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ARTICLE

Glucose tolerance is negatively associated with circulating progenitor cell levels

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Abstract
Aims/hypothesis Circulating progenitor cells participate in cardiovascular homeostasis. Depletion of the pool of endothelial progenitor cells (EPCs) is associated with increased cardiovascular risk. Furthermore, EPCs are reduced in the presence of classical risk factors for atherosclerotic disease, including diabetes mellitus. This study was designed to evaluate progenitor cell levels in volunteers with different degrees of glucose tolerance.
Methods Cardiovascular parameters and the levels of circulating $CD34^+$ and $CD34^+$ kinase insert domain receptor $(KDR)^+$ cells were determined in 219 middle-aged individuals with no pre-diagnosed alterations in carbohydrate metabolism. Glucose tolerance was determined by fasting and 2 h post-challenge glucose levels, with IFG and IGT considered as pre-diabetic states.
Results $CD34^+$ and $CD34^+KDR^+$ cells were significantly reduced in individuals who were found to have diabetes mellitus, and were negatively correlated with both fasting and post-challenge glucose in the whole population. While only $CD34^+$ cells, but not $CD34^+KDR^+$ cells, were significantly reduced in pre-diabetic individuals, post-challenge glucose was an independent determinant of the levels of both $CD34^+$ and $CD34^+KDR^+$ cells.
Conclusions/interpretation Glucose tolerance was negatively associated with progenitor cell levels in middle-aged healthy individuals. Depletion of endothelial progenitors with increasing fasting and post-meal glucose may be one cause of the high incidence of cardiovascular damage in individuals with pre-diabetes.

Keywords Atherosclerosis · Cardiovascular risk · Diabetes · Endothelium · Glucose tolerance · Pre-diabetes · Progenitor cells · Stem cells

Abbreviations

EPC	endothelial progenitor cell
KDR	kinase insert domain receptor
mAb	monoclonal antibody
PE	phycoerythrin

Introduction

Type 2 diabetes is associated with excess cardiovascular morbidity and mortality [1]. However, the true contribution of hyperglycaemia to cardiovascular risk remains controversial [2]. Agreement, on the other hand, exists on the cardiovascular risk preceding overt type 2 diabetes [3]. Thus in the Paris Prospective Study, incidence of CHD mortality was twice as high in IGT patients as in NGT individuals [4]. Similar results have been confirmed in European [5, 6], Asian [7] and Japanese [8] populations. However, the question of whether modest impairment in glucose homeostasis contributes independently to cardiovascular risk or

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instead represents a marker for a more complex condition carrying excess risk is still debated.

In recent years, much emphasis has been placed on elevation of postprandial glucose [9], although mechanisms linking postprandial hyperglycaemia to cardiovascular damage are still poorly understood [10]. Post-load hyperglycaemia is, for instance, more strongly associated with several cardiovascular risk factors than fasting hyperglycaemia [11]. These factors, together with the rapid surge in glucose concentrations, may cause endothelial injury and, over time, lead to atherogenic lesions. The development of atherosclerosis may be also dependent upon defective repair of the intimal layer, which relies on the bioavailability of circulating progenitor cells. Endothelial progenitor cells (EPCs) derive from bone marrow and can be mobilised to peripheral blood in response to many stimuli [12]. Once in the bloodstream, EPCs home to the sites of ischaemia and are physically integrated into the nascent vasculature, thus contributing to compensatory angiogenesis [13]. They also constitute a pool of cells that can form a patch at sites of endothelial damage, thus contributing to ongoing endothelial turnover and repair [14]. Classic cardiovascular risk factors are associated with reduction in circulating EPCs [15, 16]; currently, this is considered to be one mechanism by which risk factors influence vascular health [17], with EPCs now listed among surrogate indicators of risk [18]. We have demonstrated that type 2 diabetes is characterised by a profound EPC depletion and dysfunction [19, 20]. Moreover, in diabetic animals, inability to mobilise EPCs after ischaemia was associated with impaired compensatory angiogenesis [21], while replenishment of the EPC pool with mobilising agents or by cell transplantation restored vascular healing [22, 23]. Taken together, these data suggest that reduction and dysfunction of EPCs in diabetes mirror a poor endogenous regenerative capacity and favour the development of vascular complications.

On the other hand, little is known about potential EPC impairment in the early stages of the natural history of type 2 diabetes. We therefore tested the hypothesis that pre-diabetic states, such as IFG and IGT, are themselves associated with alterations in progenitor cell levels, which in turn may hamper the repairing activity in response to the endothelial damage that follows metabolic injury.

Methods

Study population The study was approved by the local ethics committees and carried out in accordance with the Declaration of Helsinki as revised in 2000. Based on our previous data [15], we used the method of Dupont and Plummer [24] to calculate a sample size of around 200 individuals that would be able to detect a significant correlation between progenitor cell count and plasma glucose levels (type I error α=0.05; power=0.80; standard deviation of the regression error σ=38; standard deviation of the independent variable σ_x=71; predefined slope of the minimal linear correlation to be detected λ=0.10). The same sample size was estimated to be sufficient to detect a statistically significant 12% difference in EPC count between control and pre-diabetic individuals (expected prevalence of pre-diabetes 0.25, with α=0.05 and $1-\beta$=0.80).

Individuals were recruited at the Metabolic Outpatient Clinics of the University of Padua (n=141) and University of Pisa (n=78). Individuals participating in a screening programme for metabolic diseases and who had no sign of acute illness or infection, no immunological disease, no history of recent surgery, no uncontrolled hypertension and no established cardiovascular disease were recruited upon obtaining informed consent. All participants were at their first metabolic examination and none had any previously known abnormalities in glucose homeostasis. For all participants the following parameters were recorded: age, sex, family history for diabetes or cardiovascular disease, smoking habit (of one or more cigarettes per day), BMI, waist circumference and blood pressure (systolic and diastolic). Cardiovascular disease was ruled out using minimal criteria including personal history and physical examination.

After an overnight fast and abstention from smoking, an antecubital vein was cannulated for blood drawing. A basal blood sample was obtained for quantification of circulating EPCs and determination of lipid profile and fasting plasma glucose. After collecting basal blood samples, all participants underwent a 75 g OGTT for determination of plasma glucose 2 h after glucose load. Glucose tolerance was then defined according to the criteria set out by the American Diabetes Association [25].

Quantification of circulating endothelial progenitor cells Peripheral blood progenitor cells were analysed for the expression of surface antigens with direct two- or three-colour flow cytometry (FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA) as previously reported [15, 19, 20]. To saturate sites for unspecific binding, before staining with specific monoclonal antibodies, cells were treated with fetal calf serum for 10 min and then washed with phosphate-buffered saline and 0.5% bovine albumin. Then, 150 μl peripheral blood were stained with 10 μl FITC-conjugated anti-human CD34 monoclonal antibody (mAb) (BD Biosciences) and 10 μl phycoerythrin (PE)-conjugated anti-human kinase insert domain receptor (KDR) mAb (R&D Systems, Minneapolis, MN, USA). Control isotype IgG1 and IgG2a antibodies were obtained from BD Biosciences. The frequency of peripheral blood cells positive for the above reagents was determined by a two-

dimensional side scatter fluorescence dot plot analysis, after appropriate gating and staining with the different reagents: we initially gated $CD34^+$ peripheral blood cells in the mononuclear cell fraction and then examined the resulting population for dual expression of KDR (Fig. 1). For FACS analysis, 5×10^5 cells were acquired and scored using a FACS Calibur analyser (BD Biosciences); cell counts are then expressed as number of cells per 10^6 cytometric events. Data were processed using a software program (Macintosh CELLQuest; BD Biosciences). The instrument set-up was optimised daily by analysing the expression of peripheral blood lymphocytes labelled with FITC anti-CD4, PE anti-CD8, PECy5 anti-CD3, and allophycocyanin anti-CD45 four-colour combination. Operators, who performed all tests, were trained in flow cytometry, practised with rare event analysis and blinded to the clinical status of participants.

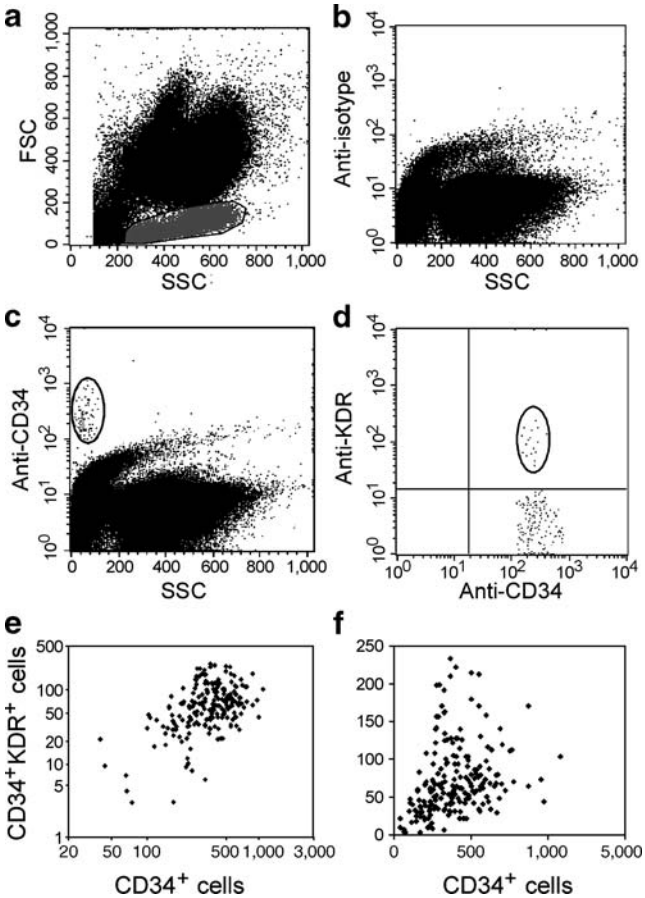

Fig. 1 Representative flow cytometry scatter plots used to determine $CD34^+$ and $CD34^+KDR^+$ cell counts. After morphological gating in the mononuclear cell fraction (**a**), $CD34^+$ cells were identified using an anti-CD34 mAb (**c**) with respect to the negative isotype control (**b**). $CD34^+KDR^+$ cells were then identified in the CD34 gate using an anti-KDR mAb in the $CD34^+$ gate (**d**). To show the extent of similarity between the two cell counts, $CD34^+$ cell count was plotted against $CD34^+KDR^+$ cells on the ln scale (**e**) as well as in the natural scale (**f**). *FSC* forward scatter, *SSC* side scatter

Substrate measurement Plasma glucose was measured on an analyser (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA, USA) employing a glucose oxidase reaction. Plasma lipids were determined according to standard enzymatic procedures. HDL-cholesterol concentration was determined after precipitation of apolipoprotein B-containing lipoproteins with phosphotungstic acid/$MgCl_2$ (Roche, Milan, Italy). LDL-cholesterol was calculated by the Friedewald formula. All determinations were performed with an autoanalyser (Hitachi/Roche, Milan, Italy).

Statistical analysis Data are expressed as means±SD. $CD34^+$ and $CD34^+KDR^+$ cell counts are expressed as cells per 10^6 events. However, due to skewed distribution, they were transformed in their natural logarithms for all statistical analyses, while plots display cell counts in the original unit of measure (cells per 10^6 events) on the ln scale. Differences between two or more groups were evaluated using Student's t test and ANOVA, respectively, with the least significant difference post hoc test; the χ^2 test was used for dichotomous variables. Correlations between cell counts and all cardiometabolic parameters were assessed with univariate regression analyses; parameters significantly correlated with cell counts were then entered in a multiple linear regression analysis, to assess their independent effects. Statistical significance was accepted at $p\leq0.05$.

Results

Characteristics of the study population Clinical and metabolic features of the study population are summarised in Table 1. Upon OGTT, 134 of 219 individuals had NGT, 61 (28%) had pre-diabetes (i.e. IFG defined as 6.1 mmol/l [110 mg/dl]≤fasting glucose<7.0 mmol/l [126 mg/dl] or IGT defined as 7.8 mmol/l [140 mg/dl]≤2 h glucose <11.1 mmol/l [200 mg/dl] or a combination of the 2) and 24 (11%) had diabetes mellitus (defined as fasting glucose ≥7.0 mmol/l [126 mg/dl] or 2 h glucose≥11.1 mmol/l [200 mg/dl]). None of the participants were taking medication at the time of the study. As can be appreciated from Table 1, participants with pre-diabetes, not unexpectedly, had an unfavourable cardiometabolic profile when compared with individuals with NGT, while diabetic participants had the worst profile.

Circulating progenitor cells in individuals with carbohydrate metabolism abnormalities Generic circulating $CD34^+$ progenitor cells decreased as a function of glucose tolerance, as they were significantly reduced in participants with pre-diabetes as compared with participants with NGT (350.0±

Table 1 Characteristics of the study sample

Characteristic	Controls (n=134)	Pre-diabetes (n=61)	Diabetes (n=24)	ANOVA p value
Age (years)	43.8±7.9	51.7±7.6[a]	55.0±8.0[a]	<0.001
Male (%)	42.5	52	67[a]	0.066
Family history of diabetes (%)	53	67	43	0.080
Family history of CVD (%)	44	66[a]	57	0.017
Smoking habit (%)	18	16	22	0.852
BMI (kg/m^2)	24.8±4.8	27.5±4.5[a]	27.5±4.8[a]	<0.001
Waist circumference (cm)	89.7±13.6	98.0±11.4[a]	101.7±11.4[a]	<0.001
Systolic blood pressure (mmHg)	121.8±13.5	128.1±13.0[a]	137.5±15.6[a,b]	<0.001
Diastolic blood pressure (mmHg)	81.6±9.9	83.2±12.5	85.6±11.9	0.220
Total cholesterol (mmol/l)	5.13±0.91	5.47±0.91[a]	4.97±0.98[b]	0.025
LDL-cholesterol (mmol/l)	3.31±0.92	3.67±0.88[a]	3.15±0.98[b]	0.019
HDL-cholesterol (mmol/l)	1.44±0.43	1.39±0.40	1.21±0.37[a]	0.055
Triacylglycerol (mmol/l)	1.0±0.55	1.52±0.75[a]	2.12±1.58[a,b]	<0.001
Fasting glucose (mmol/l)	4.81±0.52	5.87±0.78[a]	7,42±1.5[a,b]	<0.001
2 h post-challenge glucose (mmol/l)	5.71±1.16	8.32±1.78[a]	13.74±2.36[a,b]	<0.001
CD34$^+$ cells	431.8±173.7	350.0±209.7	284.2±187.5	
ln [CD34$^+$][c]	5.97±0.46	5.74±0.59[a]	5.45±0.67[a]	<0.001
CD34$^+$KDR$^+$ cells	78.5±47.0	73.0±53.5	45.6±28.5	
ln [CD34$^+$KDR$^+$][c]	4.17±0.70	4.02±0.77	3.55±0.88[a,b]	0.002

Values are presented as the means±SD.
[a] Statistically different vs control after adjusting for α-inflation (least significant difference post hoc test)
[b] Statistically different vs pre-diabetes after adjusting for α-inflation (least significant difference post hoc test)
[c] Cell counts expressed as ln [CD34$^+$]±coefficient of variation

209.7 vs 431.8±173.7 [means±SD]; p=0.002). When pre-diabetic individuals were compared with NGT individuals, those with IGT displayed a significant CD34$^+$ cell count reduction (339.5±195.0; p=0.006), while those with IFG showed a nearly significant reduction (380.5±252.5; p=0.058). CD34$^+$ progenitor cells were even lower in diabetic individuals, a further decrease that was nearly significant in comparison with pre-diabetic individuals (284.2±187.5; p<0.001 vs NGT and p=0.057 vs pre-diabetes) (Table 1, Fig. 2).

Circulating CD34$^+$KDR$^+$ EPCs also showed a progressive decline from NGT, IFG, IGT and diabetic individuals (p<0.001 for trend). Nonetheless, CD34$^+$KDR$^+$ cells were not significantly reduced in pre-diabetic individuals vs NGT (73.0±53.5 vs 78.5±47.0; p=0.226), whereas a significant reduction was apparent in diabetic individuals (45.6±28.5; p<0.001 vs NGT and p=0.016 vs pre-diabetes) (Table 1, Fig. 2).

When the lower limit for IFG was set at 5.6 mmol/l (100 mg/dl) instead of 6.1 mmol/l (110 mg/dl), the number of participants classified as IFG increased (n=59) and the CD34$^+$ cell count further decreased, becoming significantly lower vs NGT (370.5±233.5 vs 427.6±170.4; p=0.007), while the CD34$^+$KDR$^+$ cell count remained almost identical.

Natural ln-transformed counts of circulating CD34$^+$ and CD34$^+$KDR$^+$ progenitors correlated with both fasting (CD34$^+$: r=−0.219; p=0.001; CD34$^+$KDR$^+$: r=−0.144; p=0.04) and post-challenge glucose (CD34$^+$: r=−0.282; p<0.001; CD34$^+$KDR$^+$: r=−0.212; p=0.003) (Fig. 3).

In univariate regression analyses performed separately for all cardiometabolic parameters (see Table 2), CD34$^+$ cell count was significantly negatively correlated with age, HDL-cholesterol, fasting plasma glucose and 2 h post-challenge plasma glucose. When those parameters were entered as explanatory variables in a multiple linear regression analysis, only HDL and post-challenge glucose remained significantly associated with CD34$^+$ cell count.

In univariate regression, the CD34$^+$KDR$^+$ cell count was significantly correlated with fasting and post-challenge glucose. Upon multiple regression, only post-challenge glucose remained significantly associated with CD34$^+$KDR$^+$ cell counts (Table 2).

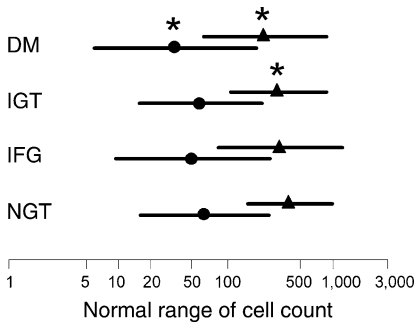

Fig. 2 CD34$^+$ (*triangles*) and CD34$^+$KDR$^+$ (*circles*) cell levels in subjects with NGT, IGT, IFG and diabetes mellitus (*DM*). *Bars* indicate normal range of cell count on the ln scale. *p<0.05 vs NGT

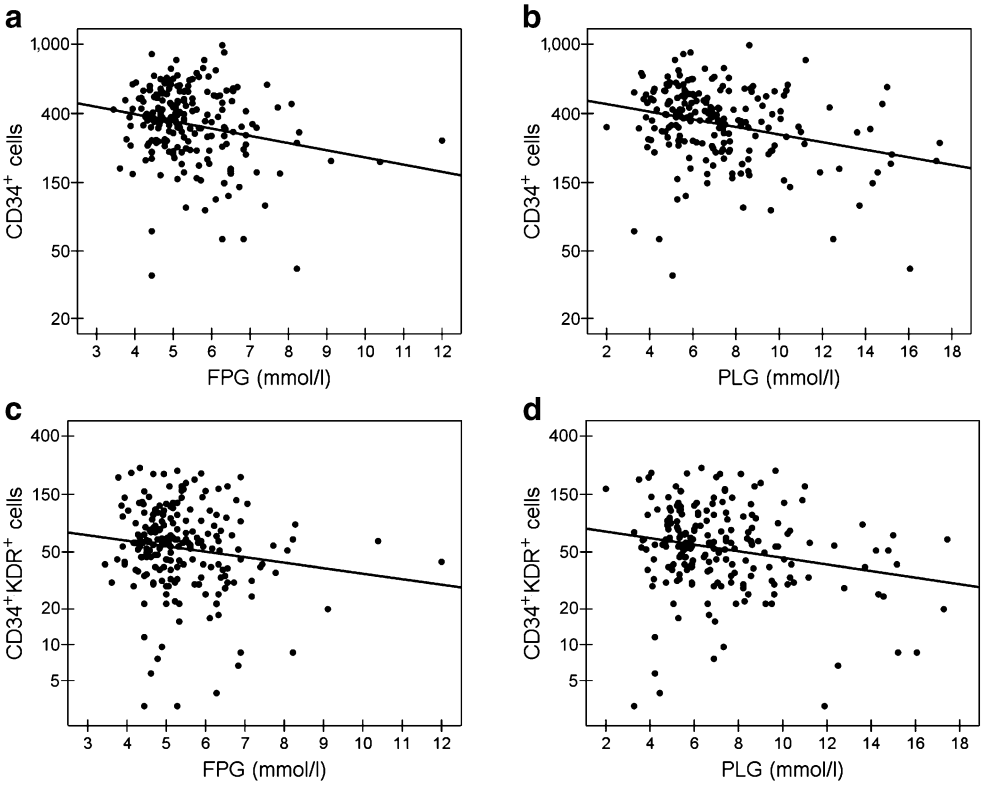

Fig. 3 Linear correlations of $CD34^+$ and $CD34^+KDR^+$ cell counts (original cell count on the ln scale) with fasting plasma glucose (*FPG*) (**a**, **c**) and post-load glucose (*PLG*) (**b**, **d**). $r=-0.219$, $p=0.001$, residual coefficient of variation (rCV)=0.62 (**a**); $r=-0.282$, $p<0.001$, rCV=0.69 (**b**); $r=-0.144$, $p=0.04$, rCV=0.59 (**c**); $r=-0.212$, $p=0.03$, rCV=0.58 (**d**)

Discussion

We demonstrate that circulating $CD34^+$ and $CD34^+KDR^+$ progenitor cell counts are inversely related to glucose tolerance independently of concomitant risk factors. In light of the comprehensive role played by circulating progenitor cells in vascular homeostasis, exhaustion of these cells may contribute to the increased cardiovascular risk that characterises pre-diabetic conditions.

The notion that a subgroup of progenitor cells from the bloodstream has the ability to repopulate the endothelium has been substantiated by a vast literature of in vitro and animal

Table 2 Univariate and multiple regression analyses to identify cardiometabolic variables significantly correlated with $CD34^+$ and $CD34^+KDR^+$ cell counts (ln-transformed)

Explanatory variable	$CD34^+$ cells		$CD34^+KDR^+$ cells	
	Univariate	Multiple	Univariate	Multiple
Age (years)	−1.19 (−0.42 to −1.96)*	−0.2 (−1.17 to 0.78)	−0.9 (−2.05 to 0.28)	–
Sex	13.1 (−2.37 to 31.0)	–	−14.27 (−30.5 to 5.73)	–
Family history DM	−2.37 (−15.9 to 13.3)	–	−7.32 (−25.0 to 14.5)	–
Family history CVD	−5.26 (−18.4 to 9.96)	–	−6.01 (−23.94 to 16.2)	–
Smoking habit	−1.69 (−19.0 to 19.4)	–	1.0 (−22.9 to 32.4)	–
Waist circumference (cm)	−0.1 (−0.69 to 0.49)	–	−0.7 (−1.47 to 0.08)	–
BMI (kg/m^2)	−0.2 (−1.75 to 1.38)	–	−0.9 (−3.0 to 1.26)	–
Systolic BP (mmHg)	−0.1 (−0.69 to 0.49)	–	−0.7 (−1.47 to 0.08)	–
Diastolic BP (mmHg)	−0.1 (0.69 to 0.49)	–	−0.4 (−1.37 to 0.58)	–
Total cholesterol (mmol/l)	−0.3 (−8.19 to 8.26)	–	−2.96 (−13.4 to 8.73)	–
LDL-cholesterol (mmol/l)	−0.2 (−8.09 to 8.36)	–	−2.96 (−13.6 to 8.94)	–
HDL-cholesterol (mmol/l)	−23.9 (−35.9 to −9.6)*	−29.3 (−40.6 to −15.7)*	−0.3 (−22.3 to 29.4)	–
TG (mmol/l)	−0.4 (−8.63 to 8.57)	–	−3.73 (−70.9 to 218.2)	–
Fasting glucose (mmol/l)	−9.97 (−15.4 to −4.14)*	−4.02 (−12.8 to 5.66)	−9.15 (−17.0 to −0.58)*	−4.88 (−17.1 to 9.11)
2 h glucose (mmol/l)	−5.35 (−7.73 to −2.91)*	−5.26 (−8.36 to −2.05)*	−5.64 (−9.08 to −2.05)*	−4.59 (−9.15 to −0.20)*

Data are expressed as the relative percentage increase in the dependent variable per unit of increase in the explanatory variable (95%CI)
DM: diabetes mellitus, *TG*: triacylglycerol
*$p<0.05$

studies [26]. EPCs can be isolated and expanded in culture to assess their properties and functional capacities: unfortunately, those costly and time-consuming procedures cannot be applied on a large scale. Therefore, in population-based studies, progenitor cells are defined and enumerated by flow cytometry, on the basis of the expression of surface antigens, a method considered the gold standard for this purpose [27]. Even if their exact phenotype is uncertain, in the present study, EPCs were defined as $CD34^+KDR^+$ cells, first because this is becoming the most widely accepted EPC phenotype [20, 28], and second because $CD34^+KDR^+$ cell count predicted cardiovascular outcomes in two large prospective studies [29, 30]. While CD34 is an adhesion molecule expressed mainly on haematopoietic stem cells, KDR (also known as type 2 vascular endothelial growth factor receptor) is a typical endothelial marker. In support of the hypothesis that $CD34^+KDR^+$ cells correspond to EPCs, it should be noted that freshly isolated human $CD34^+KDR^+$ cells showed vascular healing properties in animal models of coronary and peripheral artery disease [31, 32]. However, the simple evaluation of progenitor cells by flow cytometry implies the conceptual abstraction that antigenic phenotypes correspond to functional phenotypes, which is not always true: thus it is likely that $CD34^+KDR^+$ cells are a mixed population that includes EPCs. We also determined total $CD34^+$ cells, as we have recently shown that this phenotype strongly correlates with all cardiometabolic parameters and provides an index of cardiovascular risk [15].

Regardless of the actual functional/antigenic correlations, both circulating $CD34^+$ and $CD34^+KDR^+$ cells have been found to be reduced in subjects with established atherosclerotic disease [16, 33]. Previous studies have also shown that $CD34^+KDR^+$ cell count is reduced early on in pre-atherosclerosis and represents an independent predictor of carotid intima-media thickness in the general population [28]. These findings indicate that depletion of this putative EPC phenotype precedes the development of overt atherosclerosis, supporting the hypothesis that such alterations are not a mere disease epiphenomenon, but play a role in the process of atherogenesis [34].

Hyperglycaemic diabetic patients also showed a marked depletion of $CD34^+$ and $CD34^+KDR^+$ cell pools, as well as a profound functional impairment of cultured EPCs [19, 20, 35]. This was hailed as a novel explanation for the extensive vascular damage in diabetic patients, also because EPC-based therapies restored vascular healing in diabetic animals [22, 23]. Less well known is the behaviour of these cells in the presence of minor abnormalities in glucose tolerance, and this despite the fact that IGT is known to be associated with increased cardiovascular risk [36, 37]. Indeed, alterations of glucose tolerance form a continuum, from NGT to overt diabetes, which progressively worsens vascular health [38]. We therefore recruited middle-aged healthy subjects at their first metabolic evaluation in order to explore the relation between progenitor cells and glucose tolerance. This approach had the advantage of studying a naïve population sample and minimising confounders, such as concomitant diseases and medications. Although, as noted before, we cannot infer that $CD34^+$ and $CD34^+KDR^+$ cells perfectly correspond to EPCs, by showing a reduction of these cells in parallel with increases in fasting and post-load glucose, we do provide evidence that carbohydrate metabolism may regulate these progenitor cell pools. Therefore, even if the results of this study may not be directly extended to the general population, we suggest that the excess cardiovascular risk associated with pre-diabetes is likely to be sustained not only by the enhanced endothelial injury effected by hyperglycaemia and concomitant metabolic risk factors, but also by impaired endothelial regeneration resulting from depletion of circulating progenitors.

Interestingly, available experimental findings suggest a link between glucose toxicity and progenitor cell alterations. For instance, mild in vitro hyperglycaemia (12 mmol/l) reduced proliferation and increased apoptosis of cultured EPCs [39, 40]; additionally, reduction of plasma glucose levels in poorly controlled diabetic individuals was followed by an increase of circulating $CD34^+$ progenitor cells [41]. In addition, we have demonstrated that stable hyperglycaemia impairs mobilisation of progenitor cells from bone marrow to peripheral blood in diabetic rats [21].

It is possible that clustering cardiovascular risk factors in patients with IGT contribute to reduce progenitor cell levels. Nonetheless, our data suggest that glucose tolerance as such, rather than the accompanying metabolic alterations, determined cell reduction. In fact, in the multiple analysis, the 2-h plasma glucose remained significantly associated with cell counts, regardless of other cardiovascular parameters and risk factors. Epidemiological and mechanistic studies have repeatedly suggested that increased 2 h post-OGTT and postprandial glucose may also confer great cardiovascular risk in pre-diabetic patients, as well as in diabetic patients [10, 42, 43]. Acute hyperglycaemia impairs endothelial function probably through induction of oxidative stress [44]. Although this was an association study not designed to prove cause–effect relationships, our demonstration that progenitor cells declined in parallel with rises in post-load glucose may indicate that excessive glycaemic elevations, as may occur after ingestion of a meal, reduce endothelial progenitors, also possibly via oxidative stress. The subsequent depletion of the EPC pool would result in the inability to maintain adequate vascular homeostasis and to stimulate compensatory angiogenesis where needed, ultimately leading to an elevated risk of cardiovascular events.

In the present study we have found that, as compared with individuals with NGT, $CD34^+$ cells were significantly lower in pre-diabetic participants and further reduced in those with

diabetes, suggesting that the decline in $CD34^+$ progenitors follows the natural history of the development of diabetes, as well as the concomitant surge in cardiovascular risk. $CD34^+KDR^+$ cells also declined progressively with worsening glucose tolerance, but they were not significantly reduced in pre-diabetic individuals, as they were in diabetic individuals. Despite the cross-sectional nature of this study and the acknowledged difficulty of the antigenic-into-functional translation, it is tempting to speculate that depletion of generic $CD34^+$ cells precedes reduction of the specific $CD34^+KDR^+$ population in the development of type 2 diabetes. This would agree with our observation that $CD34^+$ does indeed represent the progenitor cell phenotype most closely correlated to cardiovascular risk [15]. Probably, the $CD34^+$ population is very heterogeneous; moreover, it has been shown to include, besides haematopoietic stem cells, a wide range of endothelial, smooth muscle, perivascular and myocardial progenitor cells [45]. All these cellular compartments may be altered by the metabolic abnormalities of the pre-diabetic state, resulting in impairment of the endogenous regenerative potential of the whole cardiovascular system [46, 47]. Finally, it should be noted that when dealing with rare events such as circulating progenitor cells, single antigen flow cytometry proved to be more accurate and precise than double-antigen analysis [15]: this may be one reason for the non-significant results with the $CD34^+KDR^+$ phenotype.

In conclusion, we have shown that $CD34^+$ and $CD34^+KDR^+$ cell counts are inversely related to glucose tolerance and that reduction of these putative endothelial progenitors may occur early in the natural history of type 2 diabetes, since $CD34^+$ cells were already reduced in participants with pre-diabetes. Progenitor cell exhaustion appears to be influenced by the degree of post-OGTT hyperglycaemia, although the causal link responsible for such an association remains to be elucidated. Should our data reflect an actual deficiency of functional EPCs, we propose that the excess of cardiovascular risk in individuals with IGT derives not only from the endothelial insult triggered by multiple metabolic disturbances, but also from an impaired regenerative capacity of the endothelium. This concept would indicate possible novel therapeutic and preventive strategies aimed at improving endogenous endothelial healing.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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