Circulating progenitor and angiogenic cell frequencies are abnormally static over pregnancy in women with preconception diabetes: A pilot study

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Abstract

Type 1 and 2 diabetes decrease the frequencies and functional capacities of circulating angiogenic cells (CAC). Diabetes also elevates gestational complications. These observations may be interrelated. We undertook pilot studies to address the hypothesis that preconception diabetes deviates known gestational increases in CACs. Cross-sectional study of type 1 diabetic, type 2 diabetic and normoglycemic pregnant women was conducted at 1st, 2nd, and 3rd trimester and compared to a 6mo postpartum surrogate baseline. Circulating progenitor cells (CPC; CD34+CD45dimSSlow) and CACs (CD34+CD45dimSSlow expressing CD133 without or with KDR) were quantified by flow cytometry and by colony assay (CFU-Hill). In pregnant normoglycemic women, CD34+CD45dimSSlow cell frequency was greater in 1st and 3rd trimester than postpartum but frequency of these cells was static over type 1 or 2 diabetic pregnancies. Type 1 and type 2 diabetic women showed CACs variance versus normal controls. Type 1 diabetic women had more total CD34+KDR+ CACs in 1st trimester and a higher ratio of CD133+KDR+ to total CD133+ cells in 1st and 2nd trimesters than control women, demonstrating an unbalance in CD133+KDR+ CACs. Type 2 diabetic women had more CD133+KDR+ CACs in 1st trimester and fewer CD133+KDR- CACs at mid-late pregnancy than normal pregnant women. Thus, pregnancy stage-specific physiological fluctuation in CPCs (CD34+) and CACs (CD133+KDR+ and CD133+KDR-) did not occur in type 1 and type 2 diabetic women. Early outgrowth colonies were stable across normal and diabetic pregnancies. Therefore, preconception diabetes blocks the normal dynamic pattern of CAC frequencies across gestation but does not alter colony growth. The differences between diabetic and typical women were seen at specific gestational stages that may be critical for initiation of the uterine vascular pathologies characterizing diabetic gestations.

Introduction

Various circulating, bone marrow-derived, vasculotrophic cell types support vessel repair and neoangiogenesis [1]. Circulating angiogenic cells (CACs), originally termed circulating endothelial progenitor cells (EPCs), were considered a single lineage that differentiated into mature endothelium. EPCs were phenotypically defined as Lin-CD34+CD45- or CD45dim and reactive with either CD133 or KDR (Kinase insert Domain Receptor; VEGFR2 or CD309). These populations gave rise to two types of colonies in culture [1,2]. Although early outgrowth colonies, known as "colony forming units (CFU)-Hill" grew with endothelial cell-like morphology, they are now known to include myeloid cells (macrophages and monocytes) and to have frequent T cell contamination. Cells forming these mixed colonies however, are still thought to promote angiogenesis and vascular repair [3]. Early outgrowth colonies are used to predict patient cardiovascular risk [4]. The other colony type (later outgrowth colonies or outgrowing endothelial cells), were much rarer and displayed properties resembling endothelial cell lines. Later outgrowth colonies emerge only after long time culture and it uncertain whether this population differentiates *in vivo*. Concepts regarding EPCs have been considerably refined. Heterogeneous circulating vasculotrophic cells are now recognized, most of which localize to perivascular spaces rather than differentiating into endothelium. CPC and CAC represent cell populations enriched in monocytes that exert their angiogenic effects via paracrine mechanisms. Here we addressed the dual impacts of diabetes and pregnancy on CPCs (CD34+CD45dimSSlow), CACs (CD34+CD45dimSSlow expressing CD133 and/or KDR) and on early outgrowth colonies.

Healthy pregnancy is a physiological state heavily dependent upon maternal utero-placental and systemic angiogenesis and vascular remodeling. Early maternal angiogenesis is driven by nidatory hormones and conceptus implantation. Subsequently, angiokines are secreted into the maternal circulation by the conceptus-derived placenta [5–8] and by maternal decidual leukocytes [9,10]. In healthy pregnancies, CPCs (CD34+) and CACs (CD34+KDR+ and CD133+KDR+) are reported to increase by 2nd and into 3rd trimester then decline to nulligravida levels by 48h postpartum [11,12,13]. Little is known regarding CPC or CAC functions during maternal uterine or systemic vascular remodeling and expansion that typifies normal pregnancy. It is reported that the menstrual cycle and menopause alter CD34+KDR+ CACs frequency via estrogen-based mechanisms [14]. In the common pregnancy complications of preeclampsia [12,15] and new onset gestational diabetes [13,16], CAC frequencies are lower than in normoglycemic pregnant women and colony growth is slower. Preeclampsia, an acute, multi-organ vascular syndrome of late pregnancy, is associated with high maternal plasma sFLT1 [17], a decoy receptor for vascular endothelial growth factor (VEGF). Pregnant diabetic women have elevated preeclampsia risk, regardless of their underlying diabetic etiology [18–20]. No publications report CACs levels during pregnancy in normotensive women with established preconception diabetes.

Non-pregnant, type 1 and type 2 diabetic individuals exhibit vascular and endothelial dysfunctions [21, 22]. The two "hits" of reduced CAC frequency and impaired regenerative capacity [23–27] are thought to compromise vascular health [26– 28], predict cardiovascular disorders [20, 27, 29] and limit vascular repair. We hypothesized that CPC and CAC frequencies would increase across normal pregnancy when compared their frequencies in non-pregnant women. We further postulated that preconception diabetes would diminish these gains, even in women with regular physician-supervised and individually- tailored diabetic management, and this reduction would contribute to the maternal and fetal complications characteristic of diabetic pregnancies. Our aim was to quantify CPC and CACs across pregnancy (1st, 2nd, and 3rd trimester) in type 1 and type 2 diabetic women and matched normoglycemic women

carrying singleton fetuses and attending a tertiary care obstetrical diabetes clinic. Postpartum (~6 months) was used as a surrogate time point for preconception, baseline CPC and CAC values, since our patient referrals follow conception. Differences in CPC and CAC frequencies were documented between pregnant diabetic women and women experiencing typical pregnancies and between pregnant type 1 and pregnant type 2 diabetic patients.

Material and methods

Patients and recruitment

Pregnant and postpartum women with type 1 diabetes (n = 32) or type 2 diabetes (n = 28), and healthy age and gestation stage-matched controls (n = 49) were recruited at Kingston General Hospital (Kingston, ON, Canada). Diabetic patients regularly attended the diabetic pregnancy clinic for supervised care. Exclusion criteria were more than one fetus or previous diagnosis or treatment of hypertension, polycystic ovarian syndrome, cancer and/or autoimmune disease other than diabetes. Patients who provided written consent were blind encoded to the investigators and no personal identifying information was recorded. Protocols were pre-approved by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (ANAT-030-10). Blood was collected from different participants at 6–13 weeks (1st trimester), 18–26 weeks (2nd trimester), 28–36 weeks (3rd trimester) or postpartum (~6 months) into acid citrate dextrose anti-coagulant by venipuncture (BDVacutainer, BD Bioscience; Mississauga, ON, Canada). Mononuclear cells were immediately isolated by centrifugation (400 g; 30 min; 4˚C) using Histopaque 1077 (Sigma Aldrich, St. Louis, MO, USA), recovered, washed twice (PBS; 600 g; 10 min; 4˚C), enumerated using a hemocytometer then aliquoted for flow cytometry or CFU- Hill culture.

Flow cytometry

The modified protocol of the International Society of Hematology and Graft Engineering (ISHAGE) was used to examine $1x10^5$ washed cells that had been incubated (30 min; 4˚C in dark) in 100 uL of PBS containing 1% BSA (Sigma Aldrich, Oakville, ON, Canada), 2% heat-inactivated human serum (Life Technology, Canada; FACS buffer) and antibodies directly conjugated to fluorochromes: anti-CD45 Phycoerythrin Cyanin 7 (Biolegend, San Diego, CA, USA), anti-CD34 FITC (BD Pharmigen, San Diego, CA, USA), anti-CD133 Phycoerythrin (Macs Miltenyl Biotec, Alburn, CA, USA) and anti-KDR PerCp (R&D System, Minneapolis, MN, USA) and their respectively and isotype controls in concentrations suggested by the manufacturer (Table 1). After washing in PBS, cells were fixed 2% paraformaldehyde and analysed (Beckman Coulter Cytomics FC500; Beckman Coulter, Inc., Mississauga, ON, Canada). Post-

Table 1. Antibody and isotype information.

Antibody and Isotypes	Fluorochrome	Company	Catalogue number
Anti-CD34	FITC	BD Pharmigen	S55821
Anit-CD45	Phycoerythrin Cyanin 7	Biolegend	304016
Anti-CD133	Phycoerythrin	Macs	130-090-826
Anti-KDR	PerCP	R&D System	FAB357C
IgG1	FITC	Biolegend	981802
IgG1	Phycoerythrin Cyanin 7	Biolegend	400126
IgG1	Phycoerythrin	Biolegend	400112
IgG1	PerCP	Biolegend	400148

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acquisition compensation and analyses were performed using Kaluza software (Beckman Coulter, Inc.).

Early outgrowth colonies (CFU-Hill assay)

For clonogenic assays, $5x10^6$ washed mononuclear cells were resuspended in 1.5mL CFU-Hill Basal Medium (Stem Cell Technologies Inc., Vancouver, Canada) with CFU-Hill proliferation supplement (Stem Cell Technologies Inc.) according to supplier instructions, plated on fibronectin-coated 35 mm dishes (Biocoat BD Bioscience, Mississauga, ON, Canada) and incubated (37˚C; 5% CO2). After pre-plating depletion (48 h) of adherent macrophages and mature endothelial cells, non-adherent cells were collected, re-plated at $2x10^6$ cells per well on fibronectin-coated 4-well chamber slides (Biocoat BD Bioscience) and cultured 72 h. Then, medium was removed, wells were washed in PBS and Giemsa stained (Sigma-Aldrich, Oakville, ON, Canada). Colonies, defined as a central core of round cells with peripheral elongated sprouting cells, were enumerated.

Statistical analyses

Continuous variables were tested for normality using Kolmogorov-Smirnov test. Non-parametric Kruskal-Wallis one-way analysis of variance test followed by Dunn's multi-comparisons post-test was used to compare values across pregnancy and postpartum within the same group (control, type 1 diabetes or type 2 diabetes), the CFU-Hill data and patient clinical information (family wise significance and confidence levels ≤ 0.05). Specific comparisons between control, type 1 diabetes and type 2 diabetes patients were performed using Mann-Whitney U- Test (two-tailed and 95% of confidence level). The ROUT method was used as outlier identifier (Q = 1%). Data are expressed as mean±SEM or percentage, unless otherwise stated.

Results

Age, parity, number of term deliveries, abortions or living children did not differ between patient groups. More preterm deliveries occurred in type 2 diabetic patients than in controls with, 41.71% of type 2 diabetic women delivering preterm compared to 2.70% controls (Table 2). Preeclampsia was more frequent in type 1 diabetic women (28.54%) compared to controls (0%). Weight gain, systolic and diastolic blood pressures and blood glucose at 2nd and 3rd trimester in diabetic patients matched controls. Mean HbA1C (%) was between 6.50% and 7.20% (levels >6.5% indicate diabetes) in type 1 and type 2 diabetic patients, target values recommended by the Canadian Diabetes Association (Table 2). Microalbumin to creatinine ratios (M/C) in diabetic patients were >26.63 ± 17.90 mg/mmol (normal female values < 2 mg/mmol; >30 mg/mmol defined as microalbuminuria).

CPCs were defined as CD34+ cells that had low side scatter (SS) and CD45 expression (CD34+CD45dimSSlow). CACs were defined as CD34+CD45dimSSlow cells expressing CD133 or KDR (Fig 1). In control women, frequencies of circulating CD34+CD45dimSSlow cell in 1st and 3rd trimester were higher than at 6 months postpartum (p = 0.013 and p = 0.030, respectively). Second trimester frequency did not differ to postpartum (p = 0.116; Fig 2Ai). In contrast, CD34+CD45dimSSlow cell numbers were stable in type 1 and type 2 diabetic women across pregnancy and through postpartum (Fig 2Aii and 2Aiii). When circulating CD34+CD45dimSSlow cell numbers were compared among groups (Fig 2Bi; Table 3) within a gestational stage or postpartum, circulating CD34+CD45dimSSlow cells were significantly higher at 3rd trimester of control pregnancies compared to type 1 diabetic women (p = 0.043; Fig 2Bii; Table 3). Type 2 diabetic women at 3rd trimester also had fewer CD34+CD45dimSSlow cells

Table 2. Maternal information.

Demographic

	Control (36)	Type 1 diabetes (12)	Type 2 diabetes (17)	*P*
Age	29.89 (±0.68)	27.17 (±1.30)	31.41 (±1.22)	0.101
Obstetric History				
Gravida	2.02 (±0.23)	2.00 (±0.32)	2.76 (±0.31)	0.069
Term Deliveries %[†]	44.44	33.33	41.17	0.675
Preterm Deliveries %[†]	*2.70***	*25.00*	*41.71***	*0.001*
Abortions %[†]	22.24	25.00	41.17	0.343
Living Children	0.75 (±0.18)	0.58 (±0.22)	1.176 (±0.24)	0.117
Preeclampsia%[†]	*0***	*28.54***	10.00	*0.003*
Clinical				
	Control	**Type 1 diabetes**	**Type 2 diabetes**	*P*
Weight gain (Kg)- Pregnant—non-pregnant weight				
2nd trimester	4.90 (±1.93)	11.29 (±2.85)	7.03 (±2.2)	0.219
3rd trimester	11.72 (±2.60)	10.85 (±4.45)	12.24 (±2.42)	0.779
Systolic Blood Pressure (mmHg)				
2nd trimester	114.70 (±3.71)	110.00 (±3.65)	123.00 (±3.83)	0.103
3rd trimester	111.80 (±2.80)	11.60 (±5.87)	123.70 (±7.45)	0.228
Diastolic Blood Pressure (mmHg)				
2nd trimester	75.00 (±1.52)	68.60 (±1.90)	67.30 (±2.42)	0.205
3rd trimester	67.30 (±1.97)	69.20 (±2.49)	75.00 (±4.69)	0.387
Blood Glucose (mmol/L)				
2nd trimester	5.32 (±0.11)	6.76 (±1.17)	6.93 (±0.65)	0.271
3rd trimester	4.86 (±0.89)	6.83 (±1.38)	5.68 (±0.77)	0.348
HbA1C				
2nd trimester	NAD	6.70 (±0.3) (%)	6.60 (±0.3) (%)	0.903
		50(±7.4) (mmol/mol)	49.40(±12.6) (mmol/mol)	0.928
3rd trimester	NAD	7.20 (±0.6) (%)	6.50 (±0.3) (%)	0.333
		55.17 (±16.45) (mmol/mol)	47.33 (±9.7) (mmol/mol)	0.389
Microalbumin:Creatinine ratio (mg/mmol)				
2nd trimester	NAD	69.93 (±60.29)	43.72 (±34.99)	0.697
3rd trimester	NAD	26.63 (±17.90)	143.0 (±133.7)	0.539

Data are presented as mean± SEM or percentage. NAD- no abnormality detected; Normal ranges of glucose (3.3–5.6mmol/L), HbA1C (3.6–5.0% of Hb) and microalbumin:creatinine ratio (<2.0 mg/mmol).

[†] Frequency of women within a group.

Significant values of p≤ 0.01 (**).

HbA1C conversion using: http://www.ngsp.org/convert1.asp.

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compared to control women, but this difference was not statistically significant (p = 0.130; Fig 2Biii; Table 3).

In control women, CD133+KDR- CACs appeared to be increased by pregnancy but this trend did not reach statistical significance compared to a non-pregnant state (6 months postpartum *vs* 1st p = 0.082, *vs* 2nd trimester p = 0.057 and *vs* 3rd trimester p = 0.057). No pregnancy-linked elevation in CD133+KDR- CACs occurred in type 1 or type 2 diabetes (Fig 3Ai). Indeed, CD133+KDR- CACs from 2nd and 3rd trimesters of type 2 diabetic women were significantly rarer than in control women (p = 0.028 and p = 0.004, respectively; Fig 3Bi and 3Bii; Table 3). Similar statistical differences in CD133+KDR- cell frequency at 2nd and 3rd trimesters
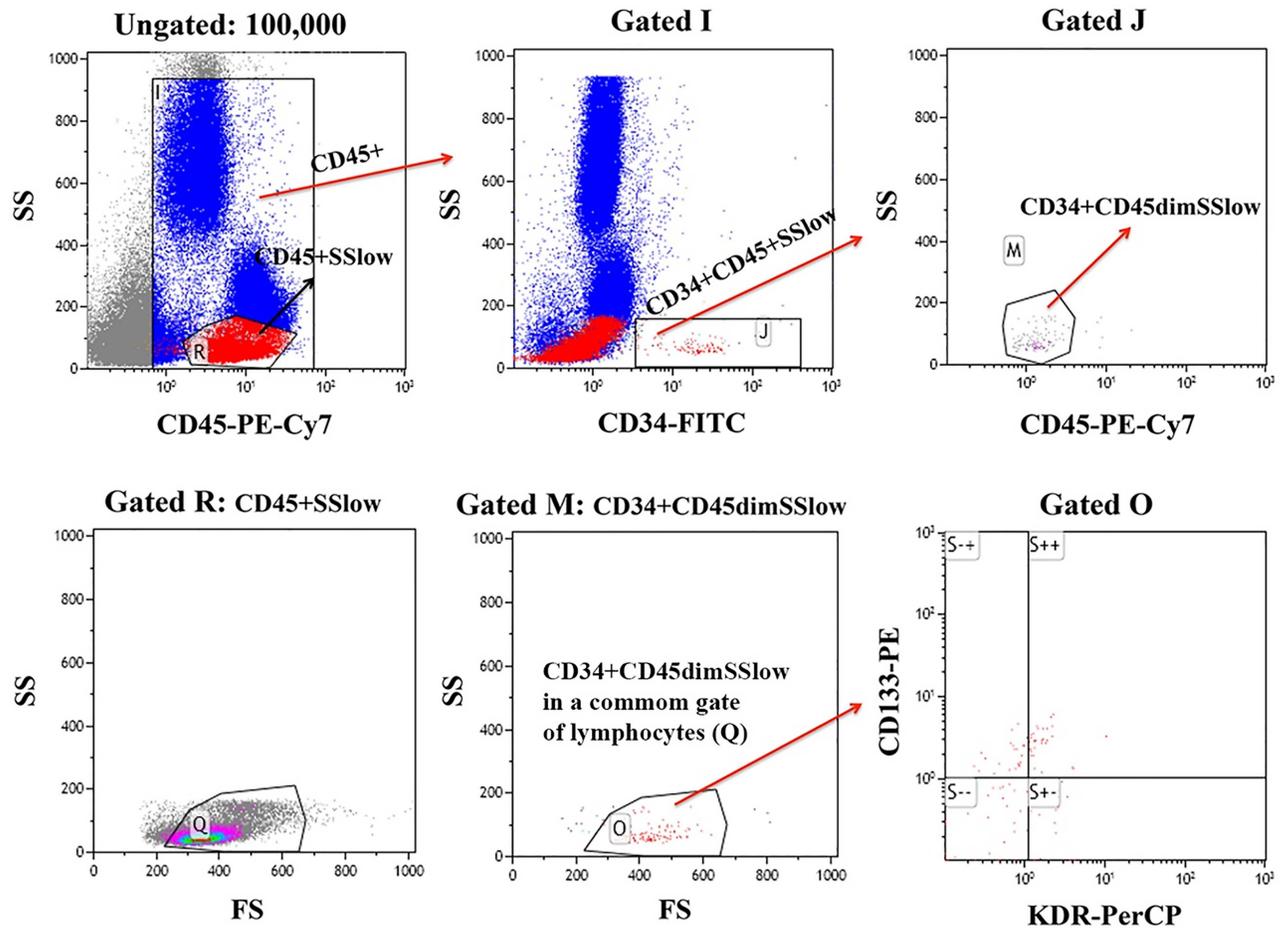
Fig 1. Gating strategy based on ISHAGE guidelines. Total CD45+ cells were gated, followed by Side Scatter (SS) *vs* CD34-FITC. The CD34+CD45+SSlow subset was selected (Gate J). A second SS *vs* CD45-PE-Cy7 gating was performed to select CD45dim cells (Gate M), and confirmed by a low Forward Scatter (R- a shared lymphocyte gate; Gate O). Analyzing CD34-FITC *vs* CD45-PE-Cy7 cells, a subset should be coincident with gate Q (lymphocyte). Expression of CD133 and/or KDR was assessed on CD34+CD45dimSSlow cells (gated O).

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were not confirmed for type 1 diabetic women (p = 0.074 and p = 0.152, respectively; Fig 3Biii and 3Biv; Table 3).

CD133+KDR+ and CD133-KDR+ CACs were less abundant in the circulations of control and diabetic women than CD133+KDR- cells. Neither KDR+ CAC subset differed statistically across pregnancy and postpartum within a group (Fig 3Aii and 3Aiii; Table 3). Generally, diabetic women (type 1 and type 2) had more circulating CD133+KDR+ CACs than controls (Fig 3Aii; Table 3). CD133+KDR+ CACs were significantly more abundant in type 2 diabetic women at 1st trimester compared to controls (p = 0.032; Fig 3Ci). This difference was not observed in 1st trimester type 1 diabetic women (p = 0.091; Fig 3Cii). The CD133-KDR+ CAC subset was constant across pregnancy or postpartum within a group, and it did not significantly differ among groups (Fig 3Aiii; Table 3). CD34+ cells that did not express CD133 or KDR were also analyzed. Numbers of these hematopoietic progenitor cells did not vary across pregnancy and postpartum in any group (Fig 3Aiv; Table 3). Additionally, no differences were found comparing circulating CD34+CD133-KDR- cell numbers between diabetic women (type 1 or type 2) and control women (Table 3). At 3rd trimester however, type 2 diabetic women had elevated CD34+CD133-KDR- cell numbers compared to type 1 diabetic women (p = 0.030; Table 3).
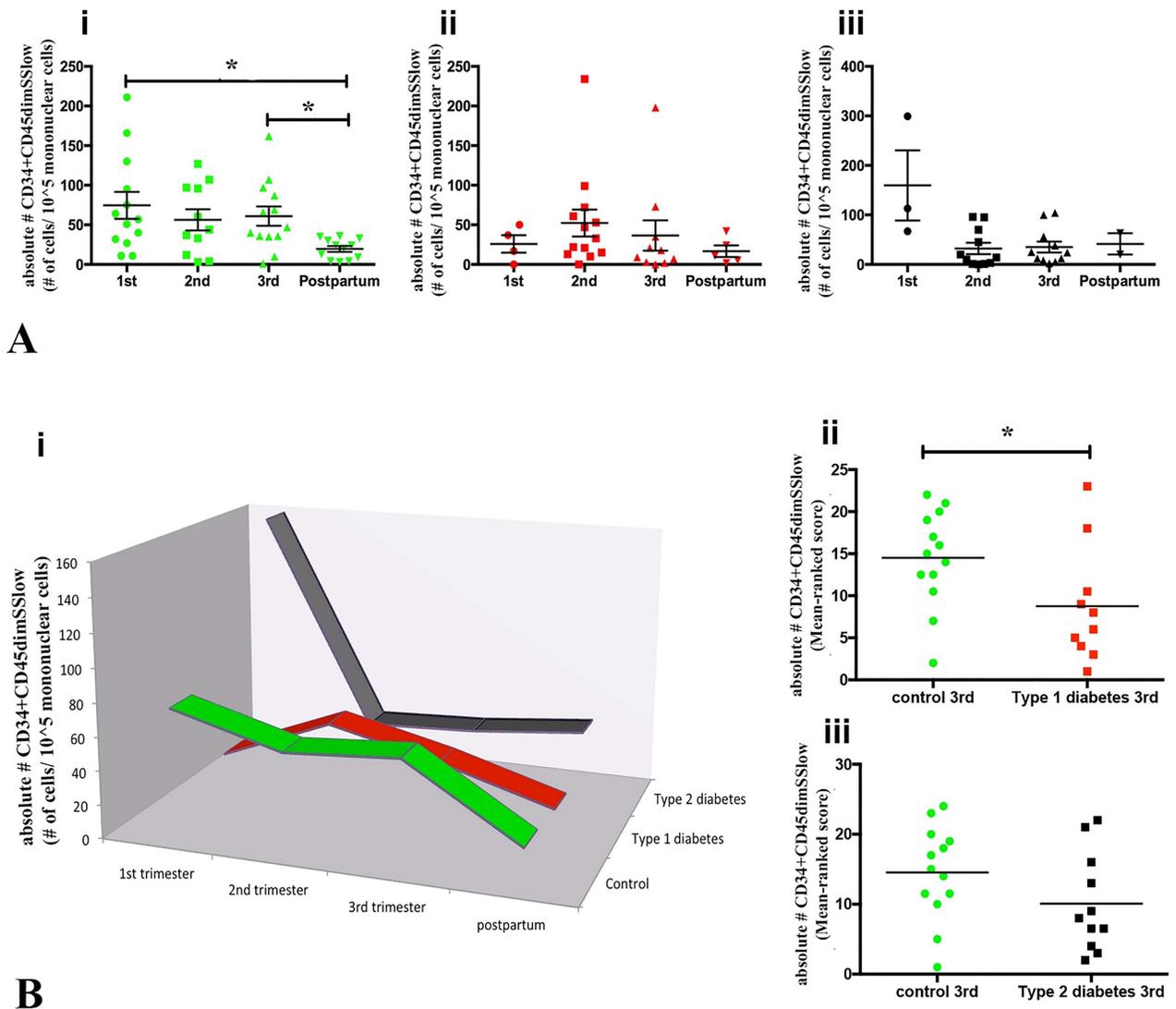
Fig 2. Total Circulating Progenitor Cell (CPC) analysis through pregnancy and postpartum in control, type 1 and type 2 diabetic women. (A) Absolute numbers of circulating CD45dimCD34+SSlow cells across pregnancy and postpartum from control (**i**), type 1 (**ii**) and type 2 (**iii**) diabetic women. In control women, CD45dimCD34+SSlow cells were significantly higher in the 1st (p = 0.013) and 3rd (p = 0.030) trimester compared to postpartum, but not in type 1 and type 2 diabetic women. **(B)** The 3D-line chart demonstrates the fluctuations in CD45dimCD34+SSlow cells over pregnancy and in a non-pregnancy state (postpartum) in control (green), type 1 (red) and type 2 (dark gray) diabetic pregnancies (**i**), while (**ii**) and (**iii**) represent the absolute numbers of CD45dimCD34+SSlow cells per 10^{5} mononuclear cells from control (green), type 1 (red) and type 2 (black) (**iii**) diabetic women at 3rd trimester. CD45dimCD34+SSlow cells were significantly more abundant in control compared to type 1 (p = 0.043), but not type 2 diabetic women (p = 0.130). Data in **A** were compared using Kruskal-Wallis test; **Bii** and **Biii** using Mann-Whitney test, considering p<0.05. Data in **Bii** and **Biii** are shown as a rank score graphic.

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The frequency of total CD34+KDR+ CACs (sum of CD133+KDR+ and CD133-KDR+) was elevated at 3rd trimester in control women, and significantly higher than in 2nd trimester (Fig 4Ai; p = 0.013). These data corroborate the results of others who measured CD34+KDR+ cell number or frequency during pregnancy [11, 13]. Interesting, in type 1 and type 2 diabetes, no change in frequency or number of these cells was observed over pregnancy (Fig 4Aii and 4Aiii). First trimester circulatory responses may be critical in diabetic women, since CD34+KDR+ CACs were elevated versus controls. Type 1 diabetic women had significantly more CD34

Table 3. CPC and CAC numbers per 10^5 blood mononuclear cells across pregnancy and postpartum.

Circulating CD34+CD45dimSSlow cells				P value		
	Control	Type 1 diabetes	Type 2 diabetes	Control *vs type 1*	Control *vs* type 2	Type 1 *vs* 2
1st trimester	74.62 (±16.94)	26.00(±11.01)	159.7(±70.92)	0.097	0.139	0.057
2nd trimester	56.36 (±13.29)	11.01(±17.06)	32.27(±11.45)	0.659	0.112	0.245
3rd trimester	*61.00(±12.18)**	*36.60(±19.23)**	35.09(±11.05)	0.043	0.130	0.436
Postpartum	19.58 (±3.64)	16.80 (±7.34)	41.50(±21.50)	0.703	NSA	NSA
Circulating CD34+133+KDR- cells				**P value**		
	Control	Type 1 diabetes	Type 2 diabetes	Control *vs type 1*	Control *vs* type 2	Type 1 *vs* 2
1st trimester	48.08 (±15.29)	2.00 (±2.00)	99.00(±95.50)	0.178	0.673	0.300
2nd trimester	*48.78(±13.64)**	17.75 (±7.09)	*7.00 (±3.02)**	0.074	0.028	0.625
3rd trimester	*39.58(±11.64)***	16.29 (±9.67)	*3.50(±2.30)***	0.152	0.004	0.775
Postpartum	2.66 (±2.42)	0.75 (±0.47)	0.50 (±0.50)	0.725	NSA	NSA
Circulating CD34+CD133+KDR+ cells				**P value**		
	Control	Type 1 diabetes	Type 2 diabetes	Control *vs type 1*	Control *vs* type 2	Type 1 *vs* 2
1st trimester	*4.30 (±1.50)**	13.00 (±9.00)	*31.33(±14.62)**	0.091	0.032	0.400
2nd trimester	3.33 (±0.79)	23.25 (±12.68)	14.14 (±9.20)	0.278	0.447	0.213
3rd trimester	10.00(±2.47)	26.14 (±17.88)	9.70 (±5.32)	0.853	0.369	0.348
Postpartum	1.88 (±0.45)	5.50 (±3.663)	3.50 (±1.50)	0.581	NSA	NSA
Circulating CD34+CD133-KDR+ cells				**P value**		
	Control	Type 1 diabetes	Type 2 diabetes	Control *vs type 1*	Control *vs* type 2	Type 1 *vs* 2
1st trimester	4.53 (±2.83)	5.66 (±1.20)	16.67(±14.71)	0.075	0.519	0.800
2nd trimester	4.33 (±3.83)	4.75 (±2.17)	4.71 (±1.79)	0.153	0.184	0.630
3rd trimester	3.83 (±1.46)	4.71(±1.91)	5.80 (±2.00)	0.534	0.377	0.904
Postpartum	3.11(±0.67)	3.50 (±1.19)	2.00 (±1.00)	0.988	NSA	NSA
Circulating CD34+CD133-KDR- cells				**P value**		
	Control	Type 1 diabetes	Type 2 diabetes	Control *vs type 1*	Control *vs* type 2	Type 1 *vs* 2
1st trimester	17.69 (±4.26)	14.00(±7.55)	12.67 (±4.97)	0.883	0.921	> 0.99
2nd trimester	11.67 (±4.15)	10.92(±3.04)	24.14 (±9.06)	0.958	0.423	0.308
3rd trimester	12.50 (±4.88)	*4.42(±2.52)**	*19.40 (±5.68)**	0.111	0.259	0.030
Postpartum	17.22 (±2.98)	10.75(±9.42)	35.50 (±24.50)	0.179	NSA	NSA

Mann-Whitney-U test (data not normally distributed: Kolmogorov–Smirnov). Data are shown as means of absolute numbers (±SEM). P values refer to comparisons between Control-type 1 diabetes; control-type 2 diabetes; or type1-type 2 diabetes.

Significance: **p ≤ 0.05 (*), p ≤ 0.01 (**)**.

Not statistically analyzed (NSA) due to low number of type 2 diabetes postpartum samples.

+KDR+ cells at 1st trimester compared to control (Fig 4Bi; p = 0.033); CD34+KDR+ cells also increased in type 2 diabetic women, but not a statistically different level (Fig 4Bii; p = 0.090).

The ratio of CD133+KDR+ (CD34+CD45dimSSlowCD133+KDR+) to total CD133+ (CD34+CD45dimSSlowCD133+) was analysed to compare KDR acquisition by CD133+ CACs over normal and diabetic pregnancies. Postpartum control women, but not type 1 or type 2 diabetic women, had a higher ratio of CD133+KDR+/total CD133+ cells compared to pregnant women (1st, 2nd and 3rd trimesters). In control women, this ratio was significantly different at 1st trimester compared to non-pregnant (postpartum) (Fig 5Ai–5Aiii; **p = 0.047**). Comparing type 1 and type 2 diabetic women with control, the CD133+KDR+/CD133+ cell ratios were higher in type 1 diabetic women at 1st and 2nd trimester compared to control women (p = 0.007 and p = 0.020, respectively; Fig 5Bi–5Biv). At 3rd trimester, the CD133+KDR +/CD133+ cell ratio was higher in both type 1 and type 2 diabetic women (Fig 5Bv and 5Bvi),
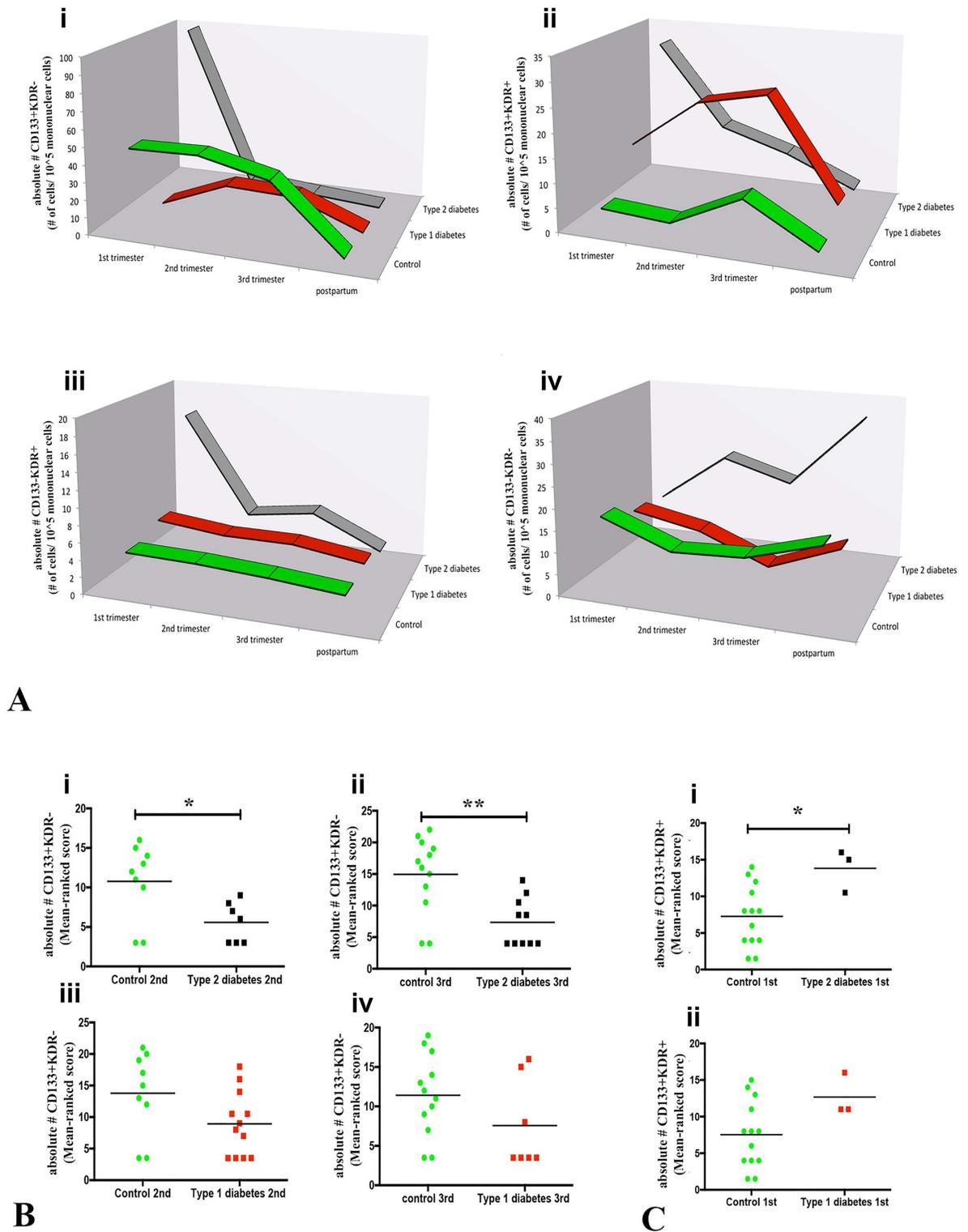
Fig 3. CAC (CD133+KDR-, CD133+KDR+, CD133-KDR+) analyses through pregnancy and postpartum in control, type 1 and type 2 diabetic women. (A) The 3D-line chart demonstrates fluctuations in the average of CD133+KDR- (**i**), CD133+KDR+ (**ii**), CD133-KDR+ (**iii**) and CD133-KDR- CACs (**iv**) over pregnancy and postpartum in control (green), type 1 (red) and type 2 (dark gray) diabetic women. **(B)** Punctual analysis comparing absolute numbers of CACs within specific gestational periods or postpartum demonstrated that CD133+KDR- CACs were lower in 2nd (**i;** p = 0.028) and 3rd (**ii;** p = 0.004) trimesters of type 2 diabetic (black) women compared to control (green). CD133+KDR- CACs appeared to be fewer in type 1 diabetic women 2nd (**iii;** p = 0.074) and 3rd (**iv;** p = 0.152) trimesters but the differences were not statistically significant versus controls. **(C)** CD133+KDR + CAC numbers were significantly higher in 1st trimester type 2 diabetic women (**i;** p = 0.032) than in controls. This difference was

not present in type 1 diabetes although the overall pattern of type 1 diabetes fluctuations resembled that of type 2 diabetes (**ii**; p = 0.091). (**B**) and (**C**) data were compared using Mann-Whitney test, considering p<0.05; and they are shown in rank score graphic.
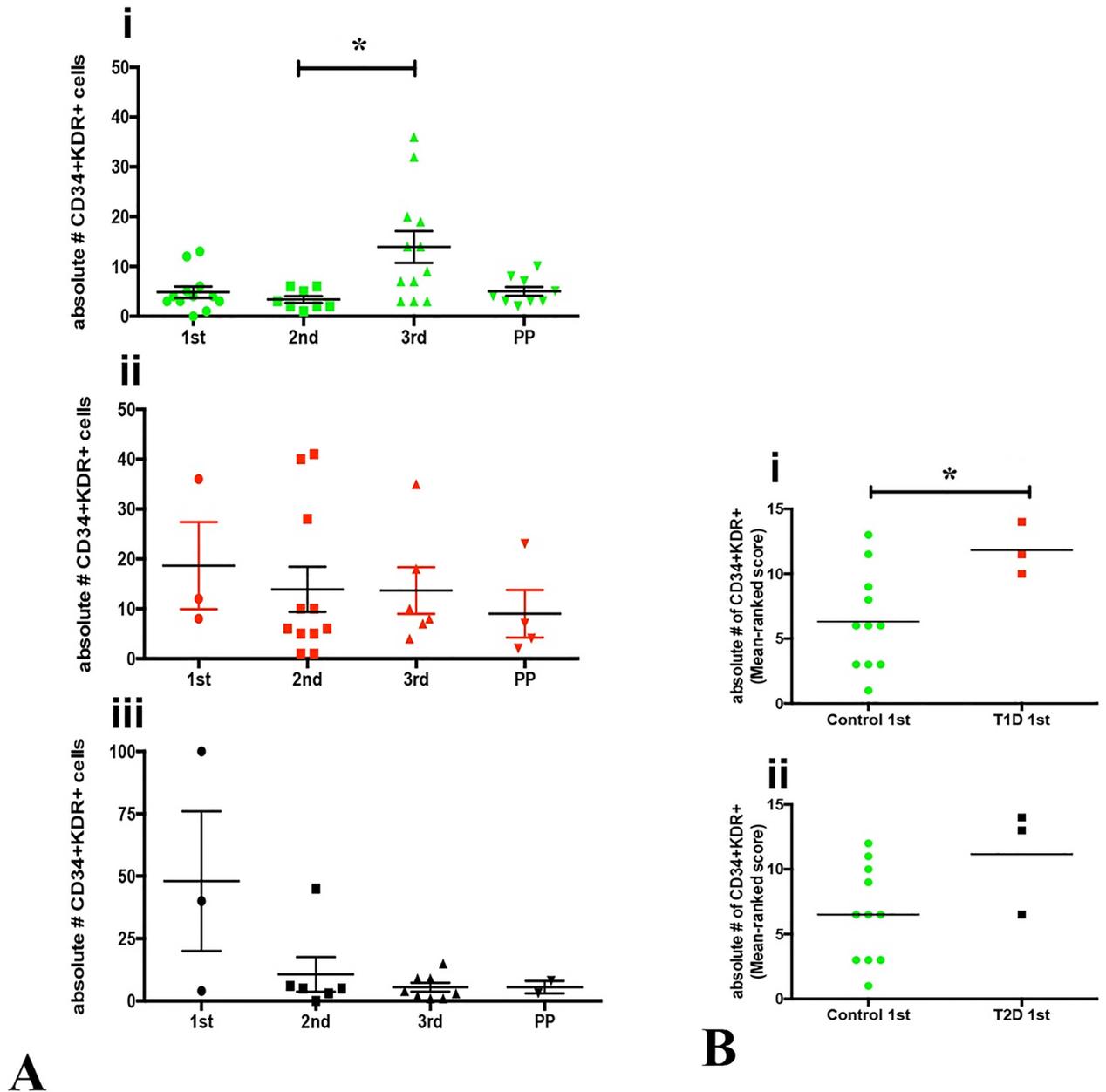
Fig 4. Total CD34+KDR+ cells across pregnancy and postpartum in control, type 1 and type 2 diabetic women. (A) Absolute numbers (per 10^5 mononuclear cells) of circulating CD45dimCD34+SSlowKDR+ (CD34+KDR+) cells across pregnancy and postpartum from control (**i**), type 1 (**ii**) and type 2 (**iii**) diabetic women. In control women, CD34+KDR+ cells were elevated in 3rd trimester and significantly higher compared to 2nd trimester (p = 0.013; **i**). Such fluctuation was not observed in type 1 and type 2 diabetic women (**i, ii**). (**B**) Comparisons between control (green) and type 1 (red) (**i**) and type 2 diabetes (black) (**ii**) at 1st trimester demonstrated that type 1 (p = 0.033), but not type 2 diabetic women (p = 0.090) had significantly more CD34+KDR+ CACs than control women. No differences were found when comparing control and diabetic (type 1 and type 2) women at 2nd, 3rd trimester and postpartum stages. Data in **A** were compared using Kruskal-Wallis test; **B** using Mann-Whitney test, considering p<0.05. Data in **Bi** and **Bii** were shown as a rank score graphic.
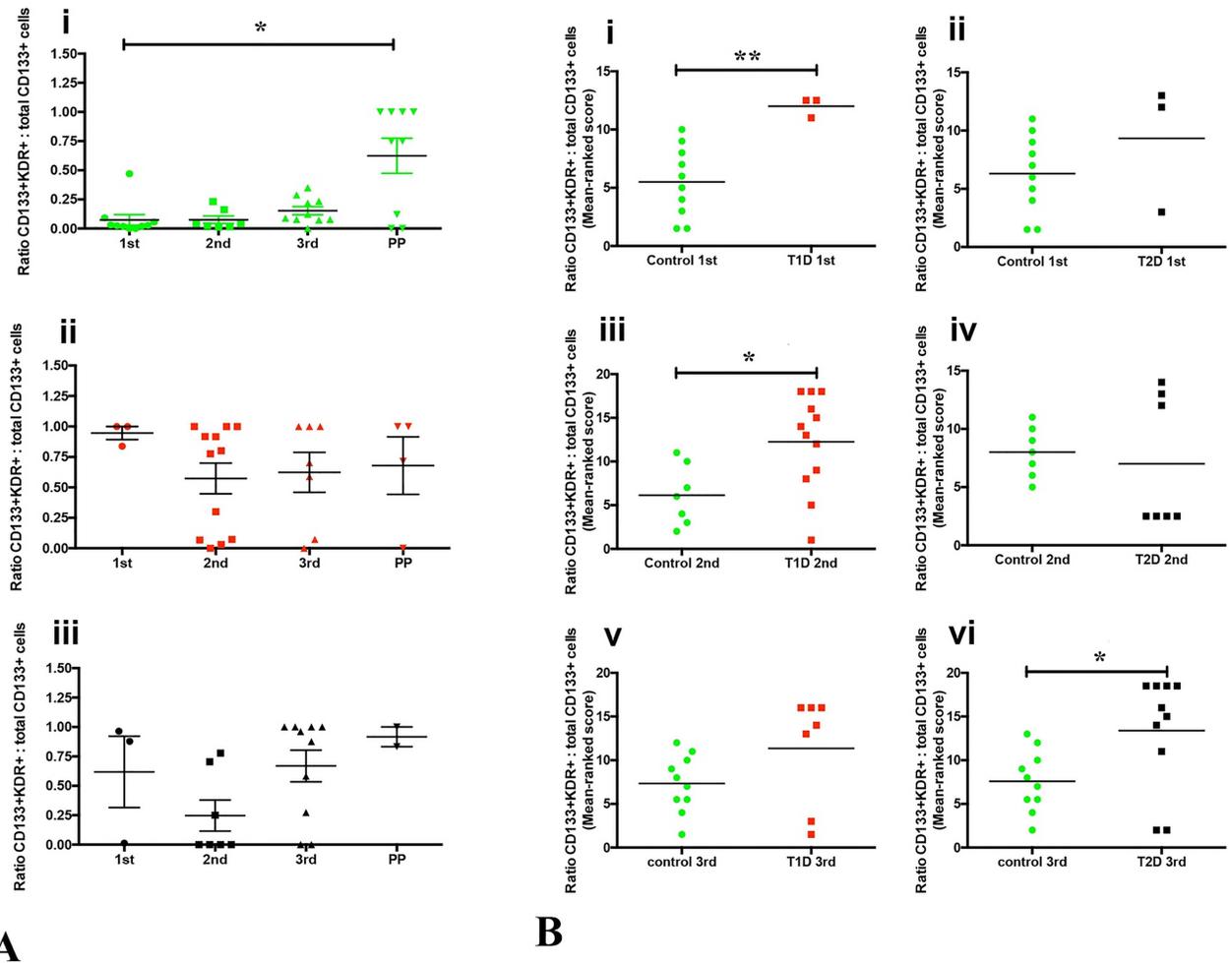
Fig 5. CD133+KDR+ to total CD133+ cell ratios across pregnancy and postpartum in control, type 1 and type 2 diabetic women. (A) Ratio of CD45dimCD34+SSlowCD133+KDR+ (CD133+KDR+) to total CD45dimCD34+SSlowCD133+ (CD133+) cells across pregnancy and postpartum from control (**i**), type 1 (**ii**) and type 2 (**iii**) diabetic women. In control women, the CD133+KDR+/ CD133+ ratio revealed that the proportion of CD133+KDR+ is relatively low during pregnancy (1st, 2nd and 3rd trimester) compared to non-pregnant women (postpartum). CD133+KDR+/ CD133+ ratio at 1st trimester in control women was statistically lower compared to postpartum (p = 0.047; **i**). This ratio was relatively higher in type 1 **(ii)** and type 2 **(iii)** diabetes compared to control. In **(B)**, comparisons between diabetic and control women identified that CD133+KDR+/CD133+ cell ratios were higher in pregnant but not postpartum diabetics. In type 1 diabetic women, significant differences were present in 1st (p = 0.007; **Bi**) and 2nd (p = 0.021; **Biii**) trimester, but not in 3rd trimester (p = 0.113; **Bv**). In type 2 diabetic women, 1st (p = 0.272; **Bii**) and 2nd (p = 0.688; **Biv**) trimesters were not significantly different but 3rd trimester was (p = 0.026; **Bvi**). Data in **A** were compared using Kruskal-Wallis test; **B** using Mann-Whitney test, considering p<0.05. Data in **Bi** and **Bii** were shown as a rank score graphic.

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but statistically significant only for type 2 diabetic women compared to controls (Fig 5Bvi; p = 0.026). Type 1 diabetes did not influence the postpartum CD133+KDR+/CD133+ cell ratio compared to controls.

Early outgrowth colonies (CFU-Hill) did not differ significantly in number across pregnancy and postpartum within any gestational group or between the groups (Fig 6).

Discussion

The major findings in this study were that preconception diabetes is associated with failure to respond to pregnancy-driven elevations in CPCs (CD34+CD45dimSSlow cells) and CACs
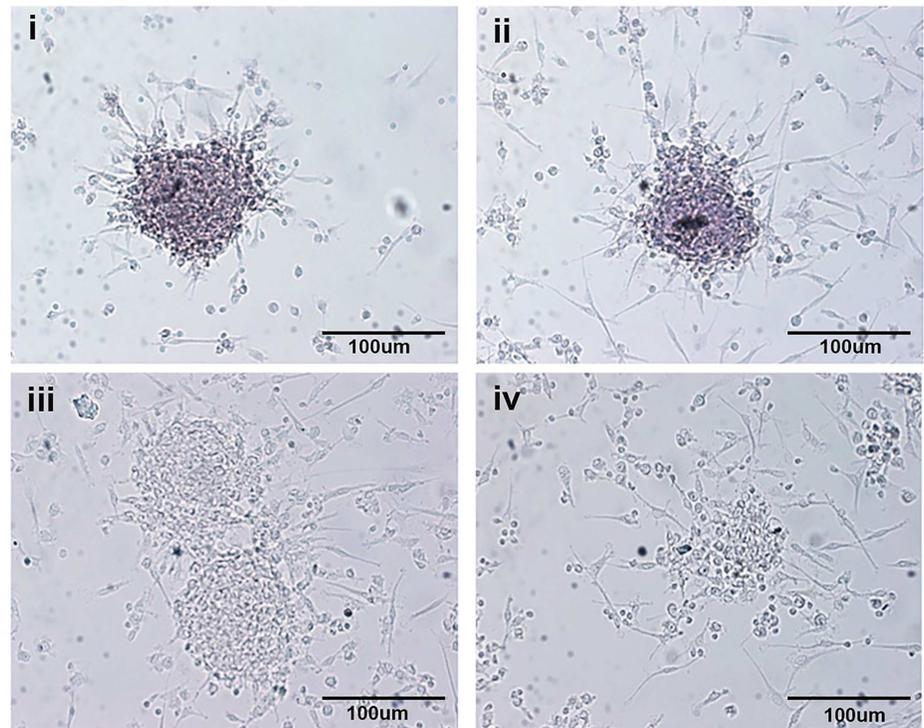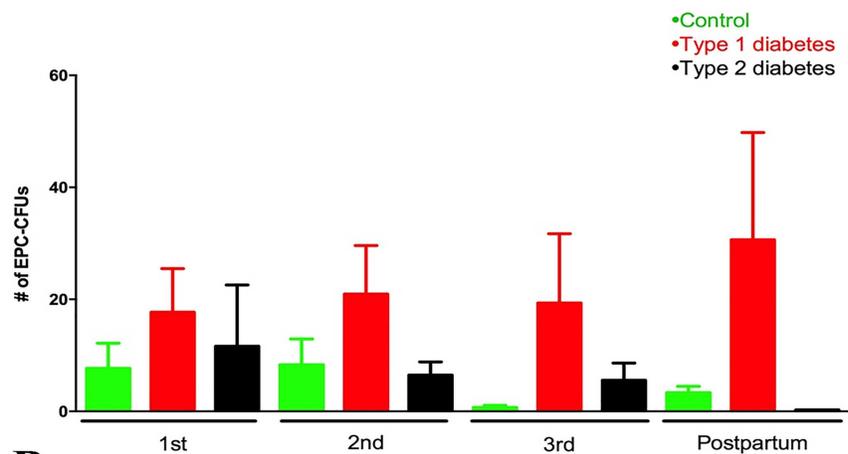
Fig 6. Early outgrowth colonies. (A) Examples of early outgrowth colonies (**i, ii** and **iii**) and a cell cluster that was not scored as a colony (**iv**). The colonies had a central core of round cells, with more elongated sprouting cells at the periphery. **(B)** Quantification of early outgrowth colonies demonstrated that diabetes (type 1: red bars; type 2: black bars) did not significantly affect the ability of CACs to form colonies compared to control women (green bars). Data were compared using Kruskal-Wallis test, considering $p < 0.05$. Bars: 100um.

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Table 4. Summary of differences between diabetes and matched controls in CPC and CAC levels.

	Type 1 diabetes	Type 2 diabetes
1st trimester	**increased** CD34+KDR+	**increased** CD34+CD133+KDR+
	increased CD34+CD133+KDR+/ total CD34+CD133+	
2nd trimester	**increased** CD34+CD133+KDR+/ total CD34+CD133+	**decreased** CD34+CD133+KDR-
3rd trimester	**decreased** CD34+CD45dimSSlow	**decreased** CD34+CD133+KDR-
		increased CD34+CD133+KDR+/ total CD34+CD133+

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(CD133+KDR+ and CD133+KDR- cells). Our findings are summarized in Table 4. Differences in frequencies of CD133+KDR- CACs between diabetic and normal women appeared to be gestationally-restricted because they did not persist postpartum (non-pregnant state). These data suggest that even in diabetic women with good glycemic control, diabetes alters CPC and CACs levels. This may be due to failure of CPC/CAC mobilization into circulation, defective maturation (i.e. acquisition of KDR expression) or more rapid consumption of CACs during maternal vascular adaptions to pregnancy. Such changes would be expected to increase risks for blood vessel-related pregnancy complications, such as preeclampsia.

The sequence of physiological changes that are induced within the maternal cardiovascular system during pregnancy suggest that several different waves of CACs recruitment from marrow to blood may be needed for gestational remodeling of different target organs. The first potential driver for CACs recruitment would be the significant expansion in plasma volume that occurs very early in human pregnancy under hormonal control. Chapman and colleagues [30] studied normal women intending to conceive during their pre-conception, menstrual cycles and at 6, 8, 10, 12 and 24 weeks post-conception. Decreased vascular resistance increased cardiac output and declining mean arterial pressure began after ovulation and prior to placental formation [30]. Between the ovulatory phase of the cycle and gestation week 6, plasma estradiol, a molecule known to mobilize CACs (CD34+KDR+ cells) [14], rose tenfold. A rise in effective renal plasma flow consistent with primary renal vasodilation was also significant by gestation week 6 [30]. During these early pregnancy weeks, the uterine lining also transforms from cycling endometrium into decidua, a process that involves extensive neoangiogenesis. The next critical angiogenic events are those that support placental development between 6 and 12 weeks of gestation. Morphological and ultrasonographic studies of human implantation sites at 8–16 weeks after conception [31, 32] report rapid hemodynamic changes within decidua and maternal spiral arteries that feed into the placental intervillous space where maternal-fetal nutrient/waste exchange occurs. Following completion of the embryonic phase (week 8 of gestation), fetal growth becomes a new driver of maternal gestational angiogenesis and by the end of the 2nd trimester, typical human fetuses weigh 820 g—1000 g which is 3x more than their placenta (280 g–300g). Although the placental weight doubles over 3rd trimester, a typical late pregnancy fetus (2500 g– 3000 g) is 6-7x heavier than its placenta (400 g–500 g) [33]. Maternal uterine vascular expansion must be needed for healthy support of this terminal phase of rapid fetal growth. In addition, stromal cell-derived factor 1 alpha (SDF1α), a crucial factor in recruitment of bone-marrow derived CD34+ cells [34] is elevated in maternal plasma during pregnancy. Levels are slightly reduced between 1st and 2nd trimesters then dramatically escalate between 2nd and 3rd trimester suggesting variable needs for CACs mobilization over gestation [35]. Changes in maternal plasma concentrations are largely due to secretions from conceptus-derived placental trophoblasts [36]. This is expected to create a concentration gradient that would preferentially localize CACs to the maternal-fetal interface.

In type 1 and type 2 diabetic women, the frequency of circulating CD34+CD45dimSSlow cells was invariant in across pregnancy and similar to postpartum. This was in stark contrast to normal women whose CD34+CD45dimSSlow cells were elevated across all trimesters of gestation, particularly in trimesters one and three. Increase in CPCs and CACs have been reported by others during normal pregnancy [12, 13, 16], although gestational age ranges, antibody reagents and cell phenotyping vary between the reports which has made understating of CPCs/CACs in pregnancy difficult. Diabetes additionally alters the balance between CD133 +KDR- and CD133+KDR+ CAC subsets across gestation. In 1st trimester, type 2 diabetic women had very high circulating numbers of CD133+KDR+ CACs, suggesting dysregulated maturation of CACs or limited recruitment of CD133+KDR+ CACs to organs undergoing the rapid vascular changes of early pregnancy. Since acquisition of KDR expression is part of CACs maturation/differentiation, the lower frequencies of CD133+KDR- cells in these patients at 2nd and 3rd trimester also supports the postulate of accelerated CAC differentiation in pregnant type 2 diabetic women.

When total CD34+KDR+ CACs (the sum of CD133+KDR+ and CD133-KDR+) were analyzed, we found that CD34+KDR+ CACs were significantly increased in normal pregnancy in 3rd trimester as previously reported by Luppi and colleagues [12]. KDR expressing CACs were at much lower levels than CD34+CD133+KDR- CACs in non-pregnant women and women in 1st or 2nd trimester. In type 1 diabetic women, however, an abnormal elevation of CD34 +KDR+ CACs was detected in 1st trimester compared to control. CD34+KDR+ CACs are markers of vascular injury although the mechanisms for their increases or decreases in the circulation of patients with cardiovascular diseases are unclear. In coronary artery disease, fewer CD34+KDR+ cells are seen [37, 38] while in heart failure or atherosclerotic disease progression more CD34+KDR+ cells occur in circulation [39, 40]. Similarly, in type 2 diabetic patients Fadini and colleagues [41] reported that KDR+ CACs are severely reduced in those with peripheral arterial disease but elevated in those with diabetic retinopathy. This "diabetic paradox" phenomenon may apply to our finding that preconception diabetes differentially altered CD133+ and KDR+ CAC numbers in a gestation stage, specific manner. The dynamic physiological changes of pregnancy (hormones, cytokines, angiokines, growth factors, etc.) may differentially modulate the frequency of CACs by controlling balances between CAC differentiation, recruitment and target tissue consumption.

In contrast to CD133+KDR+ CACs, CD133+KDR- CACs in 1st trimester of type 1 and type 2 diabetic women were at statistically comparable levels to control women. In 2nd and 3rd trimester of type 2 diabetic pregnancies however, CD133+KDR- CACs were remarkably deficient. Mechanisms to account for this were not addressed but may include failure of differentiation in marrow, failure of mobilization into circulation or excessive consumption in target organs, particularly the gravid uterus. While lower absolute numbers of CD133+KDR- CACs were also present in 2nd and 3rd trimesters of type 1 diabetic women, these differences did not reach statistical significance in comparison to either control or type 2 diabetic women.

KDR+ CACs are more differentiated than KDR- CACs and this subset responds to VEGF. To better understand the dynamic relationships between these subsets, CD133+KDR+/ total CD133+ ratios were calculated that strongly suggested the balance between the two subsets was altered by preconception diabetes. While women with type 2 diabetes had fewer CD133 +KDR- CACs at 2nd and 3rd trimester and higher CD133+KDR+ CACs at 1st trimester compared to control women, the CD133+KDR+/ CD133+ ratio demonstrated that diabetes elevates the frequency of CD133+KDR+ CACs in all stages of pregnancy in type 1 and type 2 diabetes. This finding is particularly important since control pregnant women had low levels of CD133+KDR+ cells compared to CD133+KDR- CACs, demonstrating that a coordinate recruitment and of CACs are required in pregnancy.

Pregnancy is a physiological state during which the systemic endothelium is continuously exposed to changing concentrations and compositions of circulating factors that maintain gestational success. Many of these factors also participate in CACs differentiation, recruitment into circulation and exodus from circulation. Examples of these regulatory molecules include VEGF, placental growth factor (PGF), granulocyte colony-stimulating factor (G-CSF) and granulocyte- macrophage colony-stimulating factor (GM-CSF) [42–44]. Progenitor cell mobilopathy has been characterized in diabetes and therapies based on G-CSF and GM-CSF are already in use to optimize stem-cell peripheralization and CAC recruitment into circulation [1, 45]. CAC mobilization from marrow is a complex process, regulated by a network of factors well beyond individual cytokine deficits. Further studies will be necessary to understand the physiological, pregnancy-associated drivers of CACs recruitment from marrow to blood and how these are deviated in women who are diabetic prior to conception. The apparent return of CAC numbers to control levels after pregnancy suggests that pregnancy itself does not enhance long-term negative circulatory outcomes for diabetic mothers. A much larger study would be needed to assess correlations between specific gestational pathologies and the disturbances in CAC frequencies documented in this pilot study.

Acknowledgments

Author Contributions

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