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Clinical and Biochemical Predictors of Endothelial Dysfunction in Egyptian Adolescents with Type 1 and those with Type 2 Diabetes

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Authors' contributions

This work was carried out in collaboration among all authors. Author EHZ performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MAEG and WSM managed the analyses of the study. Author NAK designed the study managed the literature searches. All authors read and approved the final manuscript."All authors read and approved the final manuscript.

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ABSTRACT

Aims: Since endothelial dysfunction precedes clinically significant diabetic vascular complications, circulating endothelial progenitor cells (EPCs) have generated interest as a biomarker of endothelial function and are considered a mirror for endogenous vasculo-regenerative capacity. So we aimed to assess EPCs count in adolescents with type 1 diabetes mellitus (T1DM) in comparison to those with type 2 diabetes mellitus (T2DM) and extend these findings to assess their relationship to other clinical and biochemical risks of endothelial dysfunction.
Patients and Methods: Fifty Egyptian adolescents were included in this study, 20 with T1DM, 20 T2DM and 10 healthy control subjects. Patients are recruited from Diabetes and Endocrinology Unit, outpatient clinic of internal medicine department Tanta University Hospital, in the period from 2017 to 2019. EPCs count was determined by Flowcytometry, anthropometric measurements and laboratory investigations were done for fasting and 2-hours post-prandial blood glucose, serum

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lipid profile, HbA1c, urinary albumin creatinine ratio, fasting C peptide, and homoeostasis model assessment of insulin resistance (HOMA- IR).
Results: In T1DM, EPCs count was significantly higher compared to T2DM (0.032) and control group (p0.001) and it was negatively correlated with age of patients and duration of diabetes but was positively correlated with HbA1c. While, the count was higher in T2DM compared to control with no statistically significant difference (p0.063) and negatively correlated with body mass index, waist circumference, blood pressure and HOMA-IR.
Conclusion: Adolescents with T2DM have distressing clinical and biochemical findings and significantly lower count of (EPCs) than adolescents with T1DM. This puts them at potential higher risk for early development of endothelial dysfunction and less power of vascular repair that may potentiate early harboring of vascular complications.

Keywords: Adolescents; diabetes, endothelial dysfunction, endothelial progenitor cell.

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM), once considered a metabolic disorder exclusively of adulthood. However, the story has changed with an alarming rise in the numbers of adolescents who develop T2DM [1]. The onset of diabetes in adolescence places them at markedly increased risk that the full spectrum of both micro- and macro vascular complications would occur in early adulthood [2].

The switch from a healthy to a dysfunctional endothelium, generally termed endothelial dysfunction, is recognized to play the central role in the development of diabetic vascular complications [3]. Indeed, despite the optimization of strategies controlling classical risk factors, including; hyperglycemia, hypertension, and dyslipidemia the rate of vascular complications is still high in patients with either types of diabetes mellitus (DM) [3].

In this context, an interesting way to prevent vascular complications is to improve the continuous replacement of dysfunctional endothelial cells. Indeed, it is now recognized that endothelial repair not only depends on proliferation of adjacent mature endothelial cells whose regenerative capacity is limited but also depends on the availability of circulating endothelial progenitor cells (EPCs), which are bone marrow-derived cells that were first described in 1997 and are able to incorporate regenerating host vessels in an area of vascular injury and to contribute to vascular repair [4].

EPCs are characterized by the expression of varying surface markers including molecules characteristic for haemopoietic progenitor cell lineage(e.g. CD34 and CD133) and endothelium-associated molecules, namely vascular endothelial (VE)-cadherin (CD144) or VE growth factor receptor-2 (VEGFR-2, CD309) and CD31 [5]. Circulating EPCs have generated interest as a biomarker of endothelial function and are considered a prognostic indicator of vascular morbidity and mortality. Therefore, reduction of EPC cell numbers is believed to promote development and/or progression of vascular diseases [6].

So far, only a few studies have been conducted in diabetic adolescents to evaluate EPCs count. Our study aimed not only to evaluate EPCs counts as an indicator of endothelial function in adolescents with type 1 diabetes mellitus (T1DM) but also to compare them with adolescents with T2DM and to assess the relationships between EPCs count and other clinical and biochemical risks of endothelial dysfunction.

2. MATERIALS AND METHODS

2.1 Study Design

This cross sectional study was carried out on a total of 40 adolescents with diabetes and 10 healthy control subjects with matched age and sex. Patients are recruited from Diabetes and Endocrinology Unit, outpatient clinic of internal medicine department Tanta University Hospital, in the period from 2017 to 2019.

2.2 Study Groups

- ***Control group****:* Ten healthy adolescents as a control group.
- ***T1DM:*** Twenty adolescents with type 1 diabetes mellitus.
- ***T2DM:*** Twenty adolescents with type 2 diabetes mellitus.

Categorization into T1DM and T2DM was based on recommendations of National Institute for Health and Care Excellence for diagnosis and

management of type 1 and type 2 diabetes in children and young people (NICE guidelines 2015) [7].

2.3 Exclusion criteria

All subjects with any of the following were excluded from the study; overt macro-vascular complications, micro-albuminuria, retinopathy and neuropathy, patients presenting with acute diabetic complications, diabetic adolescents having associated features such as retinitis pigmentosa, deafness or another systemic illness or syndrome, additional autoimmune diseases, acute inflammatory diseases, hematological diseases or neoplastic diseases and any organ failure (kidney, liver, lung, heart).

2.4 Study Work Up

In the first visit; all patients were informed about the study, and consent forms were signed by both patients and guardians, respectively. In the second visit, a complete history taking with special attention to age and clinical presentation at onset of diabetes, current and previous medications regimen and family history of diabetes.

2.4.1 Clinical and anthropometric evaluation

Body Mass Index (BMI) was calculated as body weight (kg) / body height $(m)^2$. While adolescents were standing in upright position, waist circumference (WC) was measured midway between the lowest rib and the top of the iliac crest, at the end of a normal expiration.

Egyptian Height, Weight, BMI percentile charts were used; obesity was diagnosed based on BMI ≥95th percentile for gender and age. Overweight status was defined as a BMI ≥85th percentile and <95th percentile, normal BMI (percentile 5:85). Additionally, visceral adiposity was estimated using waist circumference of ≥ 90th percentile [8]. Hypertension was diagnosed if blood pressure (BP) ≥ 90th percentile (age –sex-height) [8].

Also, fundoscopic examination, thyroid, skin (Acanthosis nigricans, insulin injection sites and lipodystrophy) and comprehensive foot examinations were done.

2.4.2 Blood collection and biochemical analysis

Fasting venous blood samples (15 mL) were obtained to estimate the hematological parameters. Blood specimens were collected in vacutainer tubes with or without ethylene-di-amine-tetra-acetic acid (EDTA) as needed. Serum and plasma were prepared and then frozen (−80◦C) for storage until analysis.

Fasting serum insulin levels and fasting plasma glucose levels were determined. The HOMA-IR was calculated based on the formula; HOMA-IR = insulin (mU/L) × glucose (mmol/L)/22.5, considering 3 as the cut-off value for the diagnosis of insulin resistance [9]. Lipid parameters were determined by the standard enzymatic methods. Low-density lipoprotein (LDL) concentration was assessed by the Friedewald equation, HbA1c level was measured using HPLC method, fasting C-Peptide and urinary albumin creatinine ratio (ACR).

2.4.3 Immunophenotyping of EPCs by flow cytometry

Venous whole blood samples (5 ml) were collected into EDTA anticoagulant tubes for analysis by flow cytometry. Freshly isolated peripheral blood mononuclear cells were washed and separated from blood of patients and controls using lysis solution for erythrocytes lysis then re-suspended in phosphate buffer saline (PBS) (pH 7.4) containing 20 uL of the appropriate antibody [10] and cells were double stained with anti-Human CD34 (CD 34L) monoclonal antibody FITC labeled (Miltenyi Biotec, Catalogue no. 130-098-142) and anti-Human CD309 (VEGFR-2 monoclonal antibody PE labeled (BD Biosciences, Catalogue no. 560872, clone 89106). (BD PharMingen, Belgium). The isotype control was used to determine nonspecific binding of the lymphocyte subset-specific antibodies and to set the cut-off between fluorescence-negative and fluorescence-positive staining [11]. The cells were analyzed using a fluorescence-activated cell scanner and Cell Quest software [FACS Caliber, Becton-Dickinson, USA] and using CellQuest Software (BD Biosciences, USA).

Analysis of EPCs was based on the surface expression of the following markers: CD34 and CD309 on the lymphocyte and monocyte gates, where the initial analysis of fluorescence-minus-one were controls and circulating progenitor cells were next identified as cells expressing CD34, and EPCs were identified as CD34+ VEGFR-2+ (CD34+CD309+) cells [11]. The results are presented as percentage of total viable mononuclear cells, Figs. 1 and 2.

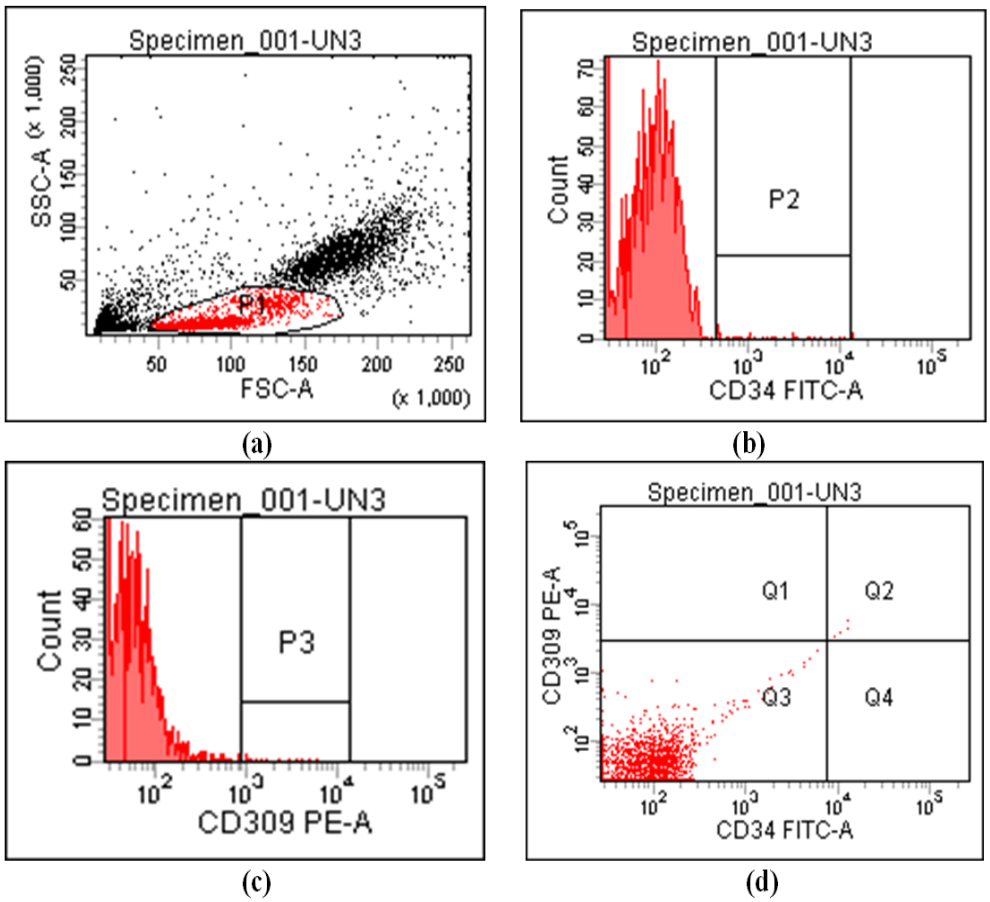

Fig. 1. Circulating endothelial progenitor cells (EPCs) were identified by flow cytometry with the expression of cell surface antigen CD34 and CD309 (KDR, VEGF-R2)
A- Forward and side light scatter (FSC-A& SSC-A) show gating on mononuclear cell
B- Histogram shows expression of (CD34) on the cell surface.
C- Histogram shows expression of (CD309) on the cell surface.
D- Representative dot-plots show double expression of (CD34 and CD309) on the cell surface that was 2% of PMNCs for adolescent with T1DM

2.5 Statistical Analysis

Once data were collected, a code sheet was developed. Organization, tabulation, presentation and analysis of data were performed by using SPSS Version 23, IBM Corp. Armonk, NY, USA. Numerical data were presented as mean and standard deviation (SD). For quantitative non-parametric data; Mann-Whitney U test and Spearman correlation were used. Categorical data were presented as number and percentage and Chi-squared test was used for statistical analysis. Spearman correlation tests were performed for estimation of the possibility of association between EPCs counts and each of the study variables. Stepwise multiple regression analysis was performed for detection of the independent risk factors for endothelial dysfunction through univariate and multivariate regression analysis. The level of significance was adopted at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 RESULTS

BMI, WC, systolic BP (SBP), diastolic BP (DBP), total cholesterol (TC), triglycerides (TG), LDL and HOMA-IR were significantly higher in T2DM compared to T1DM and control group. Considering 3 as the cut-off value for the diagnosis of insulin resistance almost all of our adolescents with T2DM had insulin resistance with HOMA-IR (5.08±1.29).

On the other hand, EPCs were significantly higher in adolescents with T1DM compared to

T2DM and control. Although it was higher in adolescents with T2DM compared to control, however the difference did not reach statistical significance, Table 1 and also shown in Figs. 1and 2.

In adolescents with T1DM; EPCs showed a significant negative correlation with age of patients and duration of diabetes, whereas they positively correlated with HbA1c. As for adolescents with T2DM; EPCs negatively correlated with age, WC, BMI, SBP, DBP and HOMA-IR. While they positively correlated with high-density lipoprotein cholesterol (HDL-C), Table 2.

On performing stepwise multiple regression analysis for identification of factors affecting endothelial progenitor cell count in adolescents with T2DM, we found that WC and HOMA-IR were the strongest independent predictors of cell count, Table 3.

3.2 DISCUSSION

Particular attention has been paid to the preclinical changes in the vascular system in diabetic adolescents in order to prevent development of overt vascular complications in early adulthood, which is mainly due to endothelial dysfunction resulting both from endothelial cell damage and impaired endothelial repair [12]. Whilst endothelial dysfunction has been shown to be a marker for risk of vascular events, there remains considerable debate about the most appropriate way to assess this [13].

Circulating EPCs are believed to contribute to endothelial repair, vascular homeostasis and compensatory angiogenesis [14]; that is why EPCs stand in unique position among all other biomarkers and considered a prognostic indicator of vascular morbidity and mortality.

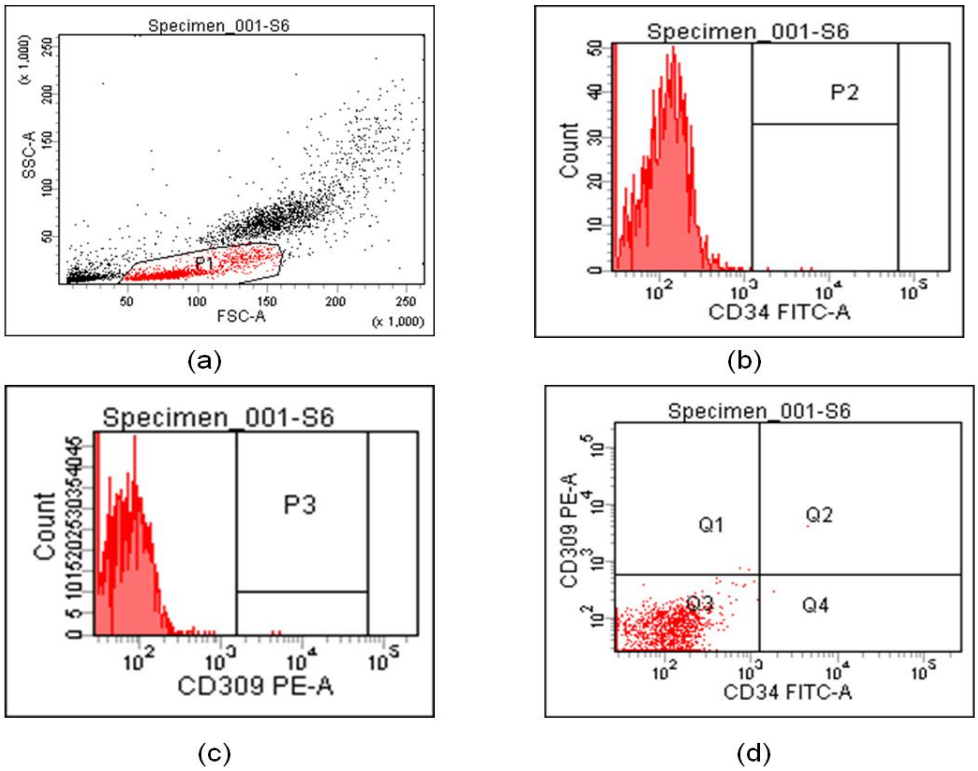

Fig.2. Circulating endothelial progenitor cells (EPCs) were identified by flowcytometry with the expression of cell surface antigen CD34 and CD309 (KDR, VEGF-R2)

A- Forward and side light scatter (FSC-A& SSC-A) show gating on mononuclear cell
B- Histogram shows expression of (CD34) on the cell surface
C- Histogram shows expression of (CD309) on the cell surface.
D- Representative dot-plots show double expression of (CD34 and CD309) on the cell surface that was 1% of PMNCs for adolescent with T1DM.

Table 1. Demographic, anthropometric and laboratory values by group (mean ± SD)

	Control	T1DM	T2DM	P value	P1	P2	P3
Sex (Female %)	5 (50%)	13 (65%)	17 (85%)	**0.117**			
Age (years)	12.80 ± 1.75	12.75 ± 1.25	16.15 ± 1.18	0.001*	0.923	0.001*	0.001*
Age at diagnosis (years)	–	9.35 ± 0.81	13.70 ± 1.49	0.001*			
WC (cm)	69.20 ± 3.08	68.80 ± 3.30	94.10 ± 3.91	0.001*	0.771	0.001*	0.001*
Height (cm)	154.90 ± 4.43	155.25 ± 6.71	161.50 ± 4.48	0.001*	0.870	0.003*	0.001*
Weight (kg)	47.49 ± 4.43	46.85 ± 6.15	77.64 ± 7.27	0.001*	0.795	0.001*	0.001*
BMI (m2/kg)	19.74 ± 0.98	19.33 ± 1.55	29.71 ± 1.53	0.001*	0.464	0.001*	0.001*
SBP	102.50 ± 4.25	102.10 ± 5.55	117.30 ± 8.23	0.001*	0.879	0.001*	0.001*
DBP	64.50 ± 3.69	66.40 ± 3.79	74.45 ± 6.52	0.001*	0.337	0.001*	0.001*
FPG (Mg/dl)	87.60 ± 8.36	139.00 ± 38.90	144.75 ± 28.13	0.001*	0.001*	0.001*	0.557
HbA1c%	–	8.12 ± 2.47	9.23 ± 2.48	0.163			
TC (mg/dl)	154.80 ± 18.77	165.90 ± 29.84	190.75 ± 32.98	0.004*	0.335	0.003*	0.010*
TG (mg/dl)	80.70 ± 15.67	99.20 ± 24.62	142.85 ± 48.50	0.001*	0.182	0.001*	0.001*
LDL (mg/dl)	71.60 ± 7.96	88.95 ± 27.17	113.30 ± 30.20	0.001*	0.092	0.001*	0.005*
HDL (mg/dl)	56.10 ± 3.60	57.15 ± 3.75	53.90 ± 8.77	0.262	0.667	0.369	0.108
C. peptide (nmol/l)	–	0.15 ± 0.10	1.93 ± 0.66	0.001*			
HOMA	–	1.46 ± 0.34	5.08 ± 1.29	0.001*			
EPCS	0.08 ± 0.09	0.33 ± 0.23	0.20 ± 0.13	0.002*	0.001*	0.063	0.032*

BMI Body Mass Index, DBP Diastolic Blood Pressure, EPCs Endothelial Progenitor Cells, FPG Fasting Plasma Glucose,HbA1c Glycosylated Hemoglobin Percentage, HDL High Density Lipoprotein, HOMA-IR Homeostasis Model Assessment-Insulin Resistance, LDL Low Density Lipoprotein, SBP Systolic Blood Pressure, TC Total Cholesterol, TG Triglycerides, WC Waist circumference.

** Significant p value < 0.05*

P1: Control & T1DM

P2: Control & T2DM

P3: T1D & T2D M

Table 2.Correlation between EPCs count and demographic, clinical and laboratory parameters in studied adolescents with T1DM and T2DM

EPCS	T1DM		T2DM	
	R	P	r	P
Age	-0.850	0.001*	-0.586	0.007*
Duration	-0.923	0.001*	-0.423	0.063
WC	-0.260	0.268	-0.642	0.002*
BMI	-0.220	0.351	-0.835	0.001*
SBP	-0.394	0.085	-0.783	0.001*
DBP	-0.305	0.191	-0.703	0.001*
FBG	0.359	0.120	-0.797	0.001*
HbA1c	0.587	0.006*	-0.420	0.065
TC	-0.042	0.859	-0.294	0.208
TG	-0.191	0.419	-0.073	0.761
LDL	0.021	0.932	-0.431	0.058
HDL	-0.251	0.285	0.592	0.006*
F. insulin			-0.548	0.012*
HOMA-IR			-0.847	0.001*

EPCs Endothelial progenitor cell count, BMI Body Mass Index, DBP Diastolic Blood Pressure,HbA1c Glycosylated Hemoglobin Percentage, HDL High Density Lipoprotein, HOMA-IR Homeostasis Model Assessment-Insulin Resistance, LDL Low Density Lipoprotein, SBP Systolic Blood Pressure, TC Total Cholesterol, TG Triglycerides, WC Waist circumference.
** Significant p value < 0.05*

Table 3. Univariate and multivariate regression analysis in adolescents with T2DM

T2D	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Age	0.612 (0.338 – 2.858)	0.322		
WC	0.325 (0.105 – 0.521)	0.001*	0.489 (0.125 – 0.769)	0.016*
BMI	0.241 (0.052 – 0.632)	0.005*	0.356 (0.201 – 3.215)	0.287
SBP	0.526 (0.125 – 0.745)	0.010*	0.589 (0.308 – 3.775)	0.189
DBP	0.531 (0.206 – 0.954)	0.007*	0.639 (0.149 – 3.214)	0.296
FBG	0.621 (0.412 – 0.856)	0.001*	0.954 (0.425 – 9.523)	0.384
HDL	2.051 (0.526 – 6.854)	0.139		
Fasting insulin	0.631 (0.125 – 2.351)	0.307		
HOMA-IR	0.355 (0.127 – 0.668)	0.001*	0.625 (0.258 – 0.941)	0.007*

BMI Body Mass Index, DBP Diastolic Blood Pressure, HDL High Density Lipoprotein, HOMA-IR Homeostasis Model Assessment-Insulin Resistance, SBP Systolic Blood Pressure, WC Waist circumference.
** Significant p value < 0.05*

Multiple studies in diabetic adults revealed that EPCs count and/or function is reduced and inversely correlated with cardiovascular risks. However, such studies were rarely conducted on diabetic adolescents. To the best of our knowledge, this is the first study to investigate EPCs count in adolescents with T1DM in comparison to those with T2DM and to assess their relationship to other clinical and biochemical risks of endothelial dysfunction.

Our study clearly demonstrates that, unlike adolescents with T1DM, adolescents with T2DM have peculiar clinical and biochemical characteristics such as obesity, dyslipidemia, elevated blood pressure and evidences of insulin resistance, all of these risks affect vascular integrity and vasculo-regenerative power.

In our study, EPCs count was higher in T1DM adolescents compared to control group, (p = 0.001). This comes in agreement with Głowinska-Olszewska et al. [12] who performed a comparative analysis of 52 adolescents with T1DM and 36 healthy sex and age matched control [11].

In contrast to our results, Hortenhuber et al. [15] found that EPCs were significantly reduced in T1DM children versus control [16]. Also,

Palombo et al. [17] studied 16 uncomplicated T1DM and 26 control subjects. They found that young subjects with relatively long-lasting uncomplicated T1DM have a significantly lower count of circulating EPCs than control [15]. This might be due to the fact that the duration of DM in their patients was longer than in ours.

Interestingly, when we evaluated EPCs count in adolescents with T2DM, it was significantly lower than T1DM (P 0.032), however it was higher than control group but without statistical significance (p 0.063). This may suggest that, despite having more distressing risks of endothelial injury, adolescents with T2DM have less vasculo-regenerative power than adolescents withT1DM.

However, studies evaluating EPCs count in adolescents especially with T2DM are scarce, Piresa et al. [18] and Jung et al. [19] investigated EPCs count in overweight and obese non diabetic adolescents and found that the count was elevated in obese and overweight compared to lean control.
While, multiple studies in adult found EPCs count was significantly lower in patients with T1DM and T2DM compared to control group, as reported by Loomans CJ et al. [20] and Lombardo et al. [21].

Not only we evaluated EPCs in our young adolescents but we also assessed their correlation with cardiovascular risk factors.

The links between EPCs count and well-established risk factors for endothelial dysfunction (e.g. Obesity, hypertension, dyslipidemia and insulin resistance) were extensively examined in adults and to some extent in obese non diabetic adolescents but is extremely scarce in adolescent with T2DM.

In our study, we found significant negative correlation between circulating endothelial progenitor cell count and waist circumference, body mass index, HOMA-IR and blood pressure of T2DM adolescents.

Piresa et al. [18] and Jung et al. [19] found that EPCs number was increased and directly correlated to BMI obese non-diabetic adolescents compared with healthy controls.

Multiple studies in adult with obesity Müller-Ehmsen et al. [22] and MacEneaney et al. [23] found that obesity have generally been associated with reduced EPCs count and there was inverse correlation between the count and BMI.

In agreement with our results, Oliveras et al. [24] and Kahn et al. [25] found inverse correlation between EPCs and blood pressure in adults.

Considering HOMA-IR, multiple studies ensured that it affects the levels of circulating EPCs. In adults (HOMA-IR) has been found to negatively correlate with EPCs count as reported by Fadini et al. [26], Cubbon et al. [27] and Kahn et al. [25] Furthermore, Chen et al. [28,29] found that the treatment with an insulin sensitizer (metformin, or thiazolidinediones) restored circulating EPC levels in diabetics .

In our study, EPCs cell count has negative correlation with age of patients and duration of diabetes. This comes in accordance with Arcangeli et al. [30] who perform a study on 111 T1DM patients without clinical vascular damage (59 children and young patients <20 years and 52 ≥ 20 years) to evaluate the count of EPCs at different ages and with different disease duration and concluded that, the number of EPCs in young (<20 years) patients was higher compared with older subjects.

Palombo et al. found young subjects with relatively long-lasting T1DM have a significantly lower count of circulating EPCs that were inversely correlated with disease duration [15].

As aging is associated with a decline in markers of cardiovascular health, so, it is not surprising that many studies also report age-related declines in both EPC number [31], Jie et al. [32], Yang et al. [33] and function [34], Xia et al. [35].

One of the most unique finding in our results is that in our T1DM patients EPCs count correlated positively with HbA1c, this is contradictory to Hortenhuber et al. [15] who found EPCs count negatively correlated with HbA1C .

However, other studies on young T1DM patients failed to demonstrate any type of relation between cell count and HbA1c Sibal et al. [36], DiMeglio et al. [37] and Głowinska- Olszewska et al [12]. This may suggest that glycemic control is not the only factor affecting the EPCs count but others such as disease duration and glucose variability may have a role.

Among the other interesting findings in our study that the multivariate regression analysis revealed that HOMA-IR and waist circumferences were the strongest effectors on endothelial progenitor

cell count in T2DM while disease duration was the most effector in those with T1DM.

Clinical and biochemical findings of our young adolescents with T2DM in addition to significantly lower count of circulating EPCs in comparison to adolescents with T1DM, makes them potentially at higher risk for early vascular complication than those with T1DM. As circulating EPCs are not only a mirror for exposure to cardiovascular risks, but they also reflect the endogenous vasculo-regenerative capacity and they are good predictor of vascular reactivity.

our result also indicate that contrary to diabetic adults who have marked low count of EPCs, adolescents with T2DM still have EPCs count higher than control. Thus, at this stage, early interventions with weight loss, controlling blood pressure, improving insulin sensitivity for adolescents with T2DM may improve endothelial integrity and delay complications. That also emphasizes the importance of early screening and diagnosis of prediabetes and T2DM in obese adolescents. Evaluation of these cells may help prediction of cardiovascular complications. They might as well serve as a therapeutic target for prevention and treatment of vascular complications.

4. CONCLUSION

In comparison to adolescents with T1DM, adolescents with T2DM had significantly lower count of (EPCs) which correlated with known clinical and biochemical risks of endothelial dysfunction. This puts them at a potential higher risk for early development of endothelial dysfunction and less power of vascular repair that may potentiate early harboring of vascular complication.

5. LIMITATION OF THIS STUDY

The small number of patients

6. RECOMMENDATIONS

- Further multi-centric studies in large sample size should be done to assess endothelial dysfunction in adolescents with T2DM and T1DM.
- Interventions aiming at weight loss, controlling blood pressure, improving insulin sensitivity must be aggressively instituted in order to reverse endothelial damage and restore endothelial integrity and delay complications in adolescents with T2DM.
- Further studies are needed to assess EPCs count in adolescents with T1DM and T2DM with vascular complications
- Further studies are needed to compare EPCs count in obese non diabetic adolescent, obese prediabetic adolescents and diabetic adolescents with and without diabetic complications.
- Further studies are needed to evaluate the effect of treatment modalities on EPCs count in diabetic adolescents.

CONSENT AND ETHICAL APPROVAL

An informed written consent was taken from each participant and the guardian before enrollment in the study. The study was ethical approved by the Institutional Review Board of Faculty of Medicine, Tanta University, Egypt (proposal code:**30669/2015**).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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