Liraglutide Preserves CD34 + Stem Cells from Dysfunction Induced by High Glucose Exposure

Annalisa Sforza
Centro Cardiologico Monzino IRCCS

Vera Vigorelli
Centro Cardiologico Monzino IRCCS

Erica Rurali
Centro Cardiologico Monzino IRCCS

Elisa Gambini
Centro Cardiologico Monzino IRCCS

Martina Arici
Università degli Studi di Milano-Bicocca

Raffaella Rinaldi
Centro Cardiologico Monzino IRCCS

Paolo Fiorina
ASST Fatebenefratelli-Sacco

Andrea Barbuti
Università degli Studi di Milano

Angela Raucci
Centro Cardiologico Monzino IRCCS

Marcella Rocchetti
Università degli Studi di Milano-Bicocca

Giulio Pompilio
Centro Cardiologico Monzino IRCCS

Stefano Genovese
Centro Cardiologico Monzino IRCCS

Maria Cristina Vinci (✉️ cristina.vinci@ccfm.it)
Centro Cardiologico Monzino IRCCS

Research Article

Keywords: GLP-1 receptor agonist, CD34+ hematopoietic stem progenitor cells, type 2 diabetes mellitus, cardiovascular disease

Posted Date: November 30th, 2021
Abstract

**Background:** Glucagon like peptide-1 receptor agonists (GLP-1RAs) have shown to reduce mortality and cardiovascular events in patients with type 2 diabetes mellitus (T2DM). Since the impairment in number and function of vasculotrophic circulating CD34⁺ hematopoietic stem progenitor cells (HSPCs) in T2D has been reported to increase cardiovascular (CV) risk, we hypothesized that one of the mechanisms whereby GLP-1 RAs exert CV protective effects may be related to the ability to improve CD34⁺ HSPC function.

**Methods:** In cord blood (CB)-derived CD34⁺ HSPC, the expression of GLP-1 receptor (GLP-1R) mRNA, receptor protein and intracellular signaling was evaluated by RT-qPCR and by Western Blot respectively. CD34⁺ HSPCs were exposed to high glucose (HG) condition and GLP-1RA liraglutide (LIRA) was added before as well as after functional impairment. Proliferation, CXCR4/SDF-1α axis activity and intracellular ROS production of CD34⁺ HSPC were evaluated.

**Results:** CD34⁺ HSPCs express GLP-1R at transcriptional and protein level. LIRA treatment prevented and rescued HSPC proliferation and CXCR4/SDF-1α axis activity from HG-induced impairment. LIRA stimulation promoted intracellular cAMP accumulation as well as ERK1/2 and AKT signaling activation. The selective GLP-1R antagonist exendin (9-39) abrogated LIRA-dependent ERK1/2 and AKT phosphorylation along with the related protective effects.

**Conclusion:** We provided the first evidence that CD34⁺ HSPC express GLP-1R and that LIRA can favorably impact on cell dysfunction due to HG exposure. These findings open new perspectives on the favorable CV effects of GLP-1 RAs in T2DM patients.

**Background**

Type 2 diabetes mellitus (T2DM) has now attained the status of a global pandemic with over 400 million individuals affected worldwide [1]. Despite glucose lowering therapies, mortality from cardiovascular disease (CVD) remains high and extremely costly for health care systems both in terms of medical expenses and disability-adjusted life years [2]. For these reasons, the development of new therapeutic strategies able to prevent CVD morbidity and mortality is crucial. Patients with T2DM are characterized by a significant decrease in circulating CD34⁺ stem/progenitor cells. CD34⁺ hematopoietic stem/progenitor cells (HSPCs) are known to possess vascular regenerative and proangiogenic capacity [3]. Their functional and numerical depletion is now considered a significant contributor to CV homeostasis impairment in diabetes. To this regard, Fadini et al. demonstrated that CD34⁺ HSPCs are reduced of about 40% in T2DM [4], and that such impairment contributes to enhanced CV risk [5]. Notably, in patients with T2DM, the reduction of CD34⁺ HSPCs number and function predicts adverse CV outcomes, defined as major cardiovascular events (MACE), and hospitalizations for CV causes [6, 7]. Recent large-scale trials have unequivocally demonstrated the ability of glucagon-like peptide 1 receptor agonists (GLP1-RAs) to reduce the risk of MACE in T2DM patients with established or at high risk of CVD [8-10]. GLP1-
RAs are now recommended by guidelines as first-line agent for prevention of CVD in T2DM patients [11, 12]. Such pleiotropic CV benefit appears to be additional to glucose-lowering effects and the mechanisms whereby they exert such striking CV protective effects are still largely unknown. At cellular and molecular level, GLP1-RA effects are mediated by GLP1-R, a Gs coupled receptor family member, which is present in various human tissues [13]. To date, there are no data describing the effects of GLP1-RAs on CD34+ HSPCs of T2D patients. We hypothesized that at least part of the unknown mechanisms whereby GLP1-RAs exert CV protective effect are mediated by its ability to improve CD34+ HSPC function. Here, by exploiting an in vitro model of diabetes, we show for the first time that CD34+ HSPCs express GLP-1R and that its stimulation by liraglutide (LIRA), a GLP1-RA, prevents and recovers the dysfunction induced by hyperglycemia.

Methods

Experimental design

We recently established a stem cell culture model of diabetes based on the use of cord blood (CB)-derived CD34+ HSPCs [14]. This method already provided a consistent and reproducible recapitulation of the major CD34+ HSPC dysfunction hallmarks in diabetes [14]. To assess the ability of GLP-1 RA to prevent CD34+ HSPC dysfunction induced by glucose overload the cells were expanded in high glucose (HG; 30mM) conditions along with 50 nM or 100 nM LIRA treatment (Figure 1A). In a different experimental setting, CD34+ HSPCs were expanded in HG condition and then treated with LIRA only after loss of glucose tolerance. (Figure 1B). Afterwards, we assessed the ability of the drug to recover a compromised phenotype. CD34+ HSPCs cultured in normoglycemic condition (NG; 30mM mannitol) were used as control. At the end of both experiments, the main dysfunctional hallmarks of the cells, namely proliferation and CXCR4/SDF-1α axis impairment, were evaluated (Figure 1).

Cell culture

Umbilical cord blood (UCB) was collected from the umbilical cord of full-term normal deliveries in collaboration with Milano Cord Blood Bank (IRCSS Ca’ Granda Foundation – Ospedale Maggiore Policlinico). The mononuclear cell fraction was obtained by density gradient centrifugation using Ficoll-Paque (Lymphoprep, Sentinel Diagnostics) and CD34+ HSPCs were immunomagnetically isolated using CD34 Microbead Kit (MiniMACS kit, Miltenyi Biotec). Isolated CD34+ HSPCs were cultured in Stem Span medium (StemCell Technologies) supplemented with 20 ng/mL of interleukin (IL)-6 (PeproTech), 20 ng/mL of IL-3 (PeproTech), 50 ng/mL of fms-like tyrosine kinase 3 (FLT3, PeproTech), and 50 ng/mL of stem cell factor (SCF, PeproTech). Cells were cultured in HG (30 mM of glucose, Sigma-Aldrich) or NG (30 mM of mannitol, Sigma-Aldrich) conditions for up to 20 days and treated or not with increasing
concentration of LIRA (50nM and 100nM; MedCHemExpress) ± selective GLP-1R antagonist exendin (9-39) (150nM EXE; MedCHemExpress)

**Cell proliferation assay**

CD34^+ HSPCs were seeded at an initial density of 2.0×10^5 cells/well and cultured for up to 20 days in NG and HG ± LIRA conditions. Cells were counted on days 5, 10, 15 and 20. Doubling time was calculated with the following formula:

\[
\text{Doubling time: } \frac{\text{duration} \times \log (2)}{\log (\text{final concentration}) - \log (\text{initial concentration})}
\]

**Migration assays**

Cell migration was determined the use of Boyden modified chamber consisting of transwell culture inserts (5-μm pore membrane; Corning Incorporated, Corning, NY). In brief, 1×10^5 cells were seeded onto the upper chamber and allowed to migrate toward the lower chamber containing, or not, stromal cell-derived factor 1 (SDF-1α 50 ng/mL; PeproTech EC Ltd.). The transwells were incubated at 37°C, 5% CO₂, for 4 hours. Migrated cells in the lower chamber were counted and migration index were calculated with the following formula:

\[
\text{Migration index: } \frac{\text{migrated cells in presence of SDF-1α}}{\text{migrated cells in absence of SDF-1α}}
\]

**Cyclic adenosine monophosphate (cAMP) quantification**

Intracellular cAMP was quantified by cAMP ELISA kit (Enzo Life Science) according to manufacturer’s instructions. Briefly, 5 x 10^5 CD34^+ HSPCs were stimulated with LIRA ± EXE and lysed in 0.1 M HCl and 0.1% Triton X-100. Sample absorbance was spectrophotometrically evaluated at 405 nm by Tecan (Infinite M200 Pro, TECAN).

**Intracellular Ca\(^{2+}\) handling**
The measurement of intracellular Ca\(^{2+}\) has been assessed through single cell and population analysis by means of confocal Nikon A1R microscope and FLUOstar Omega (BMG Labtech) multiplate reader respectively. CD34\(^{+}\) HSPCs were starved for 2 hours (with IMDM and albumin 0.1%) and incubated with the Ca\(^{2+}\)-sensitive dye Fluo-4 AM (ThermoFisher, 2µM) in Tyrode's solution (containing in mM: 154 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES/NaOH, and 5.5 d-glucose, adjusted to pH 7.35) for 1 hour.

Confocal single cell analysis was performed by plating CD34\(^{+}\) HSPCs on fibronectin/polylysine D coated glass coverslips; fluorescence (F) images (512*512 pxis) were acquired at x60 magnification every 5 seconds in basal condition and following 100 nM LIRA addition at 37°C and 5% CO\(_2\) thanks to Okolab incubator mounted on the microscope stage. Changes in single cell mean F during acquisition time were quantified through NIS-Elements analysis software following F background subtraction. Population analysis was performed by plating cells in 96-well dark plates by means of the multiplate reader equipped with an automatic injection system to inject LIRA (100 nM). F was acquired in each well every 0.74 second for 20 seconds just prior to compound injection and for 100 seconds after injection. Mean F prior compound injection was used as reference (F\(_0\)) for signal normalization (F/F\(_0\)).

**RNA extraction and RT-qPCR**

Total RNA from CD34\(^{+}\) HSPCs was isolated by using the Direct-zol RNA Kit (Zymo Research), following manufacturer's protocol. One µg of total RNA was converted to cDNA with the Superscript III kit (Life Technologies) and used to assess GLP-1R gene expression. qPCR reactions were performed with SYBR Green Supremix 2X (BIO-RAD Laboratories) on CFX96 Real-Time System PCR (BIO-RAD Laboratories). Specific GLP-1R primers (Fw: 5’-GTGTGGCCGCAATTACTAC-3’; Rv: 5’-CTTGGCAAGTCTGCATTTGA-3’) were appositely designed to evaluated mRNA expression by amplifying a region of 347 bp. The qPCR products were loaded on a 1% agarose gel with an appropriate molecular marker (PCR Marker Solution, Sigma-Aldrich). Then, the 347 bp bands were excised and purified with QIAquick Gel extraction kit (Qiagen) for subsequent Sanger sequencing analysis.

**Sanger sequencing**

The RT-qPCR products, appropriately purified from agarose, were sequenced with the help of an external service (Microsynth Biotech) by Sanger method with the use of GLP-1R Fw primer. Sequencing results were analysed by a Multiple sequence ClustalW alignment (BioEdit software) throughout the comparison of the published GLP-1R cDNA sequence (NCBI Reference sequence: NM_002062.5).

**Western Blot**

CD34\(^{+}\) stem cells and capan-1 cells were lysed in lysis buffer (50 mM TRIS-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton) added with protease inhibitors (1:10, Halt Protease Inhibitor Cocktail, Thermo Scientific).
Protein lysate was then quantified by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Forty μg and 20 μg of protein from CD34+ stem cells and capan-1 cells respectively were resolved on 10% SDS-PAGE in denaturing conditions. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) at 400 mA, 4 °C for 90 minutes. To prevent aspecific binding, the membrane was blocked with 5% bovin serum albumin (BSA) in PBS + 0,1% Tween-20 (PBST) for 1 hour. The membranes were then incubated with the primary antibodies, appropriately diluted in 3% BSA-PBST, at 4 °C O/N and with the appropriate secondary antibody linked to horseradish peroxidase (HRP) the day after for 1 hour. Specific information about antibodies and appropriate dilutions are reported in Table 1. The signal was detected by Enhanced chemiluminescence (ECL) system and quantified by Chemidoc MP Imaging System (BIO-RAD Laboratories).

Flow cytometric assays

CD34+ HSPCs were incubated for 30 minutes with allophycocyanin-conjugated monoclonal antihuman CXCR4 antibody (BD Biosciences) or with CellROX Green Flow Cytometry Assay Kit (Life Technologies) for the detection of CXCR4 and reactive oxygen species (ROS) respectively. The Gallios Flow Cytometer platform (Beckman Coulter Life Sciences) was used to analyze the samples after appropriate physical gating. At least $2 \times 10^4$ events in the indicated gates were acquired.

Statistical analysis

Results are given as mean±SEM. All experiments were performed at least in triplicate, unless stated otherwise. The data were tested for the normality by using the Shapiro–Wilk normality test. Differences between data were evaluated by 1-way, 2-way repeated-measures ANOVA followed by the post-hoc Newman–Keuls multiple comparison test, as appropriate. A value of $P \leq 0.05$ was considered significant. All statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc.).

Results

CD34+ HSPCs express GLP-1 receptor

We profiled the expression of GLP-1R in CD34+ HSPCs at both mRNA and protein levels. In order to ensure cDNA amplification of the target mRNA and exclude PCR products from DNA contamination, we designed a couple of primers spanning exon-exon junction (Figure 2A). Additionally, to confirm the correct amplification of the target template, the amplicon of 347 bp, characterized by a melting temperature of 83°C, was resolved on 1% agarose gel. The bands were then excised and sequenced. The alignment of sequenced PCR end-point products with reference coding cDNA (NM_002062.5) showed 99% of identity (Figure 2B, C and D). Finally, total protein cell lysates obtained from 3 different samples of CD34+ HSPCs were subjected to Western blot analysis. Protein cell lysate of capan-1 cells was used as positive control.
As shown in Figure 1E, GLP-1R antibody detected a unique, distinct protein band of the expected molecular weight (55 kDa) in all samples.

**The administration of GLP-1 receptor agonist liraglutide stimulates intracellular cAMP production**

GLP-1Rs are known to be coupled to activation of G\_s proteins. In pancreatic β cells the receptor agonist engagement results in activation of adenylate cyclase with consequent production of 3',5'-cyclic adenosine monophosphate (cAMP), intracellular Ca\_{2+} increase and insulin release [15]. To determine whether CD34\(^+\) HSPCs express a functional GLP-1R, we assessed intracellular cAMP production and Ca\_{2+} mobilization after LIRA stimulation.

Consistent with activation of G\_s, the treatment of cells with LIRA elicited a significant accumulation of intracellular cAMP over basal level in a time- and dose dependent manner reaching the highest value after 10 minutes of stimulation at 100 nM (Figure 3A and 2B). Notably, the addition of the selective GLP-1R antagonist exendin (9-39) (EXE) prevented intracellular cAMP accumulation at all tested LIRA concentrations, demonstrating a receptor-mediated effect (Figure 3B). Interestingly, single cell analysis showed occurrence of spontaneous Ca\_{2+} transients in CD34\(^+\) HSPCs not significantly altered by 100 nM LIRA addition (Supplementary Figure 1A). Moreover, cell population analysis showed negligible increase in intracellular Ca\_{2+} following LIRA addition (+4% over basal Ca\_{2+} level, Supplementary Figure 1B). Thus, unlike pancreatic β-cells, acute GLP-1R stimulation in CD34\(^+\) HSPCs did not significantly alter intracellular Ca\_{2+} (Supplementary Figure 1).

**GLP-1R stimulation prevents CD34\(^+\) HSPC dysfunction induced by chronic glucose overload**

We recently published that metabolic stress induced by prolonged HG exposure results in mitochondrial ROS accumulation, loss of cell proliferation ability and CXCR4/SDF1-α axis impairment [14]. Herein, we tested whether LIRA, a GLP-1RA, was able to avert all these functional damages.

Despite the chronical exposure to HG concentration, LIRA dose-dependently (50 and 100 nM), prevented cell proliferation impairment. Noteworthy, the presence of 100 nM LIRA maintained cell proliferation rate to the control values (NG) (Figure 4A and 4B). Similarly, LIRA treatment prevented CXCR4/SDF1-α axis defect promoted by HG exposure. Indeed, as shown in figure 4C and 4D, LIRA significantly maintained in dose dependent manner CXCR4 expression and the migration ability of the cells toward 50ng/mL of SDF-1α, with peak effect at 100 nM (Figure 4E). The maintenance of functional parameters despite HG presence of was associated with a significant drug-induced reduction of cell oxidative state (Figure 4F).
**GLP-1R stimulation promotes activation of ERK1/2 and PI3K signaling pathways**

Stimulation of the GLP-1R is known to activate numerous pleiotropic signaling pathways in human pancreatic islet cells including phosphatidylinositol 3-kinase (PI3K) and extracellular regulated kinases 1 and 2 (ERK1/2) [16, 17]. To determine whether the stimulation of endogenous GLP-1R expressed in CD34⁺ HSPCs was also coupled to similar signal transduction pathways, we assessed the phosphorylation of ERK1/2 and AKT (a downstream effector of PI-3 kinase). As shown in figure 5A and 5B, stimulation of the cells with 100 nM LIRA elicited a time-dependent activation of both ERK1/2 and AKT kinases.

These data indicate that CD34⁺ HSPCs express a functional GLP-1R whose stimulation is coupled to additional signaling pathways other than adenylate cyclase.

**Exendin (9-39) antagonizes LIRA effects against hyperglycemia**

In order to confirm that LIRA-induced activation of diverse pro-survival signaling pathways was acting through GLP-1R activation, aforementioned experiments were carried out in the presence or absence of EXE antagonist (150 nM) [18]. As shown in Figure 6A, EXE abrogated LIRA-dependent ERK1/2 and AKT phosphorylation as well as its protective effect on cell proliferation, measured as doubling time, (Figure 6C) and CXCR4 membrane expression (Figure 6D). Taken together, these findings support a GLP-1R-mediated effect of LIRA on intracellular pathways and functions of CD34⁺ HSPCs.

**LIRA partially recovers the functional damage induced by HG exposure in CD34⁺ HSPCs**

As we recently reported, a strong antioxidant machinery confers to CD34⁺ HSPCs a particular resistance to HG-induced oxidative stress [14]. However, after antioxidant defense exhaustion, irreversible functional alterations take place. Here, in a different experimental setting (Figure 1B), we aimed at investigating whether LIRA was also able to recover the compromised cell phenotype induced by HG exposure.

While 100 nM LIRA modestly improved HG-CD34⁺ cell growth rate (Figure 7A), we found that the same drug concentration significantly recovered CXCR4 membrane expression (Figure 7B), and restored migration ability of the cells (Figure 7C), although the latter less efficiently than when added concomitantly to HG. Again, these effects were associated with a significant drug-dependent reduction of intracellular ROS levels (Figure 7D).
Discussion

CV complications remain the major cause of morbidity and mortality of patients with DM and first-generation glucose lowering agents have proved to be inadequate [19] or only partially able to favorably impact CV prognosis [20]. Data from pivotal clinical trials provided evidence that GLP-1RAs, as LIRA and semaglutide, significantly reduced the risk in T2DM patients for MACE and all-cause mortality on top of standard of care [8-10]. Notably, it has been suggested that the observed cardiovascular benefits are unlikely to be entirely explained by the hypoglycemic effects of GLP-1RAs. Thus, the existence of additional still unknown mechanisms were postulated. CD34⁺ HSPCs are known to play a central role in the maintenance of cardiovascular homeostasis by regulating vascular repair and regeneration [21-23]. The importance of CD34⁺HSPC biological functions on cardiovascular outcome [6] is supported by the common ontological origin of vascular and hematopoietic system [24]. We hypothesized that part of the unknown mechanisms whereby GLP-1RAs improve CV outcome are related to LIRA ability to reverse the impairment of CD34⁺ HSPC function provoked by HG.

We firstly provided unprecedented evidence that CD34⁺ HSPCs express GLP-1R. The demonstration of GLP-1R expression by the identification of mRNA transcripts encoding for GLP-1R open reading frame and the use of validated antisera is often lacking and controversial in literature [25]. Therefore, to assess mRNA expression, we designed a couple of primers containing a reverse primer spanning an exon-exon junction (Figure 2A). This granted the sole amplification of GLP-1R transcripts excluding any products deriving from possible DNA contamination, as we successfully confirmed by qPCR amplification of genomic DNA (data not shown). Afterwards, qPCR products were sequenced and aligned with the reference coding cDNA (NM_002062.5) confirming their identity (Figure 2D). GLP-1R protein expression was finally detected by a top cited and validated antibody [26, 27], whose specificity was further proven in capan-1 cell lysate, a pancreatic cancer cell line expressing GLP-1R (Figure 2E) [28].

GLP-1R is a member of the secretin family or class B G protein-coupled receptors (GPCRs) [29]. Consistently with its canonical Gs mediated pathway activation, we showed that CD34⁺ HSPCs express a functional GLP-1R. In fact, LIRA elicited a time- and dose-dependent accumulation of intracellular cAMP that was abrogated by the competitive receptor antagonist EXE (Figure 3). Noteworthy, differently from what reported in pancreatic β-cells, we observed a weak intracellular intracellular Ca²⁺ mobilization, suggesting that, in CD34⁺ HSPCs, Ca²⁺-mediated signaling pathways may not be fundamental for the biological activity of the receptor [30] (Supplementary Figure 1).

In the last years, a number of preclinical and clinical studies have demonstrated that native GLP-1 as well as GLP-1 RAs exert pleiotropic effects on different tissue subsets [31]. At cardiovascular system level, they showed to improve endothelial function, reduce atherosclerosis, as well as oxidative stress and vascular and cardiac inflammation, through both GLP-1R-dependent and independent mechanisms [32]. After GLP-1R expression in HSPC was confirmed, we were puzzled to evaluate its biological effects in a diabetic environment. We recently reported that the loss of glucose tolerance in CD34⁺ HSPCs was associated with the reduction of proliferation rate, increase in mitochondrial ROS production and
CXCR4/SDF-1α axis impairment [14]. These functional deficits are known to be primarily involved in the impairment of CD34+ HSPC mobilization and migration capacity from the BM to sites of ischemia and endothelial injury in diabetic patients [33, 34]. Here we found that the concomitant administration of LIRA in HG setting was able to prevent HG-induced CD34+ HSPC dysfunction and to improve their oxidative state (Figures 4). Interestingly, in line with recent findings, the protective effects of LIRA persisted throughout the entire duration of the experiment, suggesting a sustained endosomal cyclic AMP generation induced by internalized activated receptor complex [35, 36]. GLP-1R stimulation is known to promote transactivation of multiple intracellular pathways including PI3K, and p42/44 MAPK. These pathways, which exert proliferative and cytoprotective functions [37], have been described in numerous extra glucose-lowering actions of incretins [31, 38]. In our hands, the treatment with 100 nM LIRA treatment promoted in CD34+ HSPCs a time dependent ERK1/2 and AKT phosphorylation that was completely abolished, along with the related protective effects, by the co-treatment with the GLP-1R antagonist EXE (Figures 5 and 6). Albeit we are aware that we have not provided a full demonstration of the exact mechanism by which GLP-1R activation mediates ERK1/2 and AKT phosphorylation, we think we provided enough evidence that the protective effect of LIRA in CD34+ HSPCs is mediated by GLP-1R, even if other mechanisms cannot be excluded.

According to guidelines, GLP1-RAs are now recommended to reduce the risk of cardiovascular events and mortality in T2DM patients. According to our hypothesis, this implies that LIRA cardiovascular protective effects are exerted after CD34+ HSPC dysfunction has taken hold. To assess the ability of LIRA to recover a HG-related compromised stem cell phenotype, the drug was given after the dysfunctional phenotype emerged. LIRA was able to recover HSPC CD34+ cell function, even if less efficiently than early administration (Figure 7). This observation corroborates accumulating evidences for supporting the use of GLP-1 RAs for CVD prevention in T2DM patients.

**Conclusion**

We provided first evidence that CD34+ HSPCs express GLP-1R, and that the GLP-1RA LIRA prevents proliferation and migration impairment induced by chronic HG exposure. LIRA was also able to improve, even if less efficiently, CD34+ HSPC function when previously exposed to hyperglycemic conditions. Taken together these data suggest that the reported cardiovascular benefits of GLP-RAs can at least in part be related to cytoprotective effects on CD34+ HSPCs.

**Abbreviations**

AKT/PKB protein kinase B

cAMP cyclic adenosine monophosphate

CB cord blood
CVD cardiovascular disease
ERK1/2 extracellular signal-regulated kinases 1/2
EXE exendin fragment (9-39)
GLP-1 glucagon-like peptide 1
GLP1-RAs GLP-1 receptor agonists
HG hyperglycemic
IL interleukin
LIRA liraglutide
MACE major cardiovascular events
NG normoglycemic
PVDF polyvinylidene difluoride
ROS reactive oxygen species
T2DM type 2 diabetes mellitus

Declarations

Availability of data and materials
All associated data will be available to the public, as requested

Funding
The study has been supported by Ricerca Corrente of Italian Ministry of Health (RC 2019 2764158-CA3A to Centro Cardiologico Monzino IRCCS) and 5x1000 Charity Fund.

Acknowledgments
none

Authors’ contributions
AS and VV performed the experiments and were responsible for collection and compilation of data and in writing the manuscript. ER helped in performing Western Blot analysis, compilation of the data and in manuscript writing. EG helped in flow cytometry analysis. RR contributed in performing cellular experiments. MA, AB and MR performed intracellular calcium signaling analysis. PF, AR and GP revised the manuscript. SG designed and revised the manuscript. MCV designed, performed statistical analysis, supervised and revised the manuscript. All authors read and approved the final manuscript.

Funding
The study has been supported by Ricerca Corrente of Italian Ministry of Health (RC 2019 2764158-CA3A to Centro Cardiologico Monzino IRCCS) and 5x1000 Charity Fund. Erica Rurali has been supported by FUV

Availability of data and materials
All associated data will be available to the public, as requested.

Ethics approval and consent to participate
Not Applicable

Consent for publications
Not Applicable

Competing interests
The authors declare that they have no competing interests.

References
1. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala AA, Ogurtsova K et al: Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. Diabetes Res Clin Pract2019, 157:107843.
2. American Diabetes A: 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. Diabetes Care2018, 41(Suppl 1):S13-S27.
3. Pozzoli O, Vella P, Iaffaldano G, Parente V, Devanna P, Lacovich M, Lamia CL, Fascio U, Longoni D, Cotelli F et al. Endothelial fate and angiogenic properties of human CD34+ progenitor cells in zebrafish. Arterioscler Thromb Vasc Biol 2011, 31(7):1589-1597.

4. Fadini GP, Boscaro E, de Kreutzenberg S, Agostini C, Seeger F, Dimmeler S, Zeiher A, Tiengo A, Avogaro A: Time course and mechanisms of circulating progenitor cell reduction in the natural history of type 2 diabetes. Diabetes Care 2010, 33(5):1097-1102.

5. Fadini GP: A reappraisal of the role of circulating (progenitor) cells in the pathobiology of diabetic complications. Diabetologia 2014, 57(1):4-15.

6. Fadini GP, Rigato M, Cappellari R, Bonora BM, Avogaro A: Long-term Prediction of Cardiovascular Outcomes by Circulating CD34+ and CD34+CD133+ Stem Cells in Patients With Type 2 Diabetes. Diabetes Care 2017, 40(1):125-131.

7. Rigato M, Avogaro A, Fadini GP: Levels of Circulating Progenitor Cells, Cardiovascular Outcomes and Death: A Meta-Analysis of Prospective Observational Studies. Circ Res 2016, 118(12):1930-1939.

8. Marso SP, Daniels GH, Brown-Frandsen K, Kristensen P, Mann JF, Nauck MA, Nissen SE, Pocock S, Poulter NR, Ravn LS et al. Liraglutide and Cardiovascular Outcomes in Type 2 Diabetes. N Engl J Med 2016, 375(4):311-322.

9. Marso SP, Bain SC, Consoli A, Eliaschewitz FG, Jodar E, Leiter LA, Lingvay I, Rosenstock J, Seufert J, Warren ML et al. Semaglutide and Cardiovascular Outcomes in Patients with Type 2 Diabetes. N Engl J Med 2016, 375(19):1834-1844.

10. Gerstein HC, Colhoun HM, Dagenais GR, Diaz R, Lakshmanan M, Pais P, Probstfield J, Riesmeyer JS, Riddle MC, Ryden L et al. Dulaglutide and cardiovascular outcomes in type 2 diabetes (REWINd): a double-blind, randomised placebo-controlled trial. Lancet 2019, 394(10193):121-130.

11. American Diabetes A: Cardiovascular Disease and Risk Management: Standards of Medical Care in Diabetes-2021. Diabetes Care 2021, 44(Suppl 1):S125-S150.

12. Cosentino F, Grant PJ, Aboyans V, Bailey CJ, Ceriello A, Delgado V, Federici M, Filippatos G, Grobbee DE, Hansen TB et al. 2019 ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD. Eur Heart J 2020, 41(2):255-323.

13. Wei Y, Mojsov S: Tissue-specific expression of the human receptor for glucagon-like peptide-1: brain, heart and pancreatic forms have the same deduced amino acid sequences. FEBS Lett 1995, 358(3):219-224.

14. Vigorelli V, Resta J, Bianchessi V, Lauri A, Bassetti B, Agrifoglio M, Pesce M, Polvani G, Bonalumi G, Cavallotti L et al. Abnormal DNA Methylation Induced by Hyperglycemia Reduces CXCR 4 Gene Expression in CD 34(+) Stem Cells. J Am Heart Assoc 2019, 8(9):e010012.

15. Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF: Glucagon-like peptide 1 stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. Proc Natl Acad Sci U S A 1987, 84(10):3434-3438.

16. Trumper J, Ross D, Jahr H, Brendel MD, Goke R, Horsch D: The Rap-B-Raf signalling pathway is activated by glucose and glucagon-like peptide-1 in human islet cells. Diabetologia 2005, 48(8):1534-
1540.

17. Park S, Dong X, Fisher TL, Dunn S, Omer AK, Weir G, White MF: Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. J Biol Chem 2006, 281(2):1159-1168.

18. Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Goke B: Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. J Biol Chem 1993, 268(26):19650-19655.

19. Zoungas S, Chalmers J, Neal B, Billot L, Li Q, Hirakawa Y, Arima H, Monaghan H, Joshi R, Colagiuri et al: Follow-up of blood-pressure lowering and glucose control in type 2 diabetes. N Engl J Med 2014, 371(15):1392-1406.

20. Riddle MC: Modern Sulfonylureas: Dangerous or Wrongly Accused? Diabetes Care 2017, 40(5):629-631.

21. Vinci MC, Bassetti B, Pompilio G: Endothelial progenitors: When confusion may give rise to new understanding. Int J Cardiol 2020, 318:121-122.

22. Sietsema WK, Kawamoto A, Takagi H, Losordo DW: Autologous CD34+ Cell Therapy for Ischemic Tissue Repair. Circ J 2019, 83(7):121-122.

23. Ballard VL, Edelberg JM: Stem cells and the regeneration of the aging cardiovascular system. Circ Res 2007, 100(8):1116-1127.

24. Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D: Haematopoietic stem cells derive directly from aortic endothelium during development. Nature 2010, 464(7285):108-111.

25. Drucker DJ: Incretin action in the pancreas: potential promise, possible perils, and pathological pitfalls. Diabetes 2013, 62(10):3316-3323.

26. Katsurada K, Nandi SS, Sharma NM, Zheng H, Liu X, Patel KP: Does glucagon-like peptide-1 induce diuresis and natriuresis by modulating afferent renal nerve activity? Am J Physiol Renal Physiol 2019, 317(4):F1010-F1021.

27. Yu M, Agarwal D, Korutla L, May CL, Wang W, Griffith NN, Hering BJ, Kaestner KH, Velazquez OC, Markmann JF et al: Islet transplantation in the subcutaneous space achieves long-term euglycaemia in preclinical models of type 1 diabetes. Nat Metab 2020, 2(10):1013-1020.

28. Koehler JA, Drucker DJ: Activation of glucagon-like peptide-1 receptor signaling does not modify the growth or apoptosis of human pancreatic cancer cells. Diabetes 2006, 55(5):1369-1379.

29. Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B, Drucker DJ: International Union of Pharmacology. XXXV. The glucagon receptor family. Pharmacol Rev 2003, 55(1):167-194.

30. Yabe D, Seino Y: Two incretin hormones GLP-1 and GIP: comparison of their actions in insulin secretion and beta cell preservation. Prog Biophys Mol Biol 2011, 107(2):248-256.

31. Graaf C, Donnelly D, Wootten D, Lau J, Sexton PM, Miller LJ, Ahn JM, Liao J, Fletcher MM, Yang D et al: Glucagon-Like Peptide-1 and Its Class B G Protein-Coupled Receptors: A Long March to Therapeutic Successes. Pharmacol Rev 2016, 68(4):954-1013.

32. Drucker DJ: The Cardiovascular Biology of Glucagon-like Peptide-1. Cell Metab 2016, 24(1):15-30.
33. Cencioni C, Capogrossi MC, Napolitano M: The SDF-1/CXCR4 axis in stem cell preconditioning. *Cardiovasc Res* 2012, 94(3):400-407.

34. Egan CG, Lavery R, Caporali F, Fondelli C, Laghi-Pasini F, Dotta F, Sorrentino V: Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes. *Diabetologia* 2008, 51(7):1296-1305.

35. Girada SB, Kuna RS, Bele S, Zhu Z, Chakravarthi NR, DiMarchi RD, Mitra P: Galphas regulates Glucagon-Like Peptide 1 Receptor-mediated cyclic AMP generation at Rab5 endosomal compartment. *Mol Metab* 2017, 6(10):1173-1185.

36. Jones B, Buenaventura T, Kanda N, Chabosseau P, Owen BM, Scott R, Goldin R, Angkathunyakul N, Correa IR, Jr., Bosco D et al: Targeting GLP-1 receptor trafficking to improve agonist efficacy. *Nat Commun* 2018, 9(1):1602.

37. Vinci MC, Visentin B, Cusinato F, Nardelli GB, Trevisi L, Luciani S: Effect of vascular endothelial growth factor and epidermal growth factor on iatrogenic apoptosis in human endothelial cells. *Biochem Pharmacol* 2004, 67(2):277-284.

38. Gupta V: Pleiotropic effects of incretins. *Indian J Endocrinol Metab* 2012, 16 Suppl 1:S47-56.

**Table**

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

**Figures**
Figure 1

Schematic representation of the study. CD34+ HSPCs were isolated via Mac-sorting from cord blood and characterized for GLP-1R transcript and protein expression levels, and downstream signaling pathway activation. Then, CD34+ HSPCs were expanded in HG condition with or without LIRA (A). In a different experimental setting the cells were treated with LIRA only after the loss of glucose tolerance (B). CD34+ HSPCs cultured in NG (30mM mannitol) condition were used as control (A, B). Proliferation, migration and oxidative stress were finally assessed at the end of experiments. GLP-1R= glucagone-like peptide 1 receptor; HG= high glucose; LIRA= liraglutide; NG= normal glucose.
Figure 2

GLP-1R expression in CD34+ HSPCs. mRNA expression was assessed by RT-qPCR in 3 different CD34+ HSPC biological replicates. An exon-exon spanning reverse primer was used to exclude any amplification of possible contaminating DNA; capan-1 cells were used as positive control (A). The identity of RT-qPCR products was determined by melting curve (B), agarose gel run (347 bp) (C) and finally by Sanger sequencing (D). CD34+ HSPC lysates (40 μg) deriving from three biological replicates were
immunoblotted in order to evaluate GLP-1R protein expression. Capan-1 cell lysate (20 μg) was used as positive control (E). S1, S2, S3 = samples 1, 2, 3.

**Figure 3**

GLP-1R downstream signaling pathway activation after stimulation by LIRA treatment. A) CD34+ HSPC stimulation with 100nM LIRA determined a significant time-dependent accumulation of intracellular cAMP, with a maximum peak after 10 minutes of treatment (**p ≤ 0.01; ***p ≤ 0.001 vs basal; one-way...
ANOVA). B) The addition of 150nM EXE, a competitive antagonist of GLP-1R prevented the intracellular cAMP accumulation induced by increasing concentration of LIRA (10-100 nM) (*p ≤ 0.05, **p ≤ 0.01 vs basal two-way ANOVA). EXE= exendin 9-39, LIRA= liraglutide.

Figure 4

LIRA treatment prevented CD34+ HSPC proliferation, CXCR4/SDF-1α axis impairment and ROS accumulation caused by 20 days HG exposure. A) Proliferation curve of CD34+ HSPC amplified in NG, HG
± 50nM and 100nM LIRA conditions (**p ≤ 0.01 HG vs NG; §§ p ≤ 0.01 HG 100 LIRA vs HG; two-way ANOVA). B) Doubling time of CD34+ HSPC cultured in NG, HG ± 50nM and 100nM LIRA (**p ≤ 0.01 HG vs NG; **p ≤ 0.01 HG 100 LIRA vs HG; one-way ANOVA). C) Analysis by flow cytometry of CXCR4 expression in NG, HG ± 50nM and 100nM LIRA treated CD34+ HSPCs (**p ≤ 0.001 HG vs NG; ** p ≤ 0.01 HG 100 LIRA vs HG; one-way ANOVA). D) Representative dot plot of CXCR4 cytofluorimetric analysis. E) CD34+ HSPC migration towards SDF-1α (50ng/mL) after NG, HG ± 50nM and 100nM LIRA treatment (*p ≤ 0.05 HG vs NG; *p ≤ 0.05 HG 50 LIRA vs HG; ***p ≤ 0.001 HG 100 LIRA vs HG; one-way ANOVA). F) Quantification of intracellular ROS level in NG, HG ± 50nM and 100nM LIRA treated cells (**p ≤ 0.001 HG vs NG; *p ≤ 0.05 HG 50 LIRA vs HG; ** p ≤ 0.01 HG 100 LIRA vs HG; one-way ANOVA). HG= high glucose, LIRA= liraglutide, NG= normal glucose.

Figure 5

Intracellular pathway cross-talk after GLP-1R1 stimulation with 100nM LIRA. A) Representative immunoblot of ERK1/2 and (C) AKT phosphorylation in CD34+ HSPCs treated with 100nM LIRA for 5, 10 and 15 minutes. GAPDH was used as loading control. B, D) Immunoblotting quantification presented as arbitrary units after normalization to the GAPDH protein levels of three independent experiments. AKT=
protein kinase B, ERK1/2 = extracellular signal-regulated kinases 1 and 2 GAPDH = Glyceraldehyde 3-phosphate dehydrogenase, LIRA = liraglutide, p-AKT = phospho-AKT, p-ERK1/2 = phospho-ERK1/2.

Figure 6

The GLP-1R antagonist EXE abolished ERK1/2 and AKT phosphorylation as well as the protective effects promoted by LIRA treatment. A) Representative immunoblot of ERK1/2 and AKT phosphorylation after stimulation with LIRA 100 nM ± EXE 150 nM for 10 minutes. GAPDH was used as loading control. B, C) Immunoblotting results presented as arbitrary units after normalization to the GAPDH protein levels. D) Doubling time of CD34+ HSPCs cultured in NG, HG ± 50nM and 100nM ± EXE conditions (**p ≤ 0.01 HG vs NG; ***p ≤ 0.001 HG 100 LIRA vs HG; ** p ≤ 0.01 HG 100 LIRA + EXE vs NG; one-way ANOVA. E) Analysis by flow cytometry of CXCR4 expression in NG, HG ± 50nM and 100nM ± EXE CD34+ HSPCs (*p ≤ 0.05 HG vs NG; **p ≤ 0.01 HG ± LIRA vs HG ± EXE; one-way ANOVA.***p ≤ 0.001 HG ± LIRA vs HG ± EXE).
LIRA partially recovered HG-damaged CD34+ HSPC functions. A) Proliferation curve of HG-damaged CD34+ HSPCs after 10 days stimulation with 100nM LIRA (**p≤0.001 HG vs NG; two-way ANOVA). B) Analysis by flow cytometry of CXCR4 expression level in HG-damaged CD34+ HSPCs after 10 days of LIRA treatment (***p≤0.001 HG vs NG; * p≤0.05 HG 100 LIRA vs HG; one-way ANOVA). C) Migration of HG-damaged CD34+ HSPCs after 10 days of LIRA treatment (**p≤0.01 HG vs NG; ** p≤0.01 HG 100 LIRA vs HG; one-way ANOVA). D) Quantification of intracellular ROS level by flow cytometry in HG-damaged CD34+ stem cells after 10 days of LIRA treatment (*p≤0.05 HG vs NG; * p≤0.05 HG 100 LIRA vs HG; one-way ANOVA). HG= high glucose, LIRA= liraglutide, NG= normal glucose.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
- SupplementaryFigure1.tif
- AntibodyTable.pptx