CLINICAL STUDY

Relationship between circulating endothelial progenitor cells and endothelial dysfunction in children with type 1 diabetes: a novel paradigm of early atherosclerosis in high-risk young patients

Barbara Głowińska-Olszewska*, Marcin Moniuszko^{1,3,*}, Andrzej Hryniewicz, Marta Jeznach¹, Małgorzata Rusak², Milena Dąbrowska², Włodzimierz Łuczyński, Anna Bodzenta-Lukaszyk¹ and Artur Bossowski

Department of Pediatrics, Endocrinology, Diabetology with Cardiology Division, Medical University of Białystok, Waszyngtona Street 17, 15-274 Białystok, Poland, Departments of ¹Allergology and Internal Medicine and ²Hematological Diagnostics, Medical University of Białystok, M.C. Skłodowskiej Street 24, 15-269 Białystok, Poland and ³Department of Regenerative Medicine and Immune Regulation, Medical University of Białystok, Waszyngtona Street 13, 15-269 Białystok, Poland

(Correspondence should be addressed to B Głowińska-Olszewska; Email: bglowinska@poczta.onet.pl)

*(B Głowińska-Olszewska and M Moniuszko contributed equally to this work.)

Abstract

Objective: The low number of circulating endothelial progenitor cells (EPCs) has emerged as a biomarker of cardiovascular (CV) risk in adults. Data regarding EPCs in paediatric populations with CV risk factors are limited. The aim of the study was to estimate the EPC number and its relationship with vascular function and structure in children with type 1 diabetes mellitus (T1DM).

Design and methods: We performed a comparative analysis of 52 children with T1DM (mean age 14.5 years; diabetes duration, 6.0 years; HbA1c level, 8.5%) and 36 healthy age- and gender-matched control children. EPCs were identified and analysed by flow cytometry with the use of MABs directed against CD34, CD144 (VE-cadherin) and CD309 (VEGFR-2). sICAM-1, hsCRP, thrombomodulin and adiponectin levels were also assessed. We evaluated vascular function (flow-mediated dilation (FMD)) and structure (carotid intima-media thickness (IMT)) ultrasonographically.

Results: Frequencies of CD34+ cells were similar in both groups ($P=0.30$). In contrast, frequencies of CD34+VE-cadherin+ cells were significantly higher in diabetic children compared with the healthy group ($P=0.003$). Similarly, diabetic patients tended to present with higher frequencies of CD34+VEGFR+ cells ($P=0.06$). FMD was lower (6.9 vs 10.5%, $P=0.002$) and IMT was higher (0.50 vs 0.44 mm, $P=0.0006$) in diabetic children. We demonstrated a significant relationship between CD34+VEGFR-2+ cells and BMI ($r=0.3$, $P=0.014$), HDL ($r=-0.27$, $P=0.04$), sICAM-1 ($r=0.47$, $P=0.023$) and FMD ($r=-0.45$, $P<0.001$). Similarly, frequencies of CD34+VE-cadherin+ cells were significantly correlated with BMI ($r=0.32$, $P=0.02$) and FMD ($r=-0.31$, $P=0.03$).

Conclusions: We demonstrated here that increased frequencies of EPCs observed in diabetic children are negatively correlated with endothelial function. Further studies are warranted to assess whether this phenomenon might result from effective mobilisation of EPCs in order to repair damaged endothelium in children at increased risk for atherosclerosis.

European Journal of Endocrinology 168 153–161

Introduction

Diabetes mellitus (DM) leads to long-term vascular damage to small vessels and major arteries and to impaired vascular repair. Despite advances in diabetes care, type 1 DM (T1DM) confers a two- to tenfold increased risk of death and cardiovascular disease (CVD) compared with the background population (1, 2, 3). Contributory factors include increased glucose levels, other traditional CV risk factors, arterial wall inflammation and endothelial dysfunction (4, 5). Endothelial dysfunction and damage is considered to be a major

underlying mechanism for the heightened CV burden in diabetes.

It is now recognised that endothelial repair/regeneration is not only dependent on the migration and proliferation of surrounding mature endothelial cells resident in the vascular wall but also dependent on the availability of circulating endothelial progenitor cells (EPCs) (6). A relatively novel paradigm of CVD pathogenesis is the loss of normal endothelial turnover caused by a reduction of EPCs (7). EPCs are bone marrow-derived cells that were first described in 1997 (8). It has been hypothesised that EPCs play a role in

re-endothelialisation of blood vessels damaged by ischaemia. EPCs potentially contribute to endothelial repair by homing into sites of endothelial injury at sites of ischaemia and damage and thus maintain the integrity of the endothelium (8, 9, 10, 11). EPCs are a heterogeneous population characterised by the expression of varying surface markers including endothelium-associated molecules, namely vascular endothelial (VE)-cadherin (CD144) or VE growth factor receptor-2 (VEGFR-2, CD309), CD31 and molecules characteristic for haemopoietic progenitor cell lineage (e.g. CD34 and CD133) (12, 13, 14).

Circulating EPCs have generated interest as a novel biomarker of endothelial function and are considered a prognostic indicator of CV morbidity and mortality. Therefore, reduction of EPC cell numbers is believed to promote development and/or progression of CVD (15). There are reports describing an association between higher EPC number and reduced occurrence of first major CV event (16). Some CV risk factors have been found to be associated with decreased circulating EPC counts and/or impaired function (17, 18, 19). Both type 1 and type 2 diabetes in adults are associated with a significant reduction of circulating EPCs (20, 21, 22, 23). Mechanisms linking hyperglycaemia to progenitor cell reduction include defective mobilisation of EPCs from bone marrow and their reduced survival (20, 22).

It is well established that atherosclerotic process begins in childhood and is dramatically accelerated in young patients with T1DM (24). Impaired endothelial function is now considered an early sign of atherosclerosis in children, which precedes the atherosclerotic plaque formation and has therefore become an important marker of CV risk, particularly in those with T1DM. It can be detected years before overt coronary artery disease occurs. On the other hand, flow-mediated dilatation (FMD) is an established non-invasive method of assessing endothelial function by measuring vasodilatation in the brachial artery in response to shear stress associated with increased blood flow (25). Endothelial dysfunction has been reported in patients with T1DM and T2DM, in adults and in children (23, 26, 27, 28). Progression of vascular disease is related to the degree of glycaemic control over time. Yet, the rate and timing of complication development vary from individual to individual and are not solely determined by glycaemic measurements.

So far, only a few studies have been conducted in children or young adults with present CV risk factors to examine whether a reduction of EPCs is present at early stages of disease and whether alterations in circulating progenitor subpopulations correlate with endothelial function. Therefore, in this study, we aimed not only to investigate EPC counts in children and adolescents with T1DM but also to assess mutual relationships among EPCs and parameters of endothelial function or selected biomarkers of CVD.

Materials and methods

Patients

Fifty-two children with T1DM (age 14.5 ± 2.4 years; range, 10–18 years; mean diabetes duration, 6.0 ± 3.0 years; mean HbA1c level during last 6 months, 8.5 ± 1.4) were recruited to the study. All patients were on insulin treatment, either on multiple insulin injection or pump therapy with subcutaneous insulin infusion. Inclusion criteria were as follows: duration of diabetes at least 1 year, insulin requirement at least 0.5 IU/kg per day, and age below 18 and above 9 years. The exclusion criterion was presence of any additional autoimmune disease (e.g. thyroid and celiac disease). All subjects were free from overt macrovascular complications, microalbuminuria, retinopathy and neuropathy. The control group included 36 age- and gender-matched children. All studied children were of Caucasian origin. The pubertal development was determined by the same paediatrician endocrinologist (A Bossowski), according to Tanner classification, and participants were categorised into pre-pubertal (Tanner stage 1) or pubertal (Tanner stages 2–5). Basic characteristics of the study groups is presented in Table 1.

Laboratory analyses

A blood sample of 10 ml was taken from the left cubital vein after an overnight (8–12 h) fast. For assessment of biomarkers, serum samples were collected, frozen and stored at -80°C until analyses were performed. The concentrations of sICAM-1, thrombomodulin and adiponectin were determined immunoenzymatically with the use of commercially available ELISA kits (Parameter Human Immunoassays, R&D Systems, Inc.,

Table 1 General characteristics of the study groups. Data are presented as mean $\pm$ s.d.

	Study group	Control group	P
Number of patients	52	36	
Gender (boys/girls; n (%))	24 (46)/28 (54)	16 (44)/20 (56)	
Age (years)	14.5 ± 2.4	15.1 ± 2.7	0.35
Height (m)	1.6 ± 0.1	1.6 ± 0.1	0.81
Body mass (kg)	58.9 ± 12.6	57.8 ± 13	0.71
BMI (kg/m^2)	21.9 ± 3.1	20.9 ± 2.4	0.15
Age of onset (years)	8.5 ± 3.5		
Disease duration (years)	6.0 ± 3.0		
HbA1c level (%)	8.7 ± 1.5		
HbA1c level (mean from last 6 months; %)	8.5 ± 1.4		
Tanner stage (n, %)			
1	3 (5.7)	2 (5.5)	
2	12 (23.1)	8 (22.3)	
3	17 (32.7)	10 (27.8)	
4	14 (26.9)	11 (30.5)	
5	6 (11.6)	5 (13.9)	

P values were calculated after adjustment for sex and age.

Minneapolis, MN, USA) with the use of ELx800 Automated Microplate Reader (Bio-Tek Instruments, Vermont, VT, USA). hsCRP was determined with use of the immunoturbidimetric method (Tina-quant hsCRP (Latex) HS, Roche; Hitachi 912). Concentrations of lipid parameters were determined by standard enzymatic methods (Hitachi 912). LDL concentration was assessed by the Friedewald equation. HbA1c level was measured using HPLC method.

Endothelial progenitor cells

Fresh whole-blood EDTA-anticoagulated samples (100 µl) were incubated for 30 min at room temperature with the following MABs: 20 µl FITC anti-human CD 34, 5 µl PE anti-human CD144 (VE-cadherin) or 5 µl PE anti-human CD309 (VEGFR-2) (BD PharMingen, Erembodegem, Belgium). The cells were washed twice with PBS and fixed with CellFix (BD PharMingen). Flow cytometry analysis was performed with the use of a FACSCalibur cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Analysis of EPCs was performed with the use of flow cytometry based on the surface expression of the following markers: CD34, CD144 and CD309 on the cells localised in the lymphocyte and monocyte gates. Based on the initial analysis of FMO (fluorescence-minus-one) controls, circulating progenitor cells were next identified as cells expressing CD34, and EPCs were identified as either CD34+VE-cadherin+ (CD34+CD144+) or CD34+VEGFR-2+ (CD34+CD309+) cells. Instrumental analysis was performed with CellQuest Software (BD Biosciences). Results are presented as percentage of total viable mononuclear cells. Representative plots illustrating method of delineation of CD34+ cells are presented in Fig. 1.

Ultrasound measurements

The procedure was conducted between 0800 and 1000 h after a fasting period of 8–12 h. Examinations of the brachial and carotid arteries were performed with

Hewlett Packard Sonos 4500 apparatus, using a 7.5 MHz linear transducer. Ultrasound examination of the right brachial arteries was performed in longitudinal sections 2–10 cm above the elbow, according to the guidelines (29). The principle is to induce vasodilatation in the proximal (brachial) artery by post-ischæmic (forearm) enhanced flow. All lumen diameter measurements were scanned at end diastole using the R-wave of the electrocardiogram. First scans were taken at rest and second scans during reactive hyperaemia. Increased flow was induced by deflating a pneumatic tourniquet placed on the right forearm, inflated to the pressure of 300 mmHg for 4 min. The post-ischæmic scan was performed 45–60 s after cuff deflation. FMD was derived from the percentage change of the brachial artery diameter after ischaemia of the forearm from baseline. Measurements of intima-media thickness (IMT) in the common carotid arteries (right and left) were performed as described previously, with own modification (30, 31). Measurements included end-diastolic (minimum diameter) IMT of the far walls (the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line), at the distance of more than 1 cm from the bifurcation. Analyses included the mean value of six measurements.

All the examinations were carried out and analysed by one experienced paediatric vascular ultrasonographer (A Hryniewicz), who was blinded to the participants' CV risk factor status. The intra-observer variability was 2.5% for FMD and 3.2% for IMT (evaluated in a subset of patients, $n=20$).

We obtained approval of the Ethics Committee at the Medical University of Białystok. Both parents/legal guardians and children gave their written informed consent.

Statistical analysis

Statistical analysis was performed with the use of Statistica 9.0 Software (StatSoft, Kraków, Poland). The Kolmogorov–Smirnov test of normality was used to test the distribution of variables. Unpaired Student's *t*-test

Figure 1 Representative plots illustrating method of delineation of CD34+ cells. MNCs, mononuclear cells.

Table 2 Blood pressure and basic laboratory results in the study groups. Data are presented as mean $\pm$ s.d. or median (IQR) when appropriate.

	Study group	Control group	P
Systolic BP (mmHg)	116 $\pm$ 9	114 $\pm$ 12	0.500
Diastolic BP (mmHg)	71 $\pm$ 6	69 $\pm$ 9	0.200
Total cholesterol (mg/dl)	177 $\pm$ 35	158 $\pm$ 23	0.020
Triglycerides (mg/dl)	78 (57–98)	77 (52–104)	0.800
LDL-cholesterol (mg/dl)	97 $\pm$ 30	89 $\pm$ 20	0.300
HDL-cholesterol (mg/dl)	63 $\pm$ 13	53 $\pm$ 9	0.005
sICAM-1 (ng/ml)	273 $\pm$ 78	242 $\pm$ 49	0.020
hsCRP (mg/dl)	0.4 (0.28–0.74)	0.3 (0.20–0.45)	0.041
Adiponectin (ng/ml)	16 021 (11 102–21 760)	10 799 (5954–14 445)	0.100
Thrombomodulin (ng/ml)	5.86 $\pm$ 2.4	5.25 $\pm$ 1.8	0.500

P values were calculated after adjustment for sex and age.

was used for normally distributed variables and Mann–Whitney *U* test was applied for samples not fitting parameterised distribution (EPCs, triglycerides, hsCRP and adiponectin) to compare the differences between two groups. Correlations between variables of interest were assessed by either univariate Pearson's correlation test or Spearman's rank coefficient test for parametric and non-parametric data respectively. All comparisons were adjusted for age, sex and Tanner stage. Data are expressed as either mean $\pm$ s.d. or median (interquartile range (IQR)). The level of statistical significance was set at $P < 0.05$.

Results

The type 1 diabetes patients and control group were of similar age (mean 14.5 years in T1DM group vs 15.1 years in control group) and gender (46% boys in diabetes vs 44% boys in control group) distribution, as well as of similar non-obese BMI. Blood glucose control in the diabetic group was inadequate (mean HbA1c level from last 6 months, 8.5 ± 1.4) (Table 1). Total and HDL-cholesterol levels were significantly higher in T1DM children. We also observed significantly higher levels of circulating sICAM-1 ($P = 0.02$) and hsCRP ($P = 0.04$) in diabetic children, while no differences were found for adiponectin and thrombomodulin (Table 2).

We found that CD34+ cell frequencies were similar in both groups ($P = 0.30$; Fig. 2A). In contrast, a different pattern was observed for cells with EPC phenotype. Out of varying markers delineating EPCs, VE-cadherin (CD144) and VEGFR-2 (CD309), although playing varying functional roles, are among those most frequently used for phenotypic characterisation of EPCs. In our study, we demonstrated that frequencies of CD34+ VE-cadherin+ cells were significantly higher in diabetic children compared with healthy controls ($P = 0.003$; Fig. 2B). A similar tendency that did not,

however, reach statistical significance was observed with regard to CD34+ VEGFR-2+ cell frequencies in diabetic children ($P = 0.06$; Fig. 2C). Importantly, frequencies of CD34+ VE-cadherin+ and CD34+ VEGFR+ cells were positively correlated both in healthy and in diabetic children (Fig. 3). Next, we assessed endothelial function by ultrasonographic measurements of FMD and IMT. FMD was lower (6.9 ± 3.1 vs $10.5 \pm 3.4\%$, $P = 0.002$) and IMT was higher (0.50 ± 0.07 vs 0.44 ± 0.06 mm, $P = 0.0006$) in diabetic children (Fig. 4).

Finally, we set out to analyse whether frequencies of EPCs were related to markers of endothelial dysfunction.

Figure 2 Summary of analyses of frequencies of EPCs characterised by CD34+ phenotype (A), CD34+ CD144+ (VE-cadherin+) phenotype (B) and CD34+ CD309+ (VEGFR-2+) phenotype (C). Median values are presented as black horizontal lines.

Figure 3 Correlations between frequencies of CD34+CD144+ (VE-cadherin+) and CD34+CD309+ (VEGFR-2+) cells in healthy and diabetic children.

We demonstrated a significant positive relationship between frequencies of CD34+VEGFR-2+ cells and BMI ($r=0.3$, $P=0.014$), sICAM-1 ($r=0.47$, $P=0.023$), and inverse relationship with HDL ($r=-0.27$, $P=0.04$) and FMD levels ($r=-0.45$, $P<0.001$) (Fig. 5A). Similarly, CD34+VE-cadherin+ cell frequencies correlated positively with BMI ($r=0.32$, $P=0.02$) and inversely with FMD ($r=-0.31$, $P=0.03$) (Fig. 5B). HbA1c levels were not related to EPC counts. IMT and EPC counts were not correlated in the diabetes group (Table 3).

Discussion

One of the most intriguing novel findings of our study is that EPC frequencies are not reduced in T1DM children with poor metabolic control and no obvious vascular complications. In contrast, we found that frequencies of CD34+VE-cadherin+ cells and, to a lesser extent, CD34+VEGFR+ cells, were higher in diabetic children compared with the healthy group. However, as glucose control in studied patients was less than ideal, the results of our report may not be generalisable to all children with T1DM. In the study, we also confirmed impaired endothelial function and early structural vessel changes that prove ongoing atherosclerotic process in our study population. Moreover, we reported

here increased levels of some inflammatory (hsCRP) and endothelial (sICAM-1) CVD biomarkers in diabetic children as well as lipid abnormalities that were found earlier in many other studies.

Multiple studies in adults demonstrated that EPCs count and/or function inversely correlate with risk of subsequent CVD (18, 19, 22). However, such studies have been rarely conducted in children and adolescents with a predisposition to develop vascular morbidities. In the current study, we chose to evaluate a very young population, below 18 years. To our knowledge, this is the youngest studied population with the presence of CVD risk factors. Such a study population allows to avoid participants at advanced stages of vascular injury in whom endothelial repair mechanisms might have already been activated by arterial wall damage (32). Our main results are contradictory to studies in adult populations; however, they are in agreement with the only study including healthy children. Jie *et al.* (33) demonstrated that healthy children presented with higher numbers of EPCs.

In our study, we did not limit our analysis to quantification of EPCs; instead, we extended these findings by analysing EPC counts in relation to functional measurements of endothelial function and vessel structure. This is the first study to demonstrate an inverse relationship between EPC counts and endothelial function in a very young population with T1DM. Demonstration of this correlation warrants further

Figure 4 Ultrasonography studies of vascular function (FMD) and structure (IMT). Data are presented as mean and s.d.

Figure 5 Correlations between vascular function (FMD) and frequencies of CD34+CD309+ (VEGFR-2+) cells (A) and between FMD and frequencies of CD34+CD144+ (VE-cadherin+) cells (B).

studies that could assess in detail mutual functional relations between EPCs and endothelium. DiMeglio *et al.* reported decreased levels of circulating EPCs delineated by CD34+CD133+CD31+ phenotype in a slightly older (18–22 years) small group of diabetic patients. Endothelium-dependent vasodilation was impaired similar to our study; however, it was correlated positively with reductions in circulating CD34+CD133+CD31+ cells. These cells promote endothelial integrity and may be important in vascular repair. Conversely, increased frequencies of CD34+CD45+ cells were found in the abovementioned study. These angiogenic macrophages promote new vessel growth and may indicate ongoing vessel injury (34). Sibal *et al.* in their study found reduced EPC counts and impaired endothelial function in young adults with uncomplicated T1DM. The correlation between both studied parameters was, contradictory to our results, significantly positive (23). Levels of circulating EPC were a better predictor of vascular reactivity than was the presence or absence of conventional risk factors in the study by Hill *et al.* (18).

Similar to the others (23), we did not find any relationship between IMT and EPCs; in addition, the relationship between FMD and IMT was not itself strong. However, in the recent study on young patients with long-standing T1DM (over 10 years), numbers of EPCs were an independent determinant of carotid

IMT (35). Also, a study in middle-aged patients did find a significant correlation between EPCs and IMT (36). In the light of current literature, FMD and IMT might be considered measurements assessing different parts of the same pathway of arterial wall damage, the former possibly reflecting functional abnormalities and the latter being a marker of early structural changes.

Relatively little is known about the behaviour of circulating angiogenic cell phenotypes in relation to the long-time progression of chronic vascular disease. Mild abnormalities in vascular function occurring at the beginning may in turn promote increased production and/or mobilisation of angiogenic cells into the circulation in the effort to maintain relative vascular homeostasis. This could account for the unexpected inverse association observed between EPCs and FMD in our study. The possibility that production and/or mobilisation might compensate for depletion of any cell type in mild rather than more severe stages of vascular disease is concordant with similar observations made in recent studies. In a large community-based group, Cheng *et al.* (37) demonstrated that higher EPC concentrations were modestly associated with lower peripheral arterial tone ratio. Longer time of the disease and more severe vascular injury may result in the exhaustion of bone marrow pools, decreased mobilisation and thus lower EPC counts in the peripheral circulation. Subclinical alterations in vascular function (existing in the absence of marked anatomic disease) constitute early and potentially reversible vascular disease (38, 39). Vascular dysfunction that precedes the development of anatomic atherosclerosis may yet be associated with alterations in angiogenic cell turnover (40).

Interestingly, we did not find correlations between HbA1c and hsCRP with endothelial function and endothelial progenitor subpopulations. That observation

Table 3 Correlation analysis between EPC frequencies and parameters of cardiovascular risk in diabetic patients.

	CD34+ VEGFR-2+		CD34+ VE-cadherin+	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
BMI	0.30	0.014	0.32	0.02
SBP	0.06	0.60	−0.06	0.96
DBP	0.05	0.61	0.01	0.92
Total cholesterol	−0.15	0.33	−0.19	0.93
HDL-cholesterol	−0.27	0.04	−0.24	0.14
LDL-cholesterol	−0.06	0.62	0.06	0.68
Triglycerides	−0.01	0.93	−0.02	0.87
hsCRP	−0.08	0.55	−0.12	0.38
sICAM-1	0.47	0.023	0.21	0.09
Adiponectin	0.31	0.14	0.22	0.28
Thrombomodulin	−0.13	0.52	−0.16	0.53
HbA1c (mean from last 6 months)	0.17	0.21	−0.07	0.61
IMT	0.08	0.42	0.03	0.79
FMD	−0.45	<0.001	−0.31	0.03

r, Spearman's correlation coefficient.

seems sobering because these measures are currently widely used in the clinic for assessment of risk of future microvascular and macrovascular diseases. However, other studies on young T1DM patients also failed to demonstrate these types of relations (23, 34). Altogether, these observations suggest that other factors besides 'average' of glycaemic values (such as glucose variability) may affect circulating progenitor cell numbers and vascular reactivity at a given moment in time. On the other hand, satisfactory glycaemic control in type 2 diabetic patients was associated with higher levels of circulating EPCs (41).

Another interesting finding of our study is a positive, significant correlation between EPCs and BMI. Similarly, higher number of some subpopulations of EPCs was found recently in overweight adolescents (42). In the other study conducted in children of varying weights, Kelly *et al.* found a significant association with number of circulating endothelial cells and waist circumference, systolic and diastolic blood pressure and triglyceride levels. The numbers of activated endothelial cells were lowest in normal weight children and increased over the range of weight categories (43). Studies on obese adults showed reduction in number and function of EPCs. EPCs have also been found to be reduced in pre-diabetic states (impaired fasting glucose and impaired glucose tolerance), in metabolic syndrome, with further significant reductions in number at clinical setting of type 2 diabetes (17, 20, 44, 45).

Reduced number of circulating EPCs in adults with the presence of CVD risk factors may be secondary to a variety of mechanisms occurring earlier in childhood, including exhaustion of the pool of progenitor cells in the bone marrow, reduced mobilisation of EPCs or reduced survival and/or differentiation of mobilised EPCs (46). In the only report including healthy children published so far, it has been shown that the population below 20 years presented with markedly more circulating EPCs compared with healthy adults, suggesting a higher vascular regenerative potential during childhood. In fact, both EPC numbers and clonogenic and migratory capacity decline with age (33, 47).

Having said that, the assessment of risk seems to be of little value unless there are means to ameliorate the vascular abnormality. Trigona *et al.* (48) demonstrated that children with diabetes were less active than healthy control subjects. When divided by degree of daily exercise, active children with T1DM had better vascular reactivity than children who were sedentary. There was no difference in FMD between active children with diabetes and sedentary healthy children. These data emphasise both the deleterious effects of DM on CV risk and the beneficial effects of exercise on vascular function. Walther *et al.* (49) reported that regular physical activity has a significant positive effect on number of circulating progenitor cells in the large group of healthy children. Studies on effects of physical activity on EPC numbers were not performed in diabetic

children. However, higher physical fitness was associated with a higher number of EPCs in obese children (50). Interestingly, diet and weight loss led to an increase in EPC counts in obese adults (51).

CVD begins in childhood and primary prevention must be a priority for paediatricians. Interventions to enhance vascular health are likely to be most successful early in disease before sustaining irreversible vascular damage, emphasizing the importance of studies on high-risk children and adolescents. Further assessment of cells discussed in the present paper could potentially provide a non-invasive means of assessing CV risk in clinic settings and serve as a marker of efficacy of treatments for CVD prevention and enhance the potential for discovery of novel therapies. To deal with the issue, studies evaluating the number and function of these circulating subpopulations in children and older subjects with T1DM will be important to understand how enumeration of these cells may be used as a biomarker to assess vascular damage and to enhance our understanding of the pathogenesis of vascular disease in T1DM.

This study has some methodological limitations regarding assessment of FMD. Much controversy exists as to the best method for imaging the brachial artery for reactivity studies. Multiple sets of guidelines have been published so far, and each of them proposes slightly different methodology. The current recommendations for children advise that the time of occlusion should be 4.5 min and images should be taken immediately after deflation and then after 60, 90 and 120 s (52). Our protocol is slightly different as it is based on recommendations published by Corretti *et al.* (29). This fact may lead to difficulties in comparing results generated in various centres.

In conclusion, the results of this study indicate that contrary to adult population with diabetes, diabetic children demonstrated increased frequencies of EPCs (especially delineated by CD34+CD144+ phenotype) that correlated inversely with endothelial function. Whether this phenomenon could be the result of the effective mobilisation of EPCs in the young population at increased risk for atherosclerosis in order to repair damaged endothelium will need further investigation. Taken together, higher levels of EPCs in very young patients with CVD risk factors might also reflect an unfavourable constellation, which has to be investigated in future studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by grants from Medical University of Bialystok.

Author contribution statement

B Glowńska-Olszewska and M Moniuszko designed the study, acquired data, performed statistical analysis, drafted and wrote the manuscript; A Hryniewicz performed and interpreted all ultrasonography studies; M Jeznach and M Rusak performed flow cytometry studies and interpreted these data; W Łuczyński participated in the study conception, design and contributed to a great extent to the discussion. M Dąbrowska, A Bodzenta-Łukaszyk and A Bossowski were involved in the conception, design, analysis and revising the manuscript. All authors were contributing in discussions and read and approved the final manuscript.

Acknowledgements

The authors would like to thank Prof. Paolo Beck-Peccoz, Institute of Endocrine Sciences, University of Milan, Pad. Granelli for reading this manuscript and his kind suggestions. The authors also wish to thank Mrs Ewa Fiedorczuk and Mrs Teresa Michno for excellent laboratory assistance.

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Received 20 July 2012

Revised version received 19 October 2012

Accepted 30 October 2012