomly picked and directly used for PCR amplification to verify vector insertion by T3 and T7 primers. PCR products were Sanger sequenced with the T3 primer with the help of an external service (GATC Biotech, Konstanz, Germany). Alignment (multiple sequence ClustalW alignment) and analysis of sequences were performed with BioEdit software and data analyzed and represented by QUMA software (http://quma.cdb.riken.jp/) for CpG methylation.

Chromatin Immunoprecipitation Assays

Cells were washed and cross-linked using 1% formaldehyde for 20 minutes. After stopping cross-linking by addition of 0.1 M of glycine, cell lysates were sonicated with Episonic 2000 (Epigentek Group Inc) and centrifuged for 10 minutes at 10000g. Supernatants were immunoprecipitated using the EpiTect ChIP OneDay Kit (Qiagen), following the manufacturer's protocols. The list of antibodies used is reported in Table S4. Recovered DNA fragments were amplified for the CXCR4 and NFkB-p65 genes by qPCR, primers reported in Table S5 and S6. qPCR values were normalized to input DNA and to the values obtained with immunoglobulin G isotype. The data are expressed as fold-change over NG.

E.L.I.S.A (Enzyme-Linked ImmunoSorbent Assay)

Conditioned medium from $CD34^+$ stem cells were collected after 3 days of metabolic memory experiment and the Quantikine® Colorimetric Sandwich E.L.I.S.A (R & D) direct method was used to quantify the IL-6 and TNF-α released, following the manufacturer's protocols.

cAMP Quantification

To analyze cAMP intracellular release induced by LIRA, 10^6 of $CD34^+$ stem cells were stimulated for 5, 10 and 15 minutes with LIRA 100nM at 37°C, then centrifuged and the obtained pellet lysed in 0.1M HCl+0.1% Tryton (Sigma Aldrich) solution. In order to investigate whether cAMP intracellular release after LIRA stimulation was mediated by GLP-1R activation, 10^6 of $CD34^+$ stem cells were incubated at 37°C with Exendin-3 (9-39) (EXE; TORCIS) 150nM for 10 minutes before LIRA 100nM stimulation. Cells were then centrifuged and the obtained pellet lysed in 0.1M HCl+0.1% Tryton solution. cAMP quantification was performed using Direct cAMP ELISA kit (1x96wells) (Enzo Life Sciences), following the manufacturer's protocols.

NFkB-p65 Nuclear Translocation Analysis

NFkB-p65 nuclear translocation was assessed by imaging flow cytometry with the Imagestream instrument (Image StreamX Mark II, Amnis). Cells were labeled using an anti NFkB / p65-FITC (Cell Signaling) antibody and a core dye, DRAQ5 (Abcam). For samples acquisition the instrument and the acquisition software were set as follow: channel 1 (Ch1) for the visualization of the cells in bright field, channel 2 (Ch2) for the detection of fluorescence associated with the FITC , channel 5 (Ch5) for the detection of DRAQ5 and Channel 6 (Ch6) for the “side scatter”. For each sample 10^4 single and focused events were acquired, using 488nm and 642nm lasers for the detection of NFkB-FITC and DRAQ5 respectively. The acquired images were analyzed with the IDEAS software. The analysis strategy provides for the identification of focused and single cells, identified in a graph showing the area (X axis) and the "aspect ratio" (Y axis) and positive for both NFkB-FITC and DRAQ5. To quantify the translocation of NFkB in the nucleus, the overlap of the fluorescences related to NFkB and DRAQ5 was evaluated through the application of the "Similarity Dilate index" (S.I.). This parameter provides a measure of the degree of nuclear localization of NFkB, correlating the intensity of the pixels resulting from the images in which there is fluorescence for NFkB / p65-FITC and for DRAQ5. Cells with a low S.I. will be those in which there is no correlation between the two fluorescence intensities. They will correspond to cells in which there is a predominant cytoplasmic distribution of NFkB. Conversely, cells with high S.I. will have a positive correlation between the images and will correspond to cells in which there is a nuclear distribution of NFkB.

Statistical Analysis

Results are given as mean ± SEM. All experiments were performed at least in triplicate, unless stated otherwise. All data expressed as fold-change were log2-transformed before analysis. Differences between data were evaluated by paired or unpaired Student t test (2-group comparisons), 1-way, 2-way repeated-measures ANOVA followed by the post-hoc Newman-Keuls multiple comparison test, as appropriate. Correlations were calculated using Pearson's coefficient. A value of $P \leq 0.05$ was considered significant. All statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Results

Part I

Epigenetic modifications involved in hyperglycemia-induced CD34$^+$ stem cells dysfunction

High Glucose (HG) Affects Proliferation and Oxidative State of CD34$^+$ Stem Cells

In our *in vitro* model of hyperglycemia, we cultured UCB-CD34$^+$ stem cells in 30 mmol/L of glucose. Cells were counted at days 10, 20, and 30. Twenty days after seeding the analysis of cellular growth curves showed a significant decrease in HG-CD34$^+$ stem cells proliferation when compared with their osmotic controls (30 mmol/L of mannitol; Figure1 A). This loss of glucose tolerance was associated with a significant increase in mitochondrial ROS production (Figure 1B) and with a significant reduction in the expression of the antioxidant enzymes, catalase and manganese superoxide dismutase (Figure 2A, 2B, 2D, and 2E). Conversely, the expression of p66shc gene, which is involved in ROS generation and is linked to hyperglycemic memory, was upregulated [151] (Figure 2C and 2F).

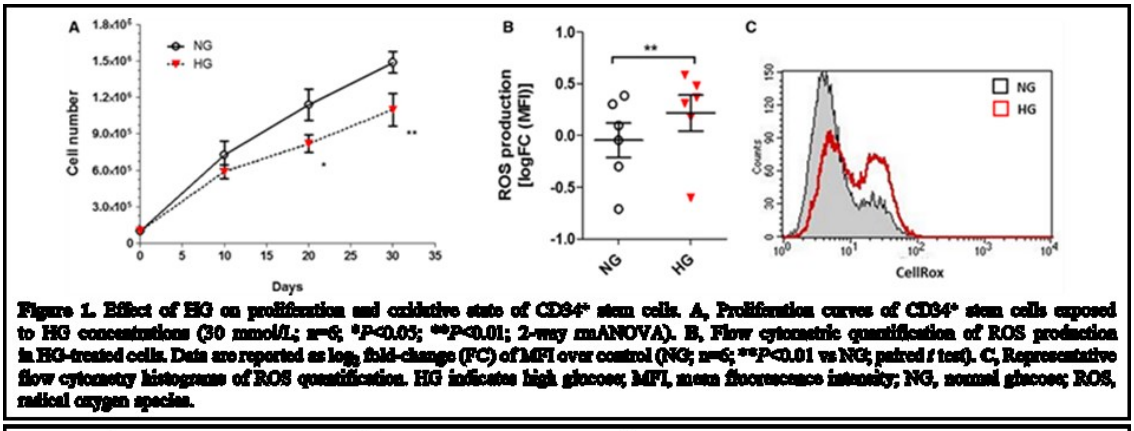

Figure 1. Effect of HG on proliferation and oxidative state of CD34⁺ stem cells. A, Proliferation curves of CD34⁺ stem cells exposed to HG concentrations (30 mmol/L; n=6; *P<0.05; **P<0.01; 2-way mANOVA). B, Flow cytometric quantification of ROS production in HG-treated cells. Data are reported as log$_2$ fold-change (FC) of MFI over control (NG; n=6; **P<0.01 vs NG; paired *t* test). C, Representative flow cytometry histograms of ROS quantification. HG indicates high glucose; MFI, mean fluorescence intensity; NG, normal glucose; ROS, radical oxygen species.

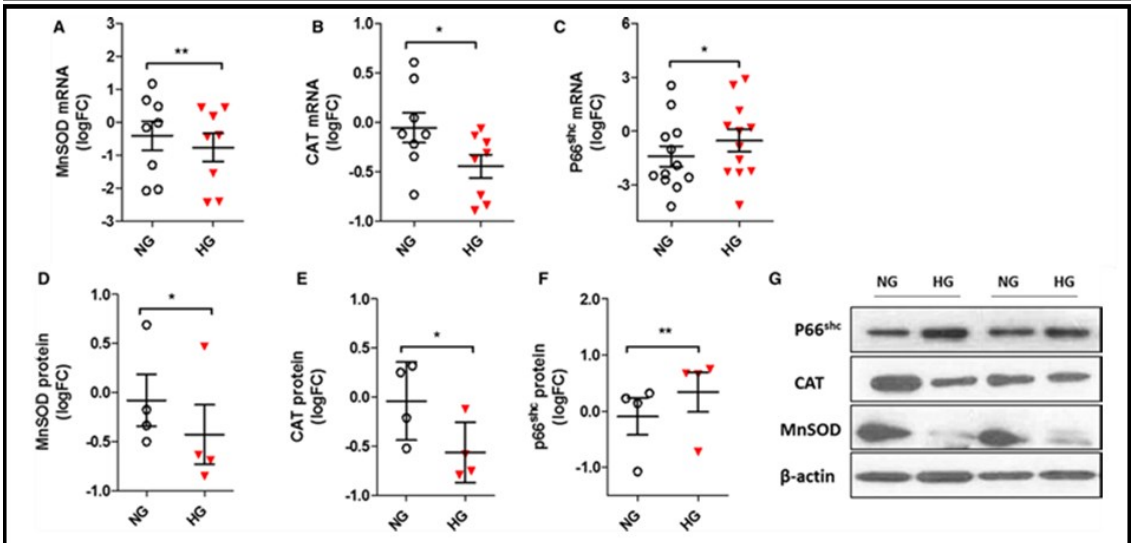

Figure 2. Effect of HG on antioxidant gene expression in CD34⁺ stem cells. A through C, Expression of MnSOD, CAT, and p66shc genes evaluated by qPCR. Data are from at least 6 independent experiments (*P<0.05; **P<0.01 vs NG; paired t test) and are expressed as log2fold-change (FC). D through F, Evaluation of MnSOD, CAT, and p66shc protein expression by western blot. Analysis of at least 3 independent experiments. Data after loading normalization (β-actin) are expressed as log2FC over control (NG; *P<0.05; **P<0.01 vs NG; paired t test). G, Representative immunoblot images are shown. CAT indicates catalase; HG, high glucose; MnSOD, manganese superoxide dismutase; NG, normal glucose; p66shc, Src homolog and collagen homologs (shc) adaptor protein.

HG Induces Persistent Impairment of CXCR4/SDF-1α Axis in CD34$^+$ Stem Cells

To investigate whether high glucose exposure could affect CD34$^+$ stem cell regenerative potential, we analyzed the CXCR4/SDF-1α axis, which is primarily involved in CD34$^+$ stem cell mobilization and migration from the BM to sites of ischemia and endothelial injury [152]. The CXCR4 receptor expression and its downstream pathways have been reported to be impaired in diabetic patients [153-155]. In our model, after 20 days, HG-CD34$^+$ stem cells exhibited a significant reduced migration toward human recombinant SDF-1α (50 ng/mL; Figure 3A). Western blot analysis of the downstream pathways revealed a reduction of AKT phosphorylation after stimulation with SDF-1α (Figure 3B). In addition, expression of CXCR4, both at mRNA and protein levels, was found to be significantly downregulated (Figure 3B and 2C). Interestingly, this impairment of the CXCR4/SDF-1α pathway induced by HG exposure was still present 3 days after recovery in NG conditions (exHG-CD34$^+$ stem cell), providing the first evidence of *in vitro*-induced metabolic memory in CD34$^+$ stem cells.

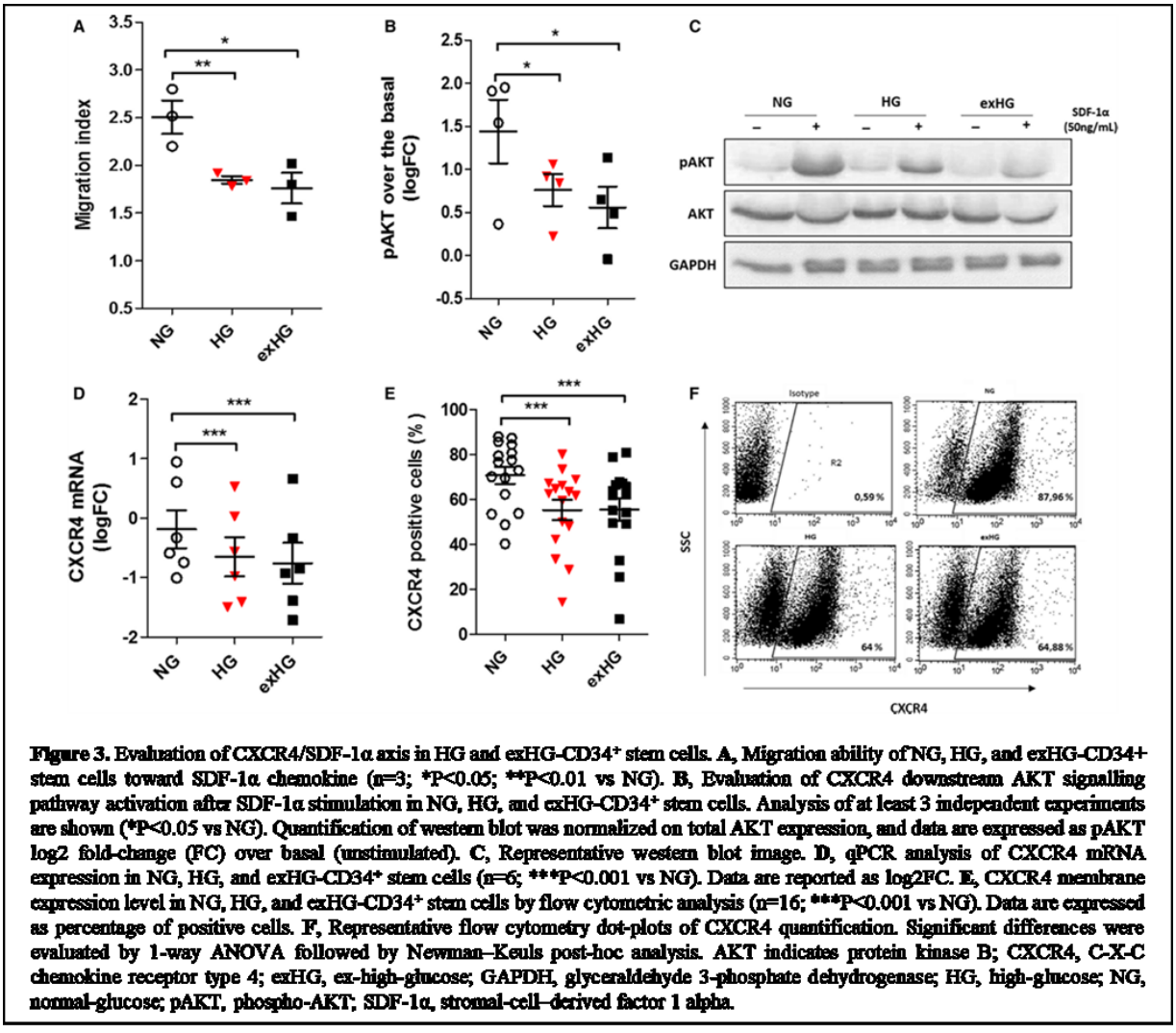

Figure 3. Evaluation of CXCR4/SDF-1α axis in HG and exHG-CD34$^+$ stem cells. **A**, Migration ability of NG, HG, and exHG-CD34+ stem cells toward SDF-1α chemokine (n=3; *P<0.05; **P<0.01 vs NG). **B**, Evaluation of CXCR4 downstream AKT signalling pathway activation after SDF-1α stimulation in NG, HG, and exHG-CD34$^+$ stem cells. Analysis of at least 3 independent experiments are shown (*P<0.05 vs NG). Quantification of western blot was normalized on total AKT expression, and data are expressed as pAKT log2 fold-change (FC) over basal (unstimulated). **C**, Representative western blot image. **D**, qPCR analysis of CXCR4 mRNA expression in NG, HG, and exHG-CD34$^+$ stem cells (n=6; ***P<0.001 vs NG). Data are reported as log2FC. **E**, CXCR4 membrane expression level in NG, HG, and exHG-CD34$^+$ stem cells by flow cytometric analysis (n=16; ***P<0.001 vs NG). Data are expressed as percentage of positive cells. **F**, Representative flow cytometry dot-plots of CXCR4 quantification. Significant differences were evaluated by 1-way ANOVA followed by Newman–Keuls post-hoc analysis. AKT indicates protein kinase B; CXCR4, C-X-C chemokine receptor type 4; exHG, ex-high-glucose; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HG, high-glucose; NG, normal-glucose; pAKT, phospho-AKT; SDF-1α, stromal-cell–derived factor 1 alpha.

HG Increases DNA Methylation of the CXCR4 Promoter in CD34$^+$ Stem Cells

A large body of evidence previously demonstrated that DNA methylation, an epigenetic modification associated with gene silencing, regulates CXCR4 expression in cancer cells [156-158]. Therefore, we hypothesized that such a repressive epigenetic modification was also involved in the downregulation of CXCR4 mRNA in HG-CD34$^+$ stem cells. We evaluated the CpG methylation status (5mCpG) of a region of the CXCR4 gene promoter encompassing −1349 to −738 nucleotides relative to the +1 transcription start site (Figure 4A). Bisulfite-treated DNA from HG and NG-CD34$^+$ stem cells were first analyzed for CXCR4 methylation density by a 2-step qPCR methylation method. This assay, designed in our laboratory for the quantitative evaluation of DNA methylation status of genes [150], showed a significant increase of CXCR4 promoter methylation in HG-CD34$^+$ stem cells (Figure 4B). Results were then validated by bisulfite Sanger sequencing, which represents the "gold-standard" technology for DNA methylation studies. According to our method, bisulfite sequencing gave similar readouts (Figure 4C and 4D). Interestingly, further analysis revealed, after normalization against bisulfite conversion efficiency by Methtools 2.05 (http://genome.imbjena.de/methtools/), a 1.8-fold increase of non-CpG methylation (5mCpN) in HG-CD34$^+$ stem cells when compared with NG-CD34$^+$ stem cells (Figure 4E and 4F). Notably, CpG methylation changes were still present after 3-days in NG concentrations (exHG-CD34$^+$ stem cells; Figure 4B through 4D), confirming that HG- and exHG-CD34$^+$ stem cells functional impairment is associated with changes in the epigenetic landscape.

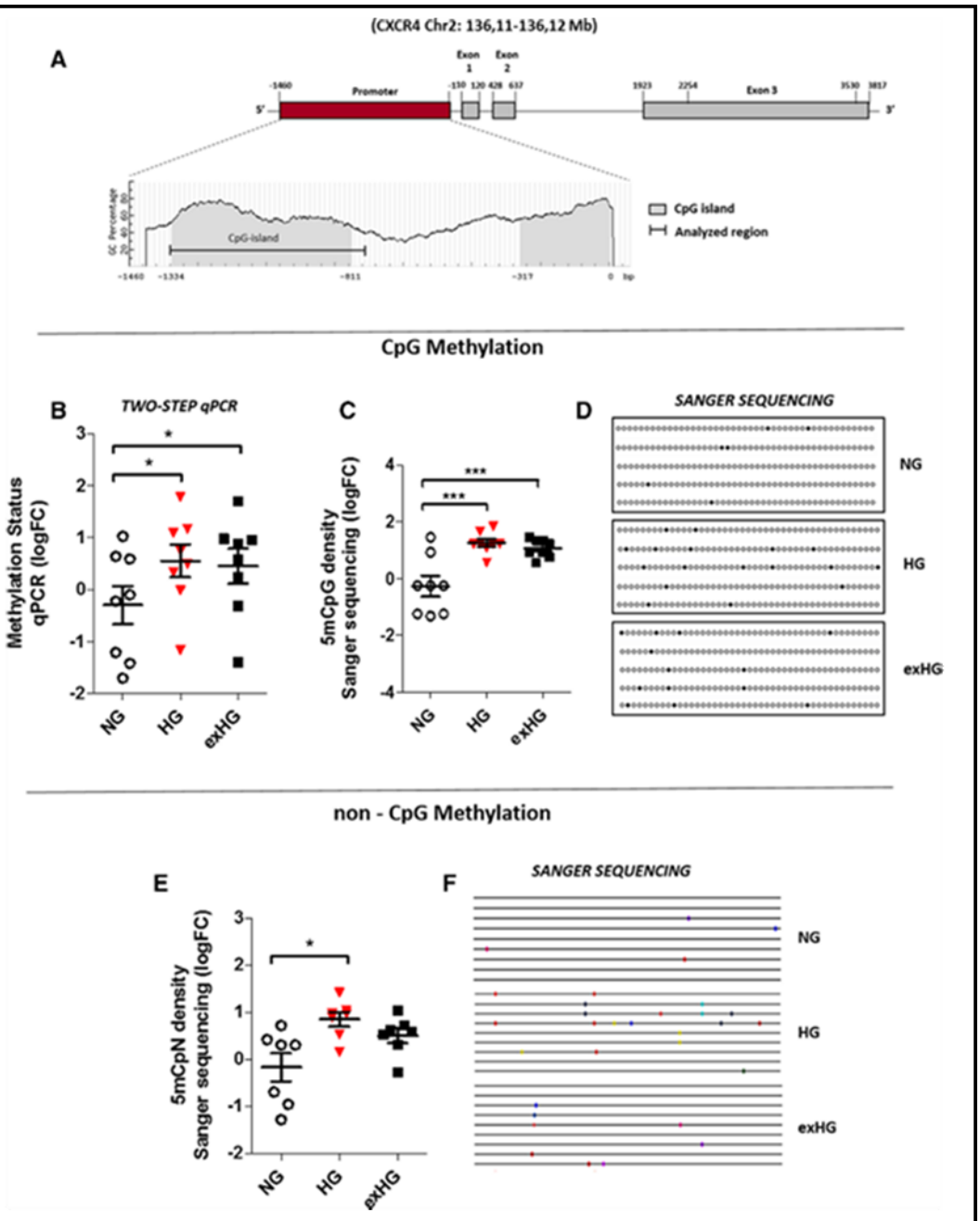

Figure 4. HG increases CXCR4 promoter methylation in CD34⁺ stem cells. A, Schematic representation of the human CXCR4 gene. The CXCR4 gene contains a promoter region (1460 nt) and 3 exons separated by intronic sequences. The analysis of the CXCR4 promoter with MethPrimer software shows the main CpG island (from −1334 to −811 bp) within the promoter and part of the second CpG island (represented in gray). The black line in the CpG island indicates the analyzed region (from −1349 to −738 bp). **B,** Quantification of CpG methylation density of the CXCR4 promoter by a 2-step qPCR method in NG, HG, and exHG-CD34⁺ stem cells (n=8; *P<0.05 vs NG). Data are expressed as log2 fold-change (FC) over NG. **C,** Quantification of CXCR4 promoter methylation levels by bisulfite Sanger sequencing in NG, HG, and exHG-CD34⁺ stem cells (n=8; ***P<0.001 vs NG). The data, expressed as log2FC over NG, are the result of 8 independent experiments where 10 colonies for each single sample were sequenced. **D,** Representative visualization of 5-bisulfite sequencing results as analyzed by QUMA software (http://quma.cdb.riken.jp/). **E,** Non-CpG methylation density of the CXCR4 promoter in NG, HG, and exHG-CD34⁺ stem cells (n=7; *P<0.05 vs NG). The data, expressed as log2FC over NG, are the results of MeMtools 2.05 software analysis after normalization on bisulfite conversion efficiency. As reported for panel C, 10 clones for each cell treatment of 8 independent experiments were sequenced. **F,** Representative visualization of 9-bisulfite sequencing results. Significant differences were evaluated by 1-way ANOVA followed by Newman-Keuls post-hoc analysis. CXCR4 indicates C-X-C chemokine receptor type 4; 5mCpG, methylation at the carbon 5 position of a cytosine ring in CpG (5'-cytosine-phosphate-guanine-3'); 5mCpN, methylation at the carbon 5 position of a cytosine ring in 5'-C-phosphate-nucleotide-3'; exHG, ex-high-glucose; HG, high-glucose; NG, normal-glucose; qPCR, quantitative polymerase chain reaction.

Increased DNA Methylation is Associated with a More-Inactive Chromatin Conformation and Reduced Engagement of RNA Polymerase II to the CXCR4 Promoter

To further evaluate whether increased methylation of the CXCR4 promoter was associated with alterations in histone modification pattern, we performed a chromatin immunoprecipitation assay on repressive histone 3 lysine 27 trimethylation, often associated with DNA methylation, and on the activating acetyl-H4 histone modifications. Interestingly, we found a significant increase of repressive histone 3 lysine 27 trimethylation modification and reduced pan-acetyl-H4 level in HG-CD34$^+$ stem cells (Figure 5B and 5C) confirming that HG treatment correlated with a more-inactive chromatin conformation of the CXCR4 promoter, contributing to the reduction of the gene expression.

Consistently, when we plotted the qPCR data of CXCR4 expression against their respective DNA promoter methylation percentage and performed correlation analysis, we found that CXCR4 mRNA content negatively correlated with promoter methylation in CD34$^+$ stem cells (Pearson r=−0.76; P=0.004; Figure 6A). Moreover, to determinate whether overall epigenetic modifications reduced RNA polymerase II recruitment to the CXCR4 promoter in HG-CD34$^+$ stem cells, we performed chromatin immunoprecipitation assay with an antibody specific for RNA polymerase II. Quantitative real-time PCR analysis of the CXCR4 gene after chromatin immunoprecipitation assay, with 3 couples of primers landing up- and downstream of the transcription start site, displayed a significant reduction of RNA polymerase II binding to the CXCR4 gene in HG-CD34$^+$ stem cells compared with NG-CD34$^+$ stem cells (Figure 6B).

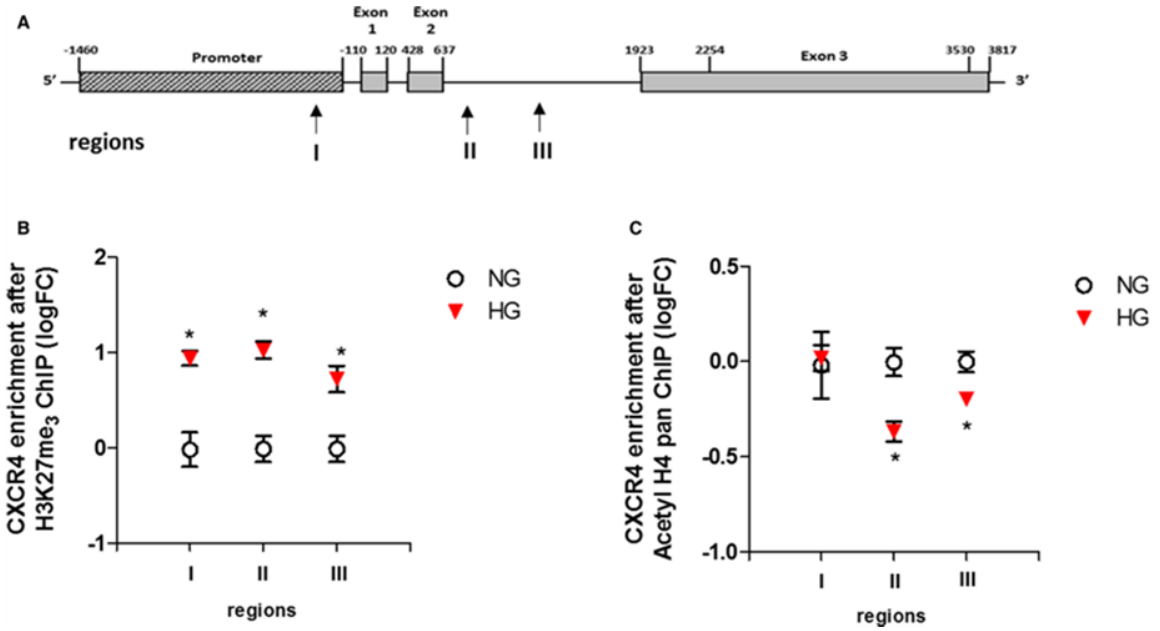

Figure 5. CXCR4 promoter methylation associates with a closer chromatin conformation. **A,** Schematic representation of the CXCR4 gene with mapped sequences (arrows, region I, II, and III). **B and C,** ChIP of the CXCR4 promoter by H3K27me3 and pan-acetyl H4 after HG exposure of CD34+ stem cells. The data after input normalization are expressed as log2 fold-change (FC) over the NG (n=3; *P<0.05 vs NG; paired t test). ChIP indicates chromatin immunoprecipitation; CXCR4, C-X-C chemokine receptor type 4; H3K27me3, trimethylation on lysine 27 of histone H3; pan-acetyl H4, acetyl-histone H4; HG, high-glucose; NG, normal glucose.

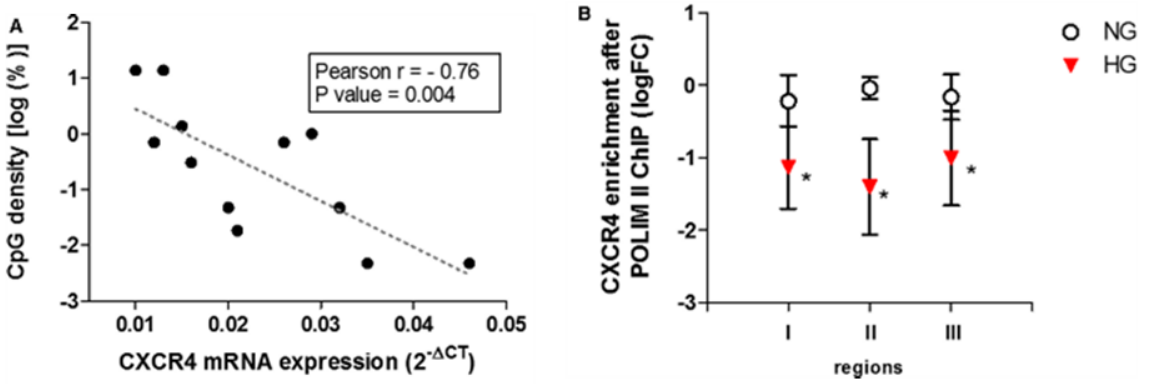

Figure 6. CXCR4 promoter methylation negatively correlates with CXCR4 mRNA expression and reduces engagement of RNA POL II to the gene. **A,** Correlation analysis between normalized quantification of CXCR4 mRNA (2−ΔCt) and log2 percentage of methylated CXCR4 promoter (Sanger sequencing). Pearson r and P value are indicated on the graph. **B,** ChIP of CXCR4 promoter by RNA POL II after HG exposure of CD34+ stem cells. The data, after input normalization are expressed as log2 fold-change (FC) over the NG (n=6; *P<0.05 vs NG; paired t test). CpG indicates methylation in 5′-cytosine-phosphate-guanine-3′; ChIP, chromatin immunoprecipitation; CXCR4, C-X-C chemokine receptor type 4; HG, high-glucose; NG, normal glucose; RNA POL II, RNA polymerase II.

HG Affects Gene Expression Pattern of the "DNA Methylation Machinery" in CD34$^+$ Stem Cells

The enzymes families that catalyze DNA methylation and demethylation process are DNA methyltransferases (DNMTs) and ten eleven translocation proteins (TETs), respectively. DNMTs family includes DNMT1, which is associated with S-phase replication foci and acts primarily as a maintenance methyltransferase, and DNMT3A and DNMT3B that are essential for *de novo* methylation [159, 160]. Interestingly, we observed an unbalance in the "DNA methylation machinery" after HG treatment. Specifically, the analysis of DNMT1 showed a significant downregulation both at mRNA and protein levels, whereas DNMT3B enzyme was increased in HG-CD34$^+$ stem cells (Figure 7A through 7F). No difference was found for DNMT3A expression (data not shown). In addition, the mRNA expression of the two DNA demethylation enzymes, TET2 and TET3, was significantly reduced (Figure 7G and 7H).

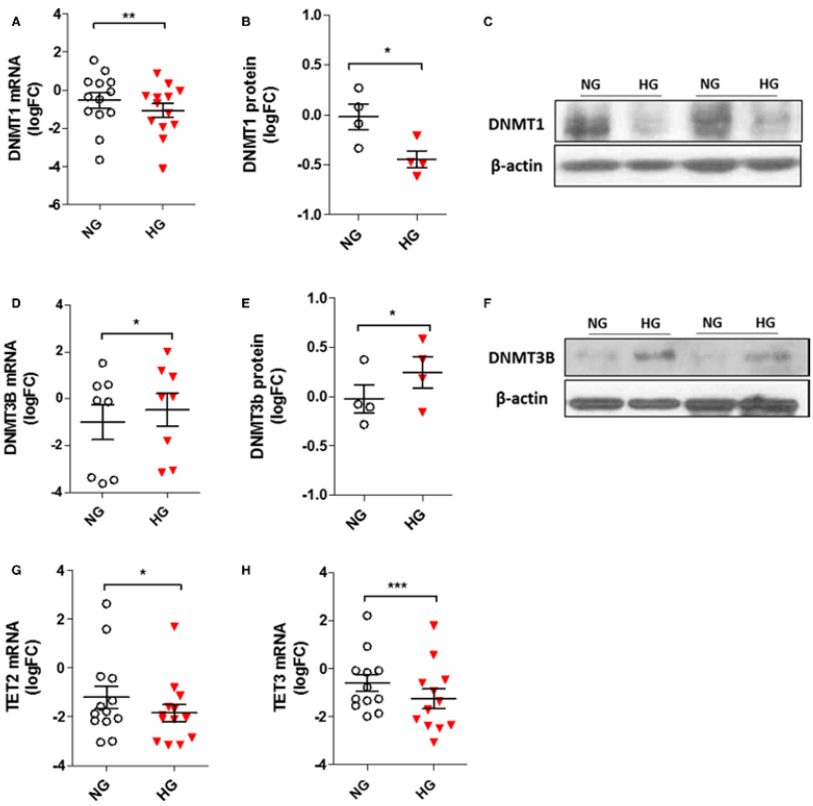

Figure 7. Effect of HG on gene-expression pattern of DNA methylation machinery in CD34$^+$ stem cells. A, D, G, and H, Analysis of DNMT1, DNMT3B, TET2, and TET3 expression in CD34$^+$ stem cells by qPCR. The data from at least 6 independent experiments (*P<0.05; **P<0.01; ***P<0.001 vs NG; paired t test) are expressed as log2 fold-change (FC). B and E, Evaluation of DNMT1 and DNMT3B protein expression by western blot. Analysis of at least 3 independent experiments. The data after loading normalization (β-actin) are expressed as log2FC over the control (NG; *P<0.05 vs NG). C and F, Representative immunoblot images are shown. DNMT1 indicates DNA methyltransferase 1; DNMT3B, DNA methyltransferase 3B; HG, high glucose; NG, normal glucose; qPCR, quantitative polymerase chain reaction; TET2, tet methylcytosine dioxygenase 2; TET3, tet methylcytosine dioxygenase 3.

Translation of Epigenetic Findings on CXCR4 Promoter of CD34$^+$ Stem Cells from Diabetic Patients

To validate our *in vitro* observations in humans, we investigated whether methylation of the CXCR4 promoter and gene expression were altered in BM-derived CD34$^+$ stem cells from T2DM patients. A cohort of NG-tolerant and T2DM male CAD patients (CAD±DM) undergoing coronary artery bypass surgery was enrolled in the study. Importantly, the 2 groups were age-matched in order to exclude any possible confounding effect of age, given that aging is associated with methylation events [161]. Clinical characteristics of patients are reported in Table S1. CD34$^+$ stem cells isolated from sternal BM biopsies were analyzed by flow cytometry for expression of the CXCR4 receptor. As shown in Figure 8A, CD34$^+$ stem cells from CAD-DM patients showed a lower expression of the CXCR4 receptor when compared with the normoglycemic CAD group. Then, we analyzed the DNA methylation density of the CXCR4 promoter by 2-step qPCR and bisulfite Sanger sequencing techniques. Consistently with our *in vitro* results, both methods revealed a significant increase of CpG methylation density at the level of the CXCR4 promoter in BM-derived CD34$^+$ stem cells from CAD-DM patients (Figure 8B trough 8D). Moreover, similarly to what we found in our *in vitro* model, when CXCR4 expression data were plotted against corresponding promoter methylation density data, a significant negative correlation was evident (Pearson r=−0.57; P=0.03; Figure 8E).

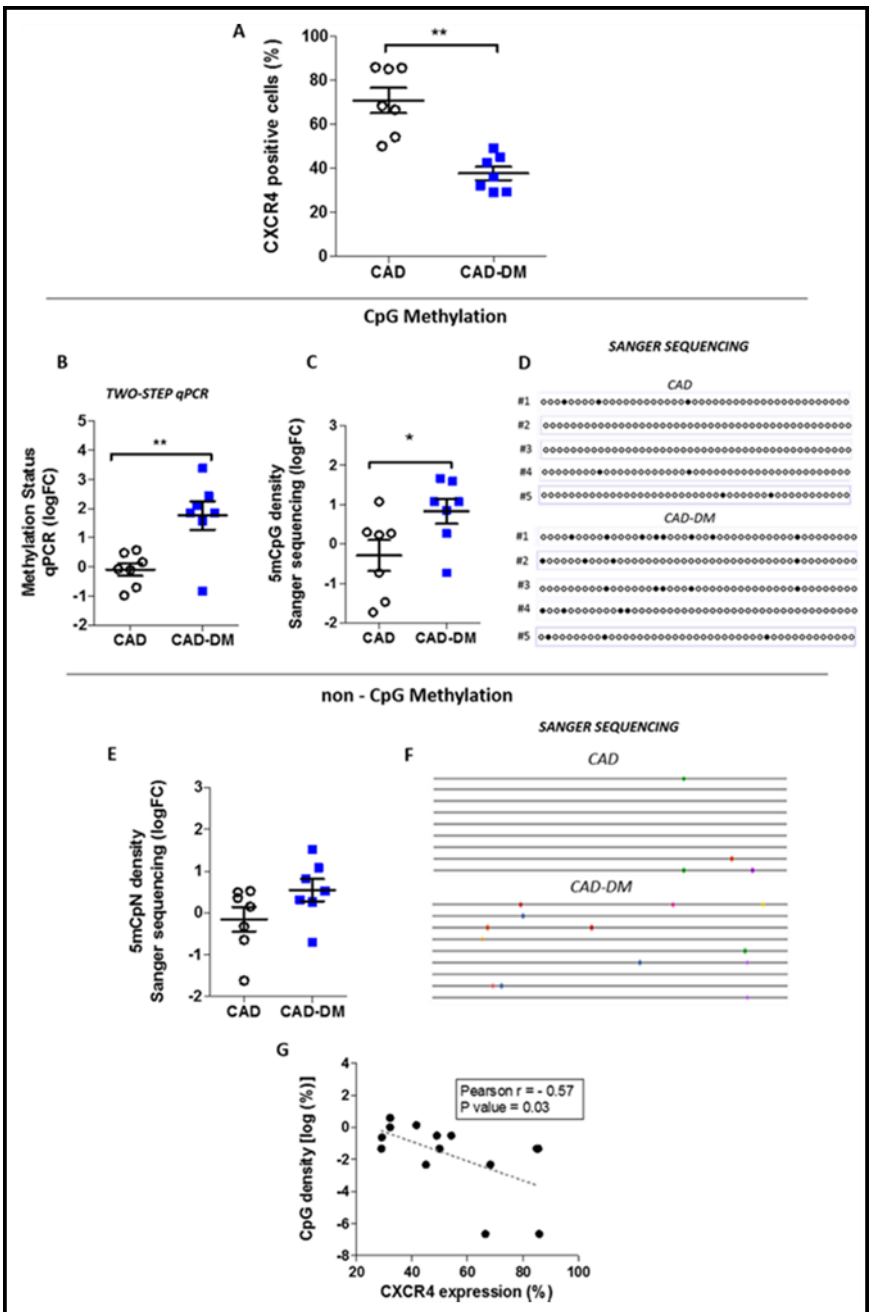

Figure 8. CXCR4 gene methylation and expression in CAD-DM patients. A, CXCR4 membrane expression level in CD34⁺ stem cells derived from CAD and CAD-DM patients by flow cytometric analysis (n=14). Data are expressed as a percentage of positive cells. B, Quantification of CpG methylation density of the CXCR4 promoter by a 2-step qPCR method in CD34⁺ stem cells of CAD and CAD-DM patients. The data are expressed as log2 fold-change (FC) over CAD. C, Bisulfite Sanger sequencing quantification of CXCR4 promoter methylation in CAD and CAD-DM patients. The data are expressed as log2 FC over CAD. Ten colonies for each patient's sample were sequenced. D, Representative visualization of 5-bisulfite sequencing results as analyzed by QUMA software (http://quma.cdb.riken.jp/). E, Non-CpG methylation density of the CXCR4 promoter in CAD and CAD-DM patients. The data, expressed as log2 FC over the CAD, are the results of Methtools 2.05 software analysis after normalization on bisulfite conversion efficiency. As reported for panel C, 10 clones from each patient's sample were sequenced. F, Representative visualization of 5-bisulfite sequencing results. Significant differences between sample patients were evaluated by unpaired t test (CAD, n=7; CAD-DM, n=7; **P<0.01; *P<0.05 vs CAD). G, Correlation analysis between percentage of CXCR4 expression and log2 percentage of CXCR4 promoter methylation (Sanger sequencing). Pearson r and P value are indicated on the graph. CXCR4 indicates C-X-C chemokine receptor type 4; 5mCpG, methylation at the carbon 5 position of a cytosine ring in CpG (5'-cytosine-phosphate-guanine-3'); 5mCpN, methylation at the carbon 5 position of a cytosine ring in 5'-C-phosphate-nucleotide-3'; CAD+DM, coronary artery disease with or without diabetes mellitus; qPCR, quantitative polymerase chain reaction.

Data published in:

- *Vigorelli V., Resta J., Bianchessi V., Lauri A., Bassetti B., Agrifoglio M., Pesce M., Polvani G., Bonalumi G., Cavallotti L., Alamanni F., Genovese S., Pompilio G., Vinci MC., "Abnormal DNA methylation induced by hyperglycemia reduces CXCR4 gene expression in $CD34^+$ stem cells" Journal of the American Heart Association, 8 (2019) Vol. 8 No 9.*
- *Bianchessi V., Lauri A., Vigorelli V., Toia M., Vinci M.C, "Evaluating the methylation status of CXCR4 promoter: A cost-effective and sensitive two-step PCR method" Analytical Biochemistry, 519 (2017) 84-91.*

Part II

Hyperglycemia epigenetically propels CD34$^+$ stem cells differentiation towards a more pro-inflammatory monocyte subpopulation

HG Induces a Senescent-Associated Secretory Phenotype (SASP) in CD34$^+$ Stem Cells

Since the decrease in proliferation induced by HG exposure (Part I; Figure 1A) was independent of the phenomenon of apoptosis (Figure 1A), we exploited qPCR to evaluate the expression of p27 and p21genes, cyclin-dependent kinase inhibitors involved in cell cycle arrest and senescence [162]. As shown in Figure 2B and 2C, HG-CD34$^+$ stem cells displayed a significant upregulation of p27 and p21 genes, which persisted despite the return in NG condition (exHG-CD34$^+$ stem cells) when compared to NG-CD34$^+$ stem cells.

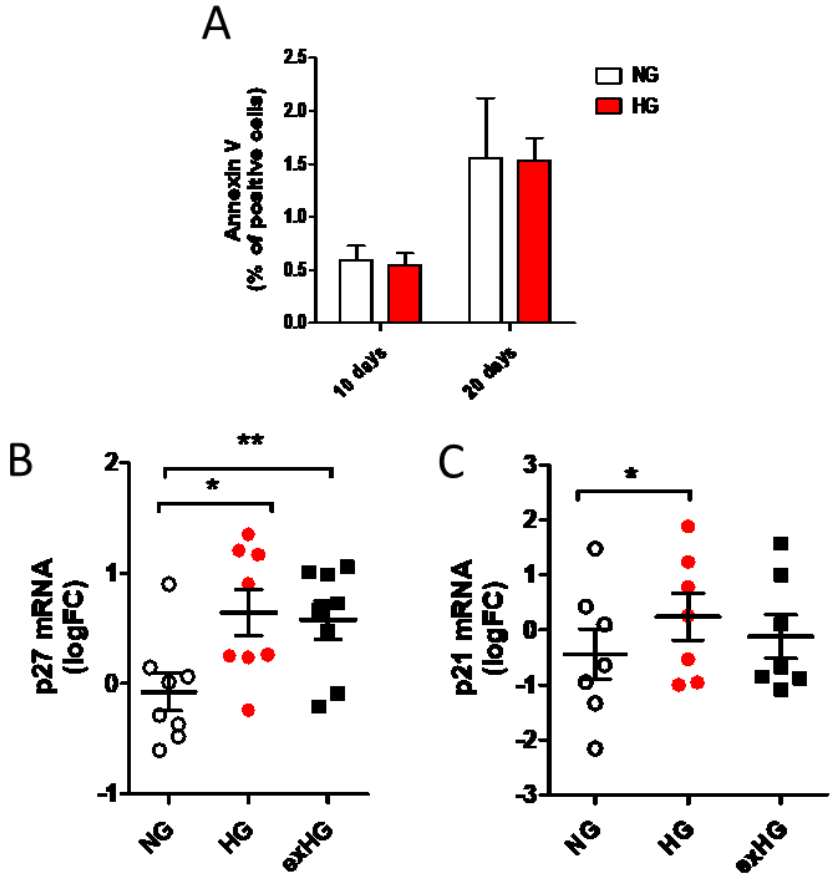

Figure 1. Flow cytometric analysis of apoptosis by Annexin V. After 10 and 20 days of culture, no significant differences were found between the two cell populations (n=3; NG vs HG; paired t-test). HG increases p27 and p21 gene expression in CD34$^+$ stem cells. **A**, qPCR analysis of p27 mRNA expression in NG, HG and exHG-CD34$^+$ stem cells (n=8 *P<0.05, **P<0.01 vs NG, 1-way ANOVA followed by Newman-Keuls post-hoc analysis.). In exHG condition p27 increased expression remains significant. **B**, qPCR analysis of p21 mRNA expression in NG, HG and exHG-CD34$^+$ stem cells (n=8 *P<0.05 vs NG, 1-way ANOVA followed by Newman-Keuls post-hoc analysis.).

Cell senescence is known to be associated with an inflammatory phenotype [163]; Thus, we investigated whether elevated glucose levels could induce the secretion of inflammatory cytokines in $CD34^+$ stem cells. Two important cytokines play a fundamental role in the inflammation of diabetic patients: IL-6 and TNFα [163]. We therefore evaluated their gene expression and secretion in cells grown under NG, HG and exHG conditions by qPCR and E.L.I.S.A. assay, respectively. Data showed a significant increased expression (Figure 2A and 2B) and secretion (Figure 2C and 2D) of TNFα and IL6 cytokines in HG-$CD34^+$ stem cells when compared with NG-$CD34^+$ stem cells. Interestingly, this HG-induced senescent/inflammatory profile persisted in exHG-$CD34^+$ stem cells.

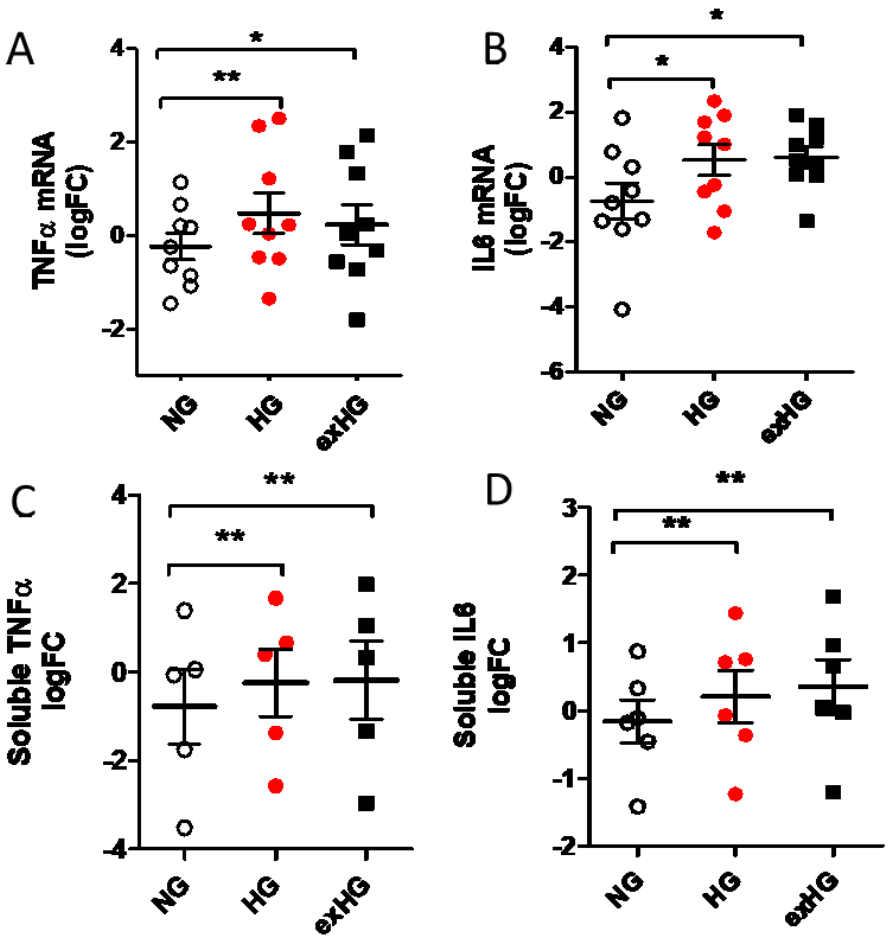

Figure 2. Quantification of TNFα and IL6 gene expression and secretion in $CD34^+$ stem cells under NG, HG and exHG conditions. **A** and **B**, qPCR quantification reveals a significant increase of TNFα and IL6 mRNA expression following exposure to HG levels and after restoration of normal glucose concentration (exHG) compared to control (NG) (log2 fold-change (FC); n=9 *P<0.05; **P<0.01 vs. NG; 1-way ANOVA followed by Newman-Keuls post-hoc analysis.). **C** and **D**, Quantification of secreted cytokines in the conditioned media showed a significant increase in TNFα and IL6 production in cells exposed to HG levels and after the restoration of a normoglycaemic environment (exHG) compared to control (NG). (C) (log2FC; n=5 *P<0.05 vs. NG; paired t-test); (D) (log2FC; n=6 **P<0.01 vs. NG; 1-way ANOVA followed by Newman-Keuls post-hoc analysis.).

HG Induces Epigenetic Activation of NFkB-p65 Gene

To mechanistically investigate the pathways involved in pro-inflammatory SASP phenotype of HG- and exHG-CD34$^+$ stem cells, we assessed the expression of the transcription factor NFkB-p65, which is primarily involved in the expression of pro-inflammatory genes. Consistently, we found a significant up-regulation of NFkB-p65 gene in HG-CD34$^+$ stem cells that persisted after the return in NG condition (exHG-CD34$^+$ stem cells) (Figure 3A). Interestingly, NFkB-p65 gene up-regulation in HG-CD34$^+$ stem cells was correlated with a significant lowering of repressive H3K9me3 epigenetic modification and with an increased recruitment of RNA Polymerase II (POLII) binding at the gene promoter (Figure 3B and 3C). Moreover, the screening of the main epigenetic enzymes revealed an increase in KAT2B expression in HG- and exHG-CD34$^+$ stem cells (Figure 3D). KAT2B is a histone acetyl-transferase also implicated in NFkB-p65 acetylation and accordingly, HG- and exHG-CD34$^+$ stem cells showed increased levels of Acetyl-K310 NFkB-p65 (Figure 3E). This post-translational modification is involved in the translocation and stabilization of NFkB-p65 at nuclear level. Consistently, Image Stream analysis confirmed increased translocation of the transcription factor NFkB-p65 at nuclear level in HG-CD34$^+$ stem cells as witnessed by greater co-localization of NFkB with the DRAQ5 DNA intercalator (Figure 3F).

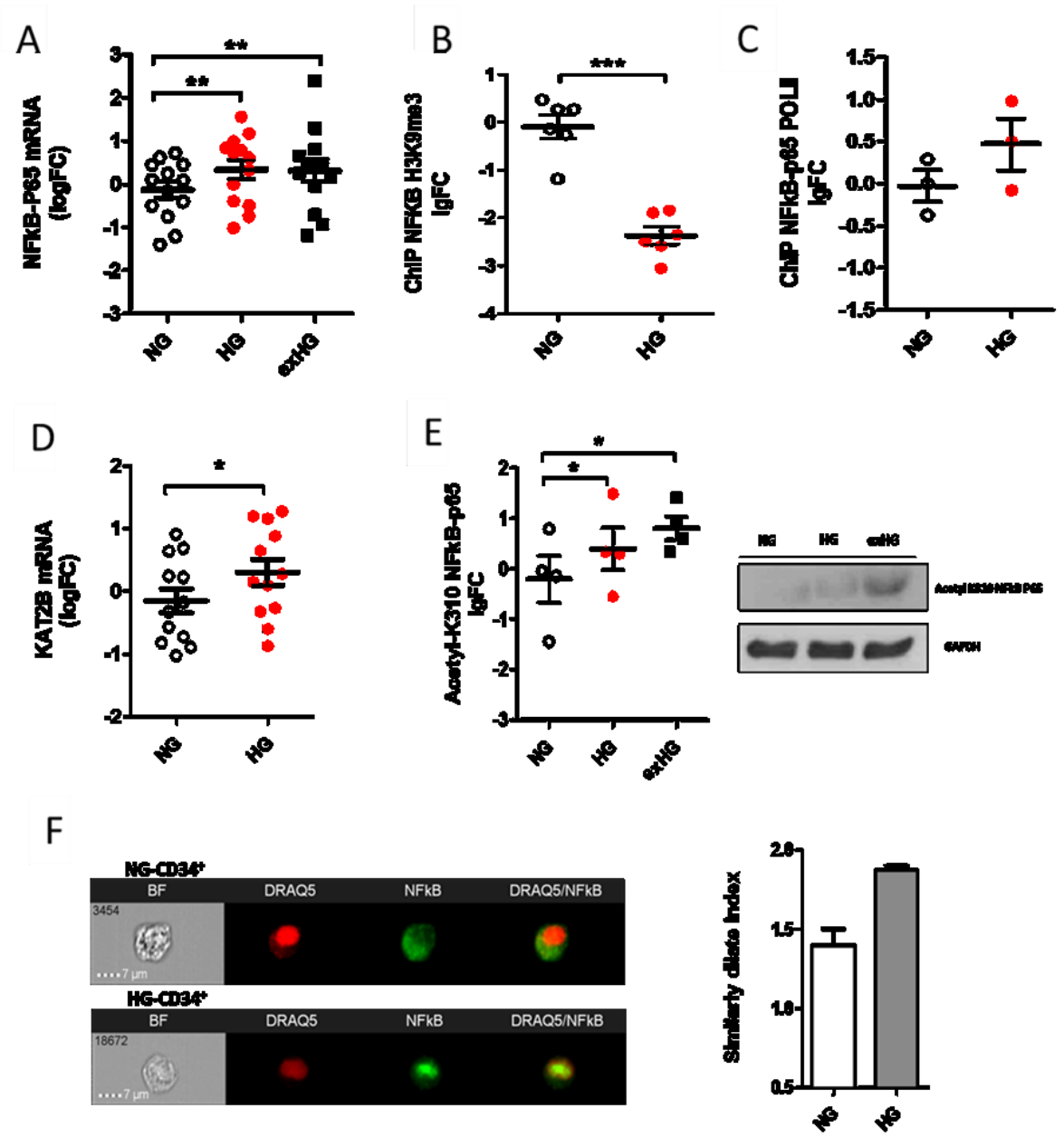

Figure 3. Effects of HG concentration on NFkB-p65 transcription factor expression and activation. **A**, qPCR revealed NFkB-p65 gene up-regulation in HG- and exHG-CD34$^+$ stem cells (log2FC; n=13 **P<0.01 vs. NG; 1-way-ANOVA). **B**, ChIP analysis to evaluate H3K9me3 levels on NFkB-p65 promoter. The analysis showed a reduction of this repressive epigenetic modification in HG-CD34$^+$ stem cells (log2FC; n=6 ***P<0.001 vs NG; paired t-test). **C**, ChIP analysis also indicated, even if still not significant, higher levels of RNA POLII recruitment on NFkB-p65 promoter after HG treatment (log2FC; n=3; paired t-test). **D**, qPCR quantification of KAT2B gene expression which resulted to be increased in HG-CD34$^+$ stem cells (log2FC; n=12 *P<0.05 vs NG; paired t-test). **E**, Evaluation of post-translational modification Acetyl-K310 in NFkB-p65 transcription factor by Western Blot. The analysis revealed higher acetylation levels in cells treated with HG concentration and after the restoration of a normoglycaemic environment (exHG) (log2FC; n=4 *P<0.05 vs NG; 1-way-ANOVA). **F**, Image Stream analysis: representative image of nuclear NFkB translocation in HG conditions compared to control (NG). The graph shows the "Similarly dilate index" (SDI) (the higher the SDI value, the greater the superimposition of the fluorescence of the NFkB marker with the fluorescence of the nuclear marker DRAQ5).

HG Exposure Induces Abnormal Expansion of Monocyte Subpopulations with High Inflammatory Phenotype

To assess whether HG exposure *per se* was able to favor $CD34^+$ stem cells differentiation into a more aggressive monocyte subpopulation, potentially contributing to the establishment of the systemic pro-inflammatory/atherosclerotic states in T2DM, we proceeded with the i*n vitro* differentiation of NG and HG-$CD34^+$ stem cells toward myeloid lineage. Results showed that, while HG exposure did not affect the number of total generated $CD14^+$ cells (Figure 4A), HG-$CD34^+$ stem cells generated higher levels of alternative pro-inflammatory monocyte ($CD14^+CD16^+$) (Figure 4B) and lower levels of classical monocyte ($CD14^+CD16^-$) (Figure 4C), a population known to play an integral role in inflammation shaping and resolution [164].

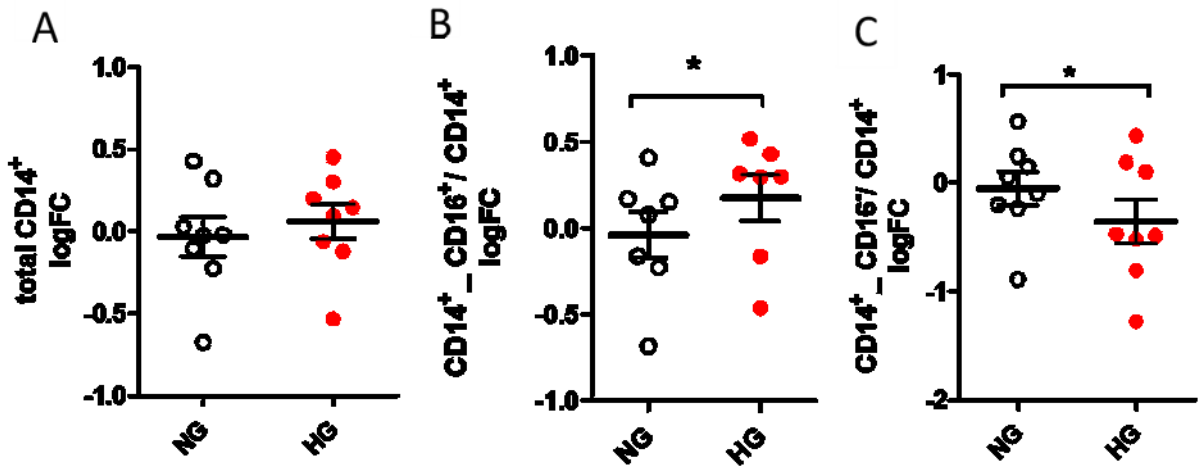

Figure 4. HG skews $CD34^+$ stem cells differentiation toward alternative pro-inflammatory monocyte. **A**, Number of $CD14^+$ total population by FACS in NG- and HG-$CD34^+$ stem cells (log2FC; n=8). **B**, Number of intermediate $CD14^+CD16^+$ population gated on total $CD14^+$ population by FACS (log2FC; n=7 *P<0.05; vs NG; paired t-test). **C**, Number of classical $CD14^+CD16^-$ population gated on total $CD14^+$ population by FACS (log2FC; n=8 *P<0.05; vs NG; paired t-test).

We further characterized the phenotype of the monocyte populations obtained *in vitro* from NG and HG-CD34^{+} stem cells. Interestingly, monocytes generated from HG-CD34^{+} stem cells maintained a senescent/inflammatory profile, with an enhanced expression of TNFα, IL6 and NFkB-p65 genes (Figure 5C, 5D and 5E).

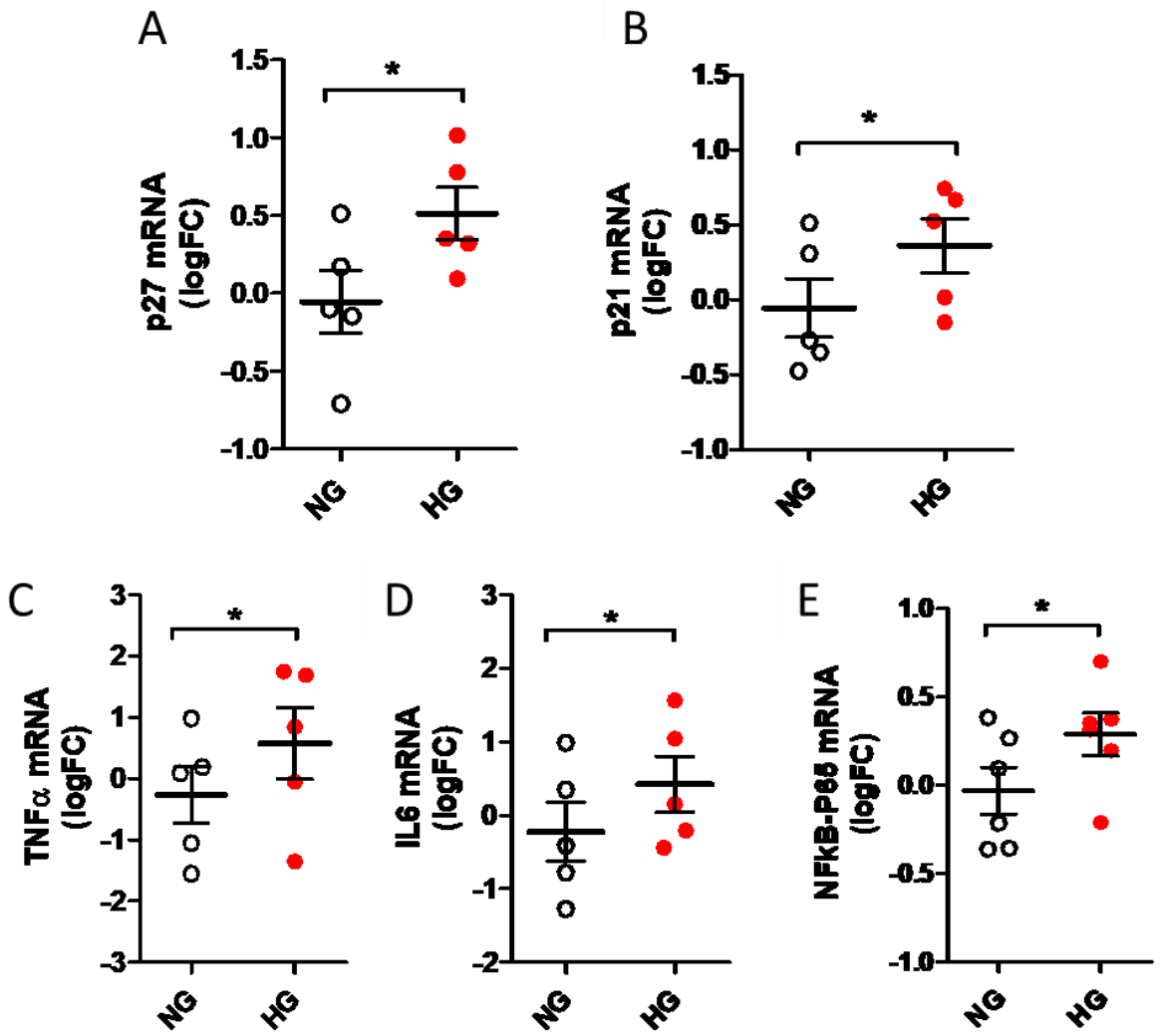

Figure 5. HG increased p27, p21, TNFα, IL6 and NFkB-p65 genes in the monocyte population differentiated from CD34^{+} stem cells. qPCR analysis of p27 (**A**), p21 (**B**), TNFα (**C**), IL6 (**D**) mRNA expression (log2FC; n=5 *P<0.05 vs NG; paired t-test). E) qPCR analysis of NFkB-p65 mRNA expression (log2FC; n=6 *P<0.05 vs NG; paired t-test).

Profile Analysis of CD34$^+$ Stem Cells from Diabetic Patients

To validate our *in vitro* findings, we investigated whether CD34$^+$ stem cells isolated from bone marrow (BM) sternal biopsies of T2DM patients with coronary artery disease (CAD-DM) displayed the same SASP phenotype. We analyzed the expression of both senescent, p27 and p21 (Figure 6A and 6B), and inflammatory markers, TNFα, IL6 and NFkB-p65 (Figure 6C, 6D and 6E). Interestingly, all these parameters resulted up-regulated, even if p21 and IL6 not significantly, in CAD-DM CD34$^+$ stem cells when compared with CD34$^+$ stem cells from CAD patients. Clinical characteristics of patients are reported in Table S2.

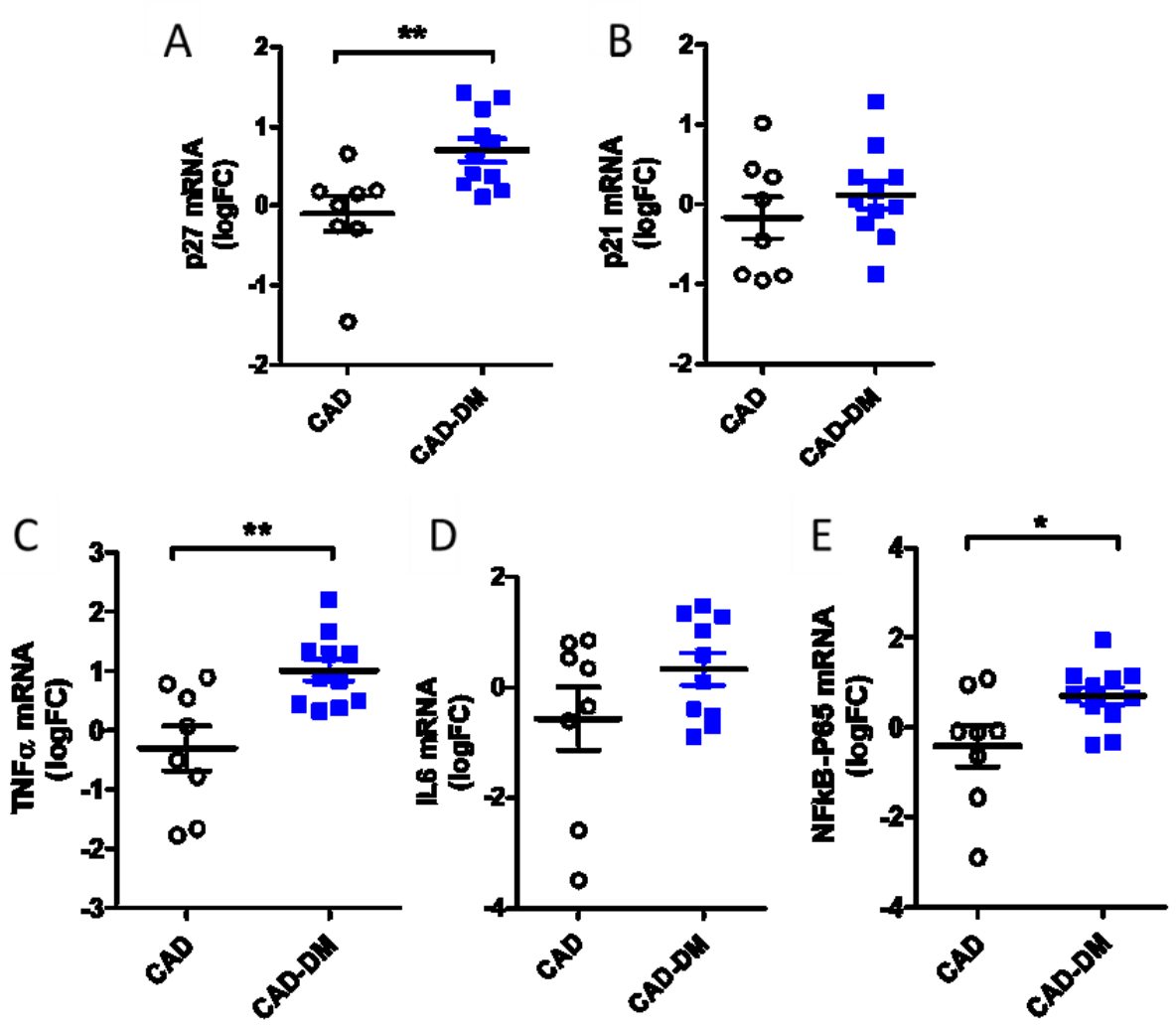

Figure 6. BM-derived CD34$^+$ stem cells from CAD-DM patients displayed a senescent/inflammatory profile compared to CAD patients. qPCR analysis of p27 (**A**), p21 (**B**), TNFα (**C**), IL6 (**D**) and NFkB-p65 mRNA expression (log2FC; n=8 CAD, n=11 CAD-DM, *P<0.05; **P<0.01 vs CAD; unpaired t-test).

We then assessed whether the SASP profile of CAD-DM-derived $CD34^+$ stem cells was sufficient to drive their differentiation into aberrant inflammatory subpopulations. We therefore proceeded with *in vitro* myeloid differentiation. Differently from our *in vitro* model of diabetes, CAD-DM $CD34^+$ stem cells generated higher levels of total $CD14^+$ cells (Figure 7A) whereas, consistently with *in vitro* data, we observed a significant expansion of alternative pro-inflammatory monocytes ($CD14^+CD16^+$) (Figure 7B) and a decreased number of classical monocytes ($CD14^+CD16^-$) (Figure 7C).

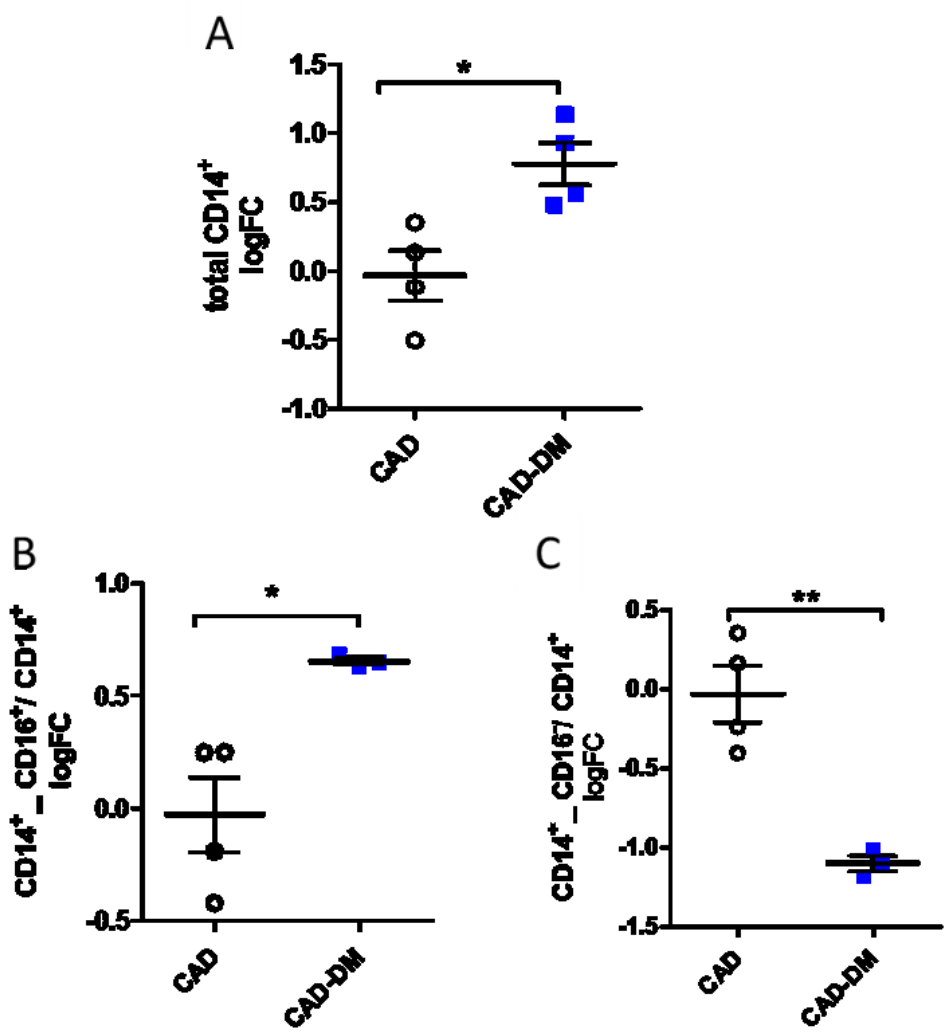

Figure 7. The differentiation of BM-derived $CD34^+$ stem cells from CAD-DM patients was skewed toward alternative pro-inflammatory monocyte. Number of total $CD14^+$ population (**A**), intermediate $CD14^+CD16^+$ population (**B**) and classical $CD14^+CD16^-$ population (**C**) analyzed by FACS (log2FC; n=4 *P<0.05; **P<0.01 vs CAD; paired t-test).

Manuscript in preparation:

- *Vigorelli et al. "Hyperglycemia epigenetically propels $CD34^+$ hematopoietic stem cell differentiation toward more pro-inflammatory monocyte subpopulations"*

Part III

Liraglutide Prevents CD34$^+$ Stem Cell Dysfunction Induced by High Glucose Concentrations

CD34$^+$ Stem Cells Express Glucagon like Peptide-1 Receptor (GLP-1R)

Besides glucose control, Liraglutide (LIRA) exerted beneficial effects by reducing major adverse CV events (MACE) and/or CV mortality in T2DM patients [138]. In order to investigate whether part of CV protective effects of LIRA were mediated by prevention of CD34$^+$ stem cells dysfunction induced by HG, we analyzed, for the first time, the expression of GLP-1R in these cells. Data showed the expression of GLP-1R in UCB-CD34$^+$ stem cells at both mRNA (Figure 1A) and surface protein (Figure 1B).

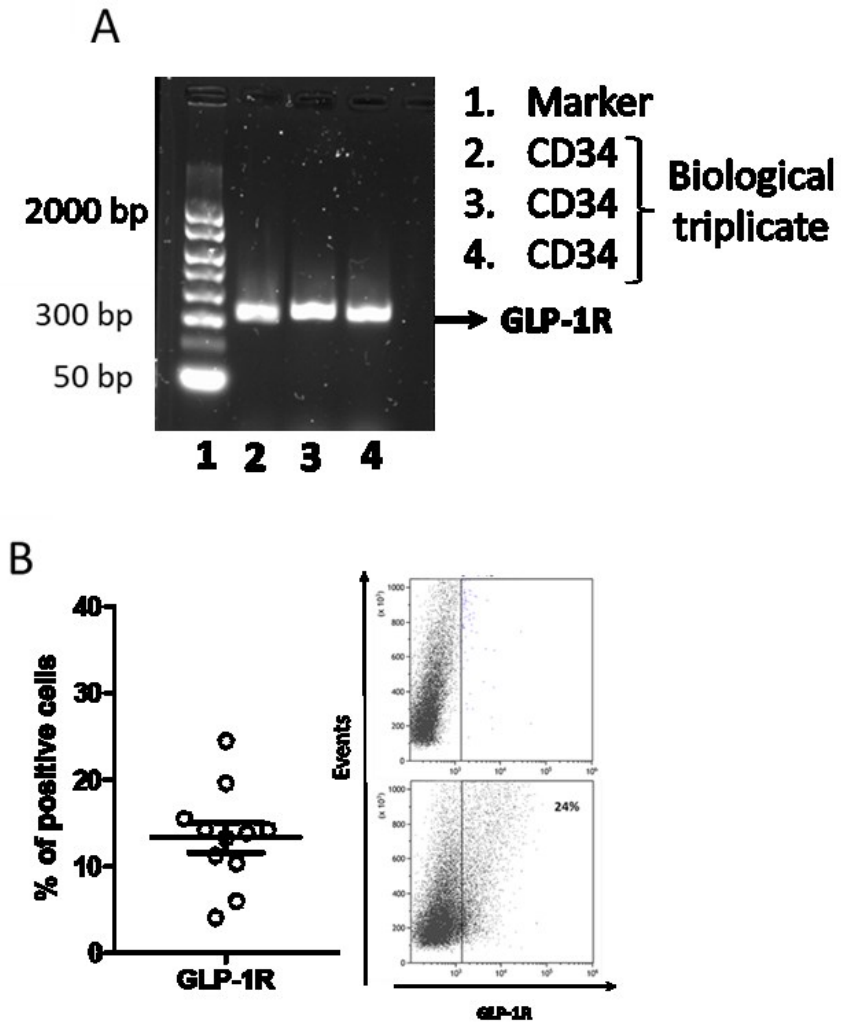

Figure 1. Evaluation of GLP-1 expression in UCB-CD34$^+$ stem cell. **A**, qPCR analysis revealed that CD34$^+$ stem cell express GLP-1 Receptor mRNA. **B**, Flow cytometer analysis show GLP-1 Receptor protein surface expression.

Furthermore, since GLP-1R can be coupled also to Gs protein [129], we verified its activation through the measurements of intracellular cAMP levels. LIRA treatment of $CD34^+$ stem cells resulted in a significant increase of cAMP levels after 5 minutes of LIRA 100nM stimulation with a further increase after 10 minutes (Figure 2A). To verify LIRA-dependent cAMP accumulation, we pre-treated cells with the selective GLP-1R antagonist Exendin (9-39) amide (EXE). Results showed that the dose-dependent increase of intracellular cAMP levels after 10 minutes of stimulation with LIRA was prevented by EXE (Figure 2B).

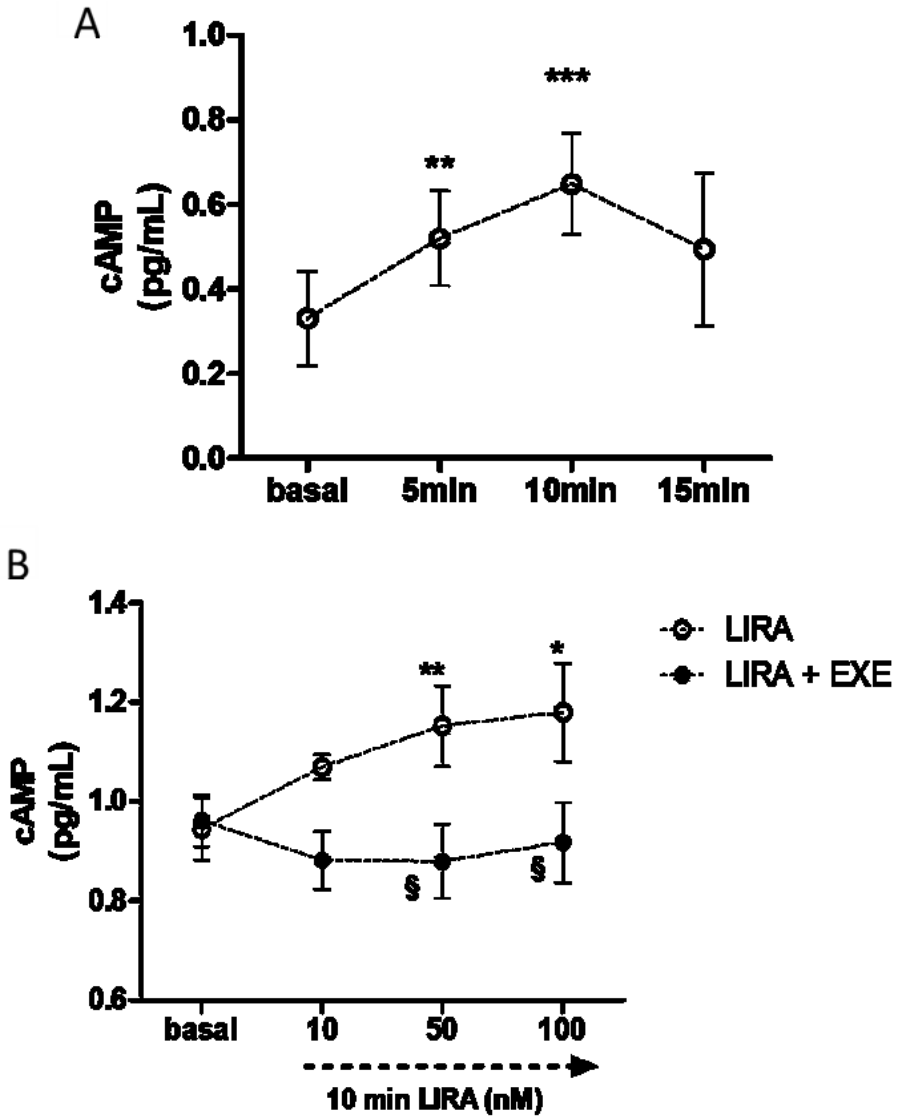

Figure 2. Evaluation of cAMP intracellular levels after LIRA stimulation. **A**, Direct cAMP E.L.I.S.A quantification revealed the increase of cAMP in $CD34^+$ stem cells after 5 and 10 minutes of LIRA 100nM treatment (n=7 **P<0.01, ***P<0.001 vs basal; 1-way ANOVA). **B**) Direct cAMP E.L.I.S.A quantification showed the inability of LIRA to promote dose-dependent intracellular cAMP increase in $CD34^+$ stem cells treated with EXE 150nM (n=4 *P<0.05, **P<0.01 vs LIRA; 2-way ANOVA).

LIRA Treatment Protects CD34$^+$ Stem Cells from the Functional Impairment induced by HG Exposure

In order to assess whether LIRA was able to prevent CD34$^+$ stem cell dysfunction induced by hyperglycemia, CD34$^+$ stem cells were cultured in HG (30mmol/L) ± LIRA (50nM and 100nM) condition up to 20 days. Cells were counted after 5, 10 and 20 days. The growth curve analysis revealed that LIRA100nM treatment was able to restore CD34$^+$ stem cells proliferation to the control level (NG) (Figure 3A). Moreover, LIRA treatment counteracted mitochondrial ROS production induced by HG in a dose-dependent manner (Figure 3B). Notably, LIRA was also able to prevent, again in dose-dependent manner, HG-induced CXCR4/SDF-1α axis impairment, as demonstrated by the maintenance of CXCR4 expression (Figure 3C) and migration ability of the cells toward SDF-1α (50 ng/mL) (Figure 3D).

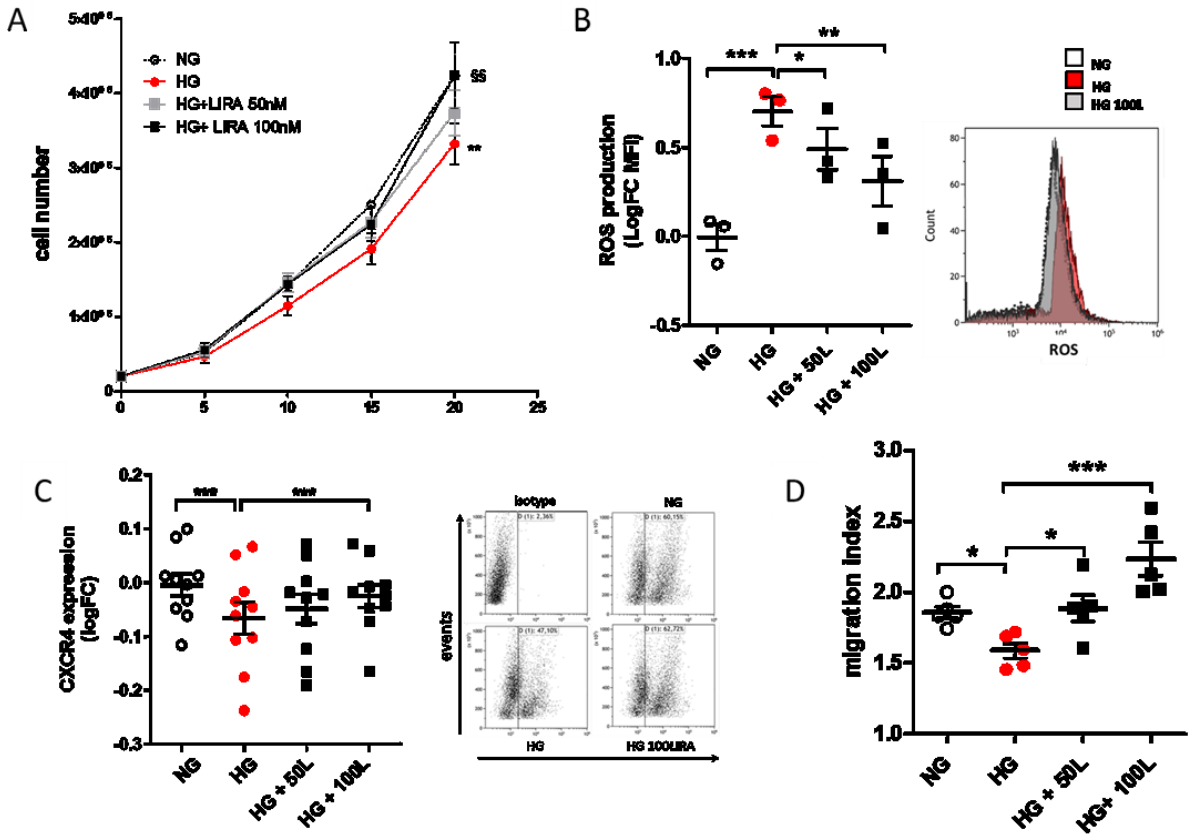

Figure 3. Effect of LIRA on proliferation, oxidative state and CXCR4/SDF1-alpha axis in CD34$^+$ stem cells cultured in HG conditions. **A**, Proliferation curves of CD34$^+$ stem cells exposed to HG (30 mmol/L) concentrations ± 50nM and 100nM LIRA (n=6; **P<0.01 vs HG; 2-way ANOVA). **B**, Flow cytometric quantification of ROS production in HG ± LIRA-treated cells and representative flow cytometry histogram of ROS quantification. Data are reported as log2 fold-change (FC) of MFI over control (NG; n=3; *P<0.05, **P<0.01, ***P<0.001 vs HG). **C**, CXCR4 membrane expression level in NG, HG±LIRA-CD34$^+$ stem cells by flow cytometric analysis (n=9; **P<0.01, ***P<0.001 vs HG) and representative flow cytometry dot-plots of CXCR4 quantification. Data are expressed as logFC over the ctr. **D**, A) Migration ability of NG and HG±LIRA-CD34$^+$ stem cells toward SDF-1α chemokine (n=5;

*P<0.05; ***P<0.001 vs HG). Significant differences were evaluated by 1-way ANOVA followed by Newman-Keuls post-hoc analysis.

LIRA Treatment Activates Pro-Survival Pathways in CD34$^+$ Stem Cells

AKT and ERK1/2 proteins drive two important signaling pathways implied in cell survival and proliferation [165, 166]. We therefore investigated whether the activation of these pathways could be involved in the protective effect of LIRA on CD34$^+$ stem cells. Interestingly, we observed increased phosphorylation of both ERK1/2 and AKT (Ser473) proteins after 5 and 10 minutes of LIRA100nM stimulation, with a following reduction after 15 minutes (Figure 4A). Consistently, LIRA activation of AKT and ERK1/2 pathways was prevented by the use of the GLP-1R antagonist EXE, 150nM (Figure 4B).

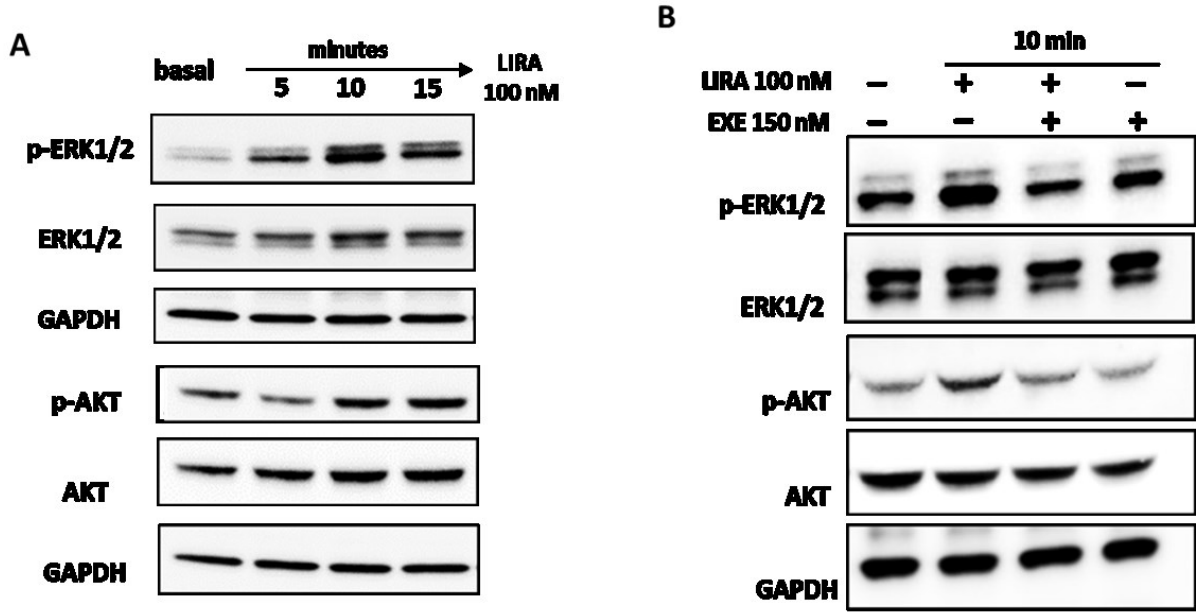

Figure 4. Evaluation of AKT and ERK 1/2 pathway involvement in LIRA protective action. **A**, Representative Western blot analysis of phospho-AKT and phospho-ERK1/2 levels in CD34$^+$ stem cells after 5, 10 and 15 minutes of LIRA100nM stimulation (n=3). **B**, Western blot analysis of phospho-AKT and phospho-ERK1/2 levels in CD34$^+$ stem cells after 10 min minutes of: LIRA100nM stimulation alone, and following EXE150nM pre-incubation a (n=3).

LIRA Treatment Prevented Up-Regulation and Nuclear Translocation of NFkB-p65 Transcription Factor in HG-Treated CD34$^+$ Stem Cells

We further investigated if LIRA treatment was able to counteract the pro-inflammatory profile induced by HG concentration in CD34$^+$ stem cells. We analyzed the expression and the nuclear translocation of the transcription factor NFkB-p65. Notably, qPCR and Image Stream analysis showed that LIRA treatment was able, although not significantly, to counteract the up-regulation of NFkB-p65 expression (Figure 5A) and its nuclear translocation in HG-CD34$^+$ cells. (Figure 5B).

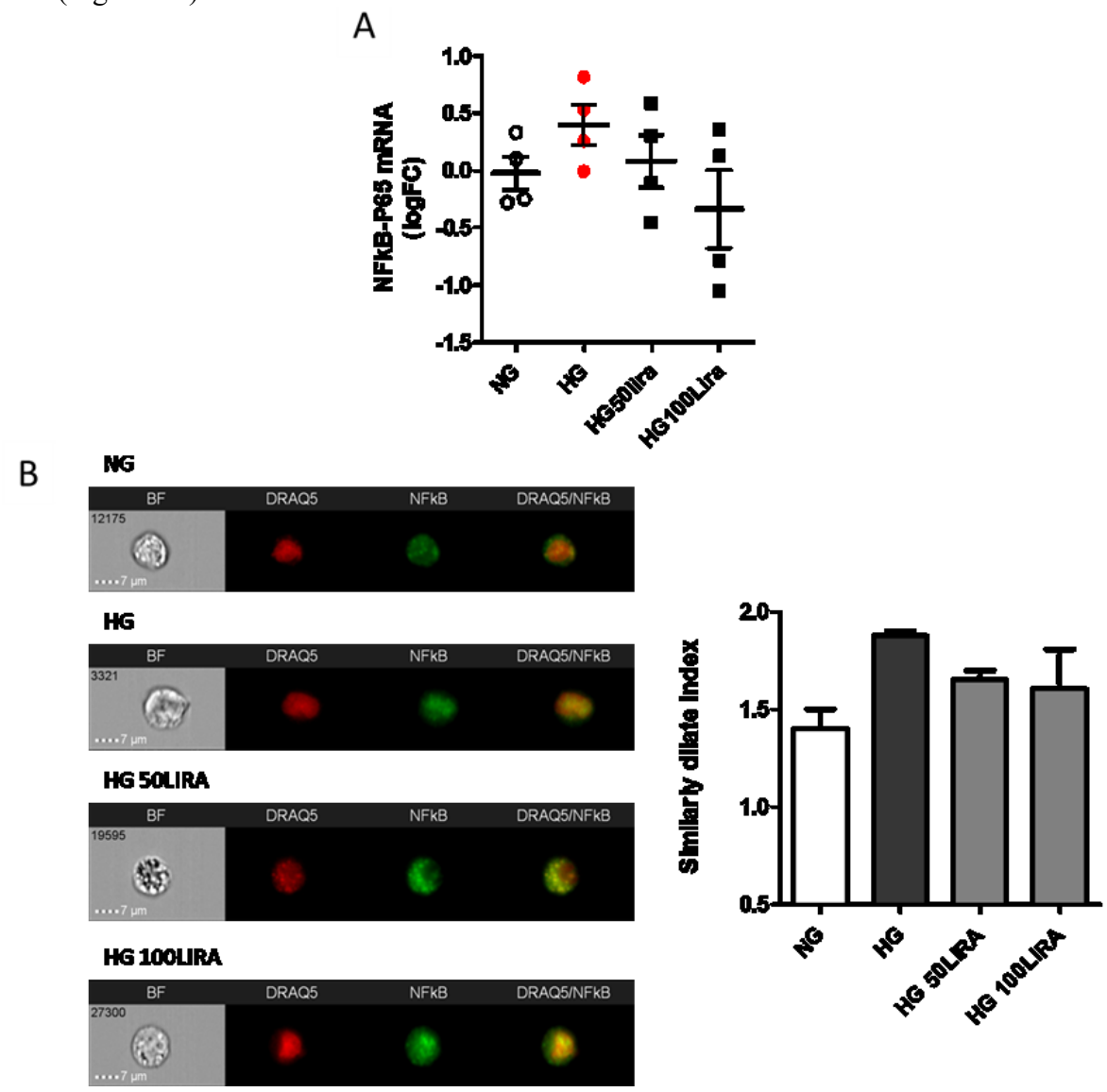

Figure 5. A, qPCR quantification of NFkB-p65 expression in CD34$^+$ stem cells in HG±LIRA treatment (n=4). **B**, Image Stream analysis: representative image of the increased translocation at nuclear level of NFkB in HG conditions compared to control (NG), which resulted to be reduced by LIRA treatment. The graph showing the "Similarly dilate index" (SDI) (the higher the SDI value, the greater the superimposition of the fluorescence of the NFkB marker with the fluorescence of the nuclear marker DRAQ5) (n=2).

LIRA Treatment Reduces Monocyte Generation from HG-Treated CD34$^+$ Cells

To conclude, in order to assess whether LIRA may also possess an anti-inflammatory action by exerting immunomodulatory activity, CD34$^+$ stem cells grown in hyperglycemic condition ± LIRA were differentiated in myeloid progenitors. The results showed that 100nM LIRA treatment did not affect the number of total CD14$^+$ positive cells (Figure 6A), but was able to reduce the expansion of alternative pro-inflammatory monocytes (CD14$^+$CD16$^+$) (Figure 6B) induced by HG exposure and to restore classical monocyte population (CD14$^+$CD16$^-$) to the control levels (NG) (Figure 6C).

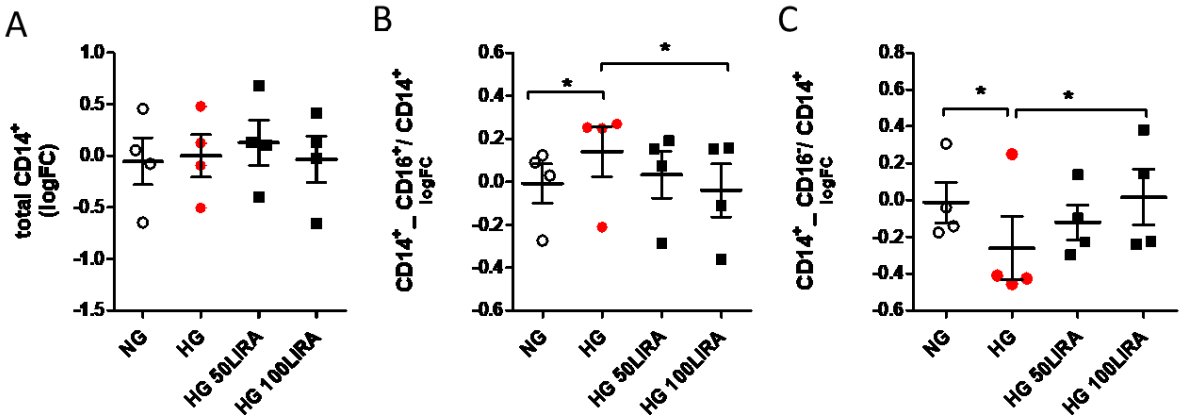

Figure 6. Effects of LIRA treatment on HG-CD34+ stem cells differentiation toward myeloid lineage. **A**, Number of CD14$^+$ total population analysed by FACS did not changed in HG±LIRA treatment (log2FC; n=4). **B**, Number of intermediate CD14$^+$CD16$^+$ population analysed on CD14$^+$ population by FACS (log2FC; n=4 *P<0.05; vs HG; paired t-test). **C**, Number of classical CD14$^+$CD16$^-$ population analysed on CD14$^+$ population by FACS (log2FC; n=4 *P<0.05; vs HG; paired t-test).

Taken together, these data suggest that part of the mechanisms by which LIRA exerts its pleiotropic CV effect could be mediated by improvement and rebalance of CD34$^+$ stem cell functions.

Discussion

Diabetes mellitus (DM) is a chronic and complex disease characterized by hyperglycemia and a systemic pro-inflammatory state, associated with a broad spectrum of micro- and macrovascular complications that constitute a major health care concern in the western world. Indeed, despite advances in glucose lowering therapies, cardiovascular disease (CVD) represents the leading cause of morbidity and mortality in type 2 diabetic (T2DM) patients [167]. To date, it is widely recognized that the depletion induced by DM in number and function of bone marrow (BM)-derived $CD34^+$ stem cells plays a central role in this scenario. This stem cell population, under normal physiological conditions, contributes to the maintenance of vascular homeostasis and regeneration. Overcoming the controversial endothelial progenitor cells (EPCs) definition, the $CD34^+$ stem cell population has been widely investigated for its CV regenerative potential and the ability to promote angiogenesis [79, 168]. In particular, Kawamoto and colleagues reported the superiority of $CD34^+$ stem cell transplantation to total mononuclear cells for the therapeutic neovascularization after myocardial infarction in terms of potency and safety [169]. Notably, it has been demonstrated that the reduction of circulating $CD34^+$ stem cells can predict adverse CV outcomes in T2DM patients, highlighting their CV protective and pro-angiogenic role [170]. Moreover, $CD34^+$ stem cells, as hematopoietic stem/progenitor cells (HSPCs), are also involved in the generation of the blood cell progenies, combining in this cell population both regenerative and immunological properties [171]. Interestingly, T2DM patients display increased inflammation, as shown by the elevation of inflammatory markers [53], the abnormal elevation of inflammatory intermediate ($CD14^{++}CD16^+$) monocytes [56, 57] and the alteration in macrophages polarization [58, 59]. In particular, emerging evidences indicate that chronic hyperglycemia promotes the reduction of circulating proangiogenic progenitors and the increased frequency of circulating pro-inflammatory cells, leading to an unbalance, which culminates in a reduction of the intrinsic vascular homeostatic capacity and in the establishment of a low-grade inflammatory status [101].

Currently, the molecular mechanisms underlying CV complications of DM are still not fully understood. However, emerging evidence suggests that epigenetic modifications might participate to modulate the etiology of DM complications, explaining the onset of the hyperglycemic memory phenomenon [109]. Epigenetic modification induced by hyperglycemia have already been observed in literature both *in vitro* and *in vivo*. Brasacchio et al observed that HG stimulation of endothelial cells induced the recruitment of the histone-lysine N-methyltransferase Set7 enzyme to the RELA gene promoter, with the subsequent increase in monomethylated H3K4 levels. Interestingly, this effect was maintained after cells

were restored in normal glucose (NG) conditions [117]. Moreover, Villeneuve et al demonstrated that vascular smooth muscle cells (VSMC) derived from T2DM *db/db* mice exhibited a persistent atherogenic and inflammatory phenotype due to decreased H3K9me3 levels [172]. However, to date, no data are available on $CD34^+$ stem cells.

The present work sought to investigate whether hyperglycemia might epigenetically induce $CD34^+$ stem cell dysfunction and propel their differentiation program toward pro-inflammatory cell subpopulations. Then, since recently a new class of glucose lowering drugs, including LIRA, have shown a reduction of major adverse CV events raising the possibility to revert hyperglycemic memory and to exert an atheroprotective/anti-inflammatory activity, we hypothesized that LIRA might exert its pleiotropic effects through the protection of $CD34^+$ stem cell function.

In our *in vitro* cell model of hyperglycemia, we show that, after 20 days of HG exposure, umbilical cord blood (UCB)-derived $CD34^+$ stem cells exhibited signs of metabolic exhaustion with a reduced proliferation associated to an increase of reactive oxygen species (ROS) production along with the reduced expression of the antioxidant gene enzymes manganese superoxide dismutase (MnSOD) and catalase (CAT). Notably, such a long period needed for the metabolic overload can be explained considering that stem and progenitor cells are known to intrinsically express high levels of antioxidant enzymes that make them more resistant than mature cells to oxidative stress [173, 174]. Interestingly, HG-$CD34^+$ stem cells also displayed increased expression of p66shc gene. P66Shc is an adaptor protein that plays a crucial role in the development of diabetic vascular complications and mainly contributes to the phenomenon of metabolic memory [151]. The mechanisms described for its pro-oxidant action include the induction of ROS generation and, in agreement with our findings, the inhibition of ROS-scavenging enzymes (i.e. MnSOD and CAT) expression [175].

In our hands, the dampening of proliferation and increased ROS production resulted in $CD34^+$ stem cells dysfunction. Specifically, HG-$CD34^+$ stem cells exhibited significant reduced migration ability toward human recombinant SDF-1α, which was associated with a decreased expression of CXCR4 receptor. It is known that the expression of chemokine receptor CXCR4 is transcriptionally regulated by promoter DNA methylation and its altered modulation is involved in diseases such as cancer [156, 157]. In line with this, our results show that CpG DNA methylation of CXCR4 promoter was influenced by HG exposure *per se* in HG-$CD34^+$ stem cells. Interestingly, both the epigenetic modification and the functional defects persisted after a 3-day recovery in normoglycemic conditions (exHG-$CD34^+$), confirming the existence of the hyperglycemic memory phenomenon in stem cells. Interestingly, the CXCR4 promoter

also displayed a significant non-CpG methylation increase in HG-CD34$^+$ stem cells. DNA methylation in mammals is predominantly reported on the cytosine of the dinucleotide sequence, CpG. However, non-CpG methylation was described in embryonic cells [176], and a recent study by Barres et al provided evidence that non-CpG methylation was present in skeletal muscle of T2DM subjects [177]. The analysis of transcription pattern of enzymes involved in DNA methylation and demethylation showed that the "DNA methylation machinery" was unbalanced by HG exposure. In particular, DNMT3B was upregulated, thus suggestin the involvement of this enzyme in the methylation increase of the CXCR4 promoter, in agreement with the findings reported by Barre et al. [175]. However, further investigations will be necessary to understand the biological role of non-CpG methylation and DNMT3B on gene transcription regulation in the diabetic context.

Moreover, DNA methylation and histone modification systems are known to be highly inter-related and mechanistically rely on each other for chromatin modulation [178]. Consistently, by using a chromatin immunoprecipitation assay, we observed that DNA methylation of the CXCR4 promoter was associated with a significant increase of repressive histone 3-lysine 27-trimethylation modification and reduced level of activating pan-acetyl-H4 in HG-CD34$^+$ stem cells. These modifications cooperate to achieve a closer chromatin structure, which was confirmed by the reduction of RNA polymerase II recruitment at the CXCR4 promoter level in HG-CD34$^+$ stem cells. These findings provide evidence that HG exposure promotes a more-inactive chromatin conformation that contributes to reduce CXCR4 expression by reducing DNA accessibility. Of note, our epigenetic findings were confirmed in bone marrow (BM)-derived CD34$^+$ stem cells of CAD-DM patients. Importantly, all the enrolled patients were selected by accurate matching for age and major risk factors, to partially overcome the study population size, which is relatively small. Overall, these data provide evidence that human CD34$^+$ stem cells can "memorize" the hyperglycemic environment in the form of epigenetic changes that, by contributing to the self-perpetuating alteration of gene expression, may be potentially responsible for progression of microangiopathy and CV events in T2DM subjects despite glycaemia correction.

It is known that the elevated levels of ROS, which are by-products of standard cellular oxidative processes, can generate different cellular responses such as senescence or apoptosis [179, 180]. In our *in vitro* model, the increase of ROS production following HG exposure was associated with p27 and p21 upregulation, two cyclin-dependent kinase inhibitors involved in cell cycle arrest and senescence. These data, in line with the study of Fitzgerald and colleagues, who demonstrated that ROS production induced p21 up-regulation in cancer cells [181], suggest that

hyperglycemia promotes stem cell senescence. Moreover, since senescence is known to associate with a secretory pro-inflammatory phenotype [163, 182], this could explain both the functional and numerical depletion of $CD34^+$ stem cells, as well as their inflammatory phenotype and aberrant differentiation. Consistently, HG-$CD34^+$ stem cells displayed increased expression and release of TNF-α and IL-6 inflammatory cytokines. These cytokines are target genes of the transcription factor NFkB that plays a key role in the regulation of the immune response, inflammation and cell proliferation [183]. Numerous studies indicated that the activation of this transcription factor represents a key event in the early stages of the pathobiology of diabetes and its complications [184-186]. Herein, our data show that HG exposure induced the upregulation of p65 subunit of the NFkB pro-inflammatory transcription factor, along with a reduction of the repressive H3K9me3 epigenetic modification and increased recruitment of RNA POLII binding at the level of gene promoter. Interestingly, KAT2B, a histone acetyl-transferase also involved in NFkB-p65 acetylation, was upregulated by HG exposure. Accordingly, HG- and exHG-$CD34^+$ stem cells showed higher level of acetyl-K310 NFkB-p65 when compared to NG-$CD34^+$ stem cells. This post-translational modification is involved in the translocation and stabilization of NFkB-p65 at nuclear level and, consistently, HG-$CD34^+$ stem cells displayed a greater NFkB-p65 nuclear localization. These data indicate that HG is *per se* able to induce a senescent associated secretory phenotype (SASP), which is likely to be mediated by epigenetic mechanisms. Of note, we found that BM-derived $CD34^+$ stem cells of CAD-DM patients were characterized by the same SASP profile.

Besides multiple vascular complications, the diabetic state is characterized by significantly accelerated rates of inflammation [56, 57, 97, 99, 187]. However, the underlying mechanisms contributing to the excessive inflammation remain poorly understood. Considering that $CD34^+$ stem cells are common precursors of both pro-angiogenic and inflammatory cells, we hypothesized that a differentiation drift induced by hyperglycemic environment could be involved in the establishment of inflammatory state of DM patients. Notably, our experiments demonstrated that HG-$CD34^+$ stem cells not only displayed senescent/inflammatory phenotype but also, when differentiated toward myeloid lineage, generated higher levels of the intermediate ($CD14^{++}CD16^+$) pro-inflammatory monocyte with a persistent senescent/inflammatory phenotype. Strikingly, the *in vitro* myeloid differentiation of BM-derived CAD-DM $CD34^+$ stem cells showed the generation of higher levels of pro-inflammatory intermediate ($CD14^{++}CD16^+$) monocytes when compared with cells derived from CAD patients, despite the use of the same differentiation-protocol condition. These data suggest that DM microenvironment may propel BM-$CD34^+$ stem cell differentiation towards more pro-

inflammatory myeloid subpopulations with consequent relevant implications in the onset of CV complications.

Recently, a new class of glucose lowering drugs, including the incretin analogous LIRA, showed potential CV protective effects: indeed, large clinical trials (NCT01179048; NCT01720446) demonstrated that they are able to reduce major adverse CV events and mortality (MACE) in T2DM patients. This raised the possibility that LIRA treatment could reverse hyperglycemic memory, because the patients enrolled in the study already had a high risk of CVD [138]. Furthermore, experimental evidence obtained both *in vitro* and in animal models has demonstrated the atheroprotective and anti-inflammatory activity of LIRA at different tissue levels [144, 188]. The cellular and molecular effects of LIRA are mediated by GLP-1R, a G_s coupled receptor family member, which is present in most tissues of human body [189]. LIRA has been shown to exert effects on a variety of stem/progenitor cells, including mesenchymal stem cells and adipocyte precursors [145, 146]. However, so far there are no studies in humans describing the cellular and molecular effects of LIRA on function and differentiation of $CD34^+$ stem cells exposed to HG. Our results provide the first evidence of GLP-1R expression and drug-induced activation in human UCB-$CD34^+$ stem cells. Moreover, LIRA, in dose-dependent manner, exerted a protective effect on $CD34^+$ stem cell functions both preventing proliferation and CXCR4/SDF1α axis impairment induced by hyperglycemia. Finally, the drug was able to counteract the up-regulation and translocation of NFkB-p65, as well as abnormal myeloid differentiation of $CD34^+$ stem cells induced by hyperglycemia, suggesting anti-inflammatory and immunomodulatory properties of the drug, even if further experiments will be necessary to finalize the data.
Among the molecular mechanisms driving the circulating progenitors proangiogenic activity, Zuccolo and colleagues recently demonstrated that SDF-1α/CXCR4 activation promotes endothelial colony forming cells migration through a biphasic Ca^{2+} increase, leading to the recruitment of the ERK1/2 and PI3K/AKT signaling [190]. Herein, it would be interesting to set up future experiments on $CD34^+$ stem cells Ca^{2+} signals to better understand the molecular mechanisms underlying both HG and LIRA action on $CD34^+$ stem cell function.

In conclusion, our data show, for the first time, that hyperglycemia-induced epigenetic alterations in human $CD34^+$ stem cells have detrimental effects on functional properties, which persist after normoglycemia recovery, as well as on the differentiation ability where seem to promote the generation of more aggressive monocyte subpopulations. Finally, independently

of metabolic control, LIRA displayed to exert *in vitro* protective effect on $CD34^+$ stem cell function and to hamper the myeloid differentiation boosted by HG exposure *in vitro*. Future investigations in patients would be worthy of attention, because if confirmed, the mechanisms contributing to the drug's CV protective effect will provide new insight for a wider use of GLP1R agonists, including primary CV prevention in DM patients.

Supplementary

Table S1. Clinical characteristics of patients included in DNA methylation analysis of CXCR4 promoter in $CD34^+$ stem cells (Results Part I).

	CAD	CAD-DM	P value
N	7	7	
AGE (years)	64,43 ± 3,963	68,43 ± 3,206	
BMI (KG/m^2)	26,71 ± 1,107	25,57 ± 0,7514	
Glycemia (mg/dL)	110,4 ± 3,330	162,3 ± 12,83	**P<0,001
LDL (mg/dL)	112,6 ± 18,34	91,50 ± 14,75	
HDL (mg/dL)	42,43 ± 3,963	50,17 ± 5,212	
Total cholesterol (mg/dL)	176,1 ± 20,96	162,3 ± 15,10	
Cardiovascular comorbidities			
Hypertension	5	5	
Dyslipidemia	4	5	
Therapies			
antihypertensive drugs	3	4	
lipid lowering drugs	4	5	

Table S2. Clinical characteristics of patients included in SASP profile analysis of $CD34^+$ stem cells and differentiation into myeloid lineage (Results Part II).

	CAD	CAD-DM	P value
N	8	11	
AGE (years)	65,82 ± 2,54	69,73 ± 2,82	
Glycemia (mg/dL)	104,9 ± 3,13	163,0 ± 8,30	***P< 0,0001
BMI (KG/m^2)	25,56 ± 0,91	26,33 ± 0,60	
LDL (mg/dL)	107,8 ± 13,06	86,20 ± 10,81	
HDL (mg/dL)	45,18 ± 3,36	47,61 ± 3,65	
Total cholesterol (mg/dL)	173,6 ± 14,77	157,3 ± 10,34	
Cardiovascular comorbidities			
Hypertension	9	9	
Dyslipidemia	7	8	
Therapies			
antihypertensive drugs	7	8	
lipid lowering drugs	8	9	

Table S3. Western Blot primary antibodies.

Antibody	Provider	Clone or antintibody ID	WB Dilution	MWt. (kDa)
MnSOD, Rabbit	Abcam	ab13533	1:5000	25
CAT, Rabbit	Abcam	ab52477	1:1000	60
p66shc, Rabbit	Abcam	ab24787	1:500	66
p-Akt, Rabbit	Cell Signaling	9271	1:1000	60
Akt, Rabbit	Cell Signaling	9272	1:1000	60
DNMT1, Rabbit	Abcam	ab19905	1:200	184-176-144
DNMT3B, Monoclonal Rabbit	Cell Signaling	D7070	1:500	110
Anti-Nf-kB p65 (acetyl K310)	Abcam	ab19870	1:1000	65
p-p44/42 MAPK (Erk1/2)	Cell Signaling	4370	1:1000	44-42
p44/42 MAPK (Erk1/2)	Cell Signaling	9102	1:1000	44-42
GAPDH, Rabbit	Cell Signaling	14C10	1:2000	37
Anti-β-Actin–Peroxidase antibody, Monoclonal Mouse	Sigma Aldrich	AC-15	1:10000	42

Table S4. ChIP assay antibodies list.

Antibody	Provider	Clone or antintibody ID	ChIP [ ]
Histone H4ac (pan-acetyl) antibody (pAb)	Active Motif	39243	5 µL
Anti-Histone H3 (tri methyl K9) antibody - ChIP Grade	QIAGEN	GAH-6204	5 µg
Anti-Histone H3K27me antibody - ChIP Grade	Abcam	ab6002	5 µg
Human RNA Polymerase II ChampionChIP™	QIAGEN	GAH-111	2 µg/ml

Table S5. Primer list of Results Part I.

Primer List		
c-DNA	Name	Sequence 5'—> 3'
	MnSOD fw	CACCACAGCAAGCACCA
	MnSOD rv	CTTGTCAAAGGAACCAAAGTCAC
	CAT fw	CTCGTGGGTTTGCAGTGAA
	CAT rv	TGGCTGTGGATAAAAGATGGA
	p66^{shc} fw	AAGTACAATCCACTCCGGAATGA
	p66^{shc} rw	GGGCCCCAGGGATGAAG
	DNMT1 fw	GTCAAACCAAAGAACCAACACC
	DNMT1 rw	GACTTCTGTGCTTCTTCTCATCT
	DNMT3A fw	GTAACCTTCCCGGTATGAACAG
	DNMT3A rw	CCTGCTTTATGGAGTTTGACCT
	TET2 fw	AGGAAGAGCAGTAAGGGACT
	TET2 rw	GAGGTGATGGTATCAGGAATGG
	TET3 fw	GGAACAACCAAAGGAGAAGGA
	TET3 rw	CTACCAGGAGCTCACCGA
	CXCR4 fw	AGCAGGTAGCAAAGTGACG
	CXCR4 rw	CCTCGGTGTAGTTATCTGAAGTG
ChIP Assay		
	Region1 CXCR4 fw	CGCCCAGTTCTTCAACCTAA
	Region1 CXCR4 rv	GCAAATAAGCCCGGAGAGAT
	Region2 CXCR4 fw	CGGGTTAACTGGATCAGTGG
	Region2 CXCR4 rv	AAATGAACAAACGGCACCTC
	Region3 CXCR4 fw	CACCCTGTGGGACAGAGC
	Region3 CXCR4 rv	GCCCCAAGTTTCATTTCCTC

Table S6. Primer list of Results Part II-III.

Primer List		
c-DNA	Name	Sequence 5'—> 3'
	p21 fw	GGAAGACCATGTGGACCTGT
	p21rv	GGATTAGGGCTTCCTCTTGG
	p27 fw	ACCTGCAACCGACGATTCTTC
	p27 rv	GGGCGTCTGCTCCACAGA
	Human IL6 fw	ACAAAAGTCCTGATCCAGTTCC
	Human IL6 rv	GACTGCAGGAACTCCTTAAAGC
	Human TNFα fw	CCCAGGGACCTCTCTCTAATCA
	Human TNFα- rv	AGCTGCCCCTCAGCTTGAG
	KAT2B/PCAF/p300-CBP-associated factor fw	ACTTGAGGCAAACCCAGGAGA
	KAT2B/PCAF/p300-CBP-associated factor rv	GCCGAGTGTGCTGACAGAAA
	NF-KB fw	CAGCCGATGAGAGCCGGCAG
	NF-KB rv	CCGGACCTCCCAGCCTGACA
	GLP-1R fw	GTGTGGCGGCCAATTACTAC
	GLP-1R rv	CTTGGCAAGTCTGCATTTGA
ChIP Assay		
	NF-KB promoter fw	GTGCAGCCTCTTCGTCCTC
	NF-KB promoter rv	GTGCACTACAGACGAGCCATT

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