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Risk factors for pre-clinical atherosclerosis in adolescents with type 1 diabetes

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ABSTRACT

Aims: To assess whether, besides "traditional" risk factors, overall oxidative stress, oxidized lipoproteins, and glycemic variability are associated with early macro-vascular damage in type 1 diabetes (T1D).
Methods: In 267 children/adolescents with T1D (130 girls, age 9.1–23.0 years) we evaluated: derivatives of reactive oxygen metabolites [d-ROMs], serum total antioxidant capacity [TAC] and oxidized LDL-cholesterol [oxLDL]; markers of early vascular damage (Lipoprotein-associated phospholipase A2 [Lp-PLA2], z-score of carotid intima-media thickness [z-cIMT] and carotid–femoral pulse wave velocity [z-PWV]); CGM metrics of four weeks preceding the visit, central systolic/diastolic blood pressures (cSBP/cDBP), and HbA1c, z-score of BP (z-SBP/z-DBP) and circulating lipids longitudinally collected since T1D onset.. Three general linear models were built with z-cIMT, z-PWV adjusted for current cDBP, and Lp-PLA2 as independent variables.
Results: The z-cIMT was associated with male gender (B = 0.491, $\eta^2 = 0.029$, p = 0.005), cSBP (B = 0.023, $\eta^2 = 0.026$, p = 0.008) and oxLDL (B = 0.022, $\eta^2 = 0.022$, p = 0.014). The z-PWV was associated with diabetes duration (B = 0.054, $\eta^2 = 0.024$, p = 0.016), daily insulin dose (B = 0.52, $\eta^2 = 0.018$, p = 0.045), longitudinal z-SBP (B = 0.18, $\eta^2 = 0.018$, p = 0.045) and dROMs (B = 0.003, $\eta^2 = 0.037$, p = 0.004). Lp-PLA2 was associated with age (B = 0.221, $\eta^2 = 0.079$, $p = 3*10^{-6}$), oxLDL (B = 0.081, $\eta^2 = 0.050$, $p = 2*10^{-4}$), longitudinal LDL-cholesterol (B = 0.031, $\eta^2 = 0.043$, p = 0.001) and male gender (B = -1.62, $\eta^2 = 0.10$, $p = 1.3*10^{7}$).
Conclusions: Oxidative stress, male gender, insulin dose, diabetes duration and longitudinal lipids and blood pressure, contributed to the variance of early vascular damage in young patients with T1D.

1. Introduction

Type 1 diabetes (T1D) is characterized by accelerated vascular aging and increased pre-clinical signs of vascular damage, compared to the general population [1]. This is consistent with the increased macro-vascular morbidity and mortality associated with this disease [2–6]. Glycaemic control assessed by HbA1c, estimated insulin sensitivity (eIS), obesity, blood pressure and lipids, have been associated with markers of arterial stiffness and atherosclerosis in youth with T1D with diverse degrees of consistency [1,7–8]. However, all the above-mentioned risk factors only partially explained the inter-individual variability in the pre-clinical markers of vascular damage in youth with T1D [1,7–8]. In fact, the bases of the early macro-vascular damage in T1D are still largely elusive [1,7–8]. Increasing our knowledge of the risk factors for early vascular damage in T1D, finding out novel potential predictors besides the traditional ones and unravelling the "residual risk", would be the first step towards improved prediction and prevention and is consequently a research priority in the field of T1D.

Oxidative stress is increased in youth with T1D and is a candidate accelerator of atherosclerosis in this patient group [9–10]. Markers of oxidative stress have been associated with the occurrence of carotid artery disease (CAD) over 20 years of diabetes duration, in 356 adults with T1D, independently of HbA1c and several traditional risk factors [11]. Oxidative stress has been associated with all-cause and cardiovascular mortality in large cohorts of patients with type 2 diabetes (T2D) [12–13], and oxidative metabolites are predictive of cardiovascular

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morbidity and mortality in the general population [14]. Oxidized lipoproteins are plausible major noxae linking oxidative stress with cardiovascular disease in T1D [15]. LDL receptors do not recognize oxLDL, which are readily bound by the scavenger receptors of macrophages [15]. Moreover, oxLDL induce the release of inflammatory modulators, leading to the promotion of monocyte migration, increased density of macrophage scavenger receptors, and increased uptake of oxLDL during foam cells formation [15]. Finally, oxLDL induce the expression of Lipoprotein-associated phospholipase A2 (Lp-PLA2), a recognized predictor of atherosclerotic cardiovascular events that is highly expressed by macrophages, T-lymphocytes and monocytes in atherosclerotic lesions, where it hydrolyzes oxidized phospholipids to yield pro-atherogenic products that are implicated in plaque formation and inflammation [15].

Glycaemic variability as accurately assessed by continuous glucose monitoring (CGM) metrics is another candidate driver of vascular damage in T1D [16]. In fact, acute glycaemic fluctuations may cause increased oxidative stress, inflammation, endothelial dysfunction and altered gene expression, eventually contributing additional risk of vascular damage after adjusting for HbA1c [16]. Up to now, a few studies in small samples of patients with T1D, of which only one composed of children and adolescents, have investigated the associations between CGM measures of glycaemic variability and markers of arterial stiffness and/or atherosclerosis, with inconclusive results [16]. A recent extensive review on the associations between CGM measures of glycaemic variability and micro- and macro-vascular complications of diabetes, concluded with a call for larger studies [16]. Analysing oxidative stress and glycaemic variability contemporarily, would be useful to assess if and at which extent the oxidative stress mediates the association of glycaemic variability with arterial stiffness and/or atherosclerosis.

In the present study, we aimed at determining the risk factors of arterial stiffness and markers of atherosclerosis in youth with T1D, testing the hypothesis that measures of overall oxidative stress (derivatives of reactive oxygen metabolites – dROMs, and total antioxidant capacity – TAC), oxidized lipoproteins and glycemic variability may explain part of the residual risk, after adjusting for HbA1c and other traditional predictors.

2. Methods

This study was conducted at the Regional Center for Pediatric Diabetes of the University Hospital of Verona, Italy. We recruited 267 children/adolescents with T1D (130 girls, age 9.1–23.0 years) followed-up since the disease onset. Inclusion criteria were the presence of T1D confirmed by the positivity of at least one of the antibodies against islet cells (ICA), insulin (IAA), glutamate dehydroxilase (GADA), islet antigen 2 (IA2A), and zinc-transporter protein 8 (ZnT8A), disease duration of at least two years, and absence of partial remission defined as insulin dose-adjusted HbA1c (HbA1c%+ 4 × insulin dose (U/kg/day) $\leq$ 9%) [17]. Another inclusion criterion was having been using CGM (either intermittently scanned CGM or real-time CGM) for at least one month before the recruitment. Exclusion criteria were the presence of any disease under drug treatment and the use of any nutraceutical or vitamin compound (excluding vitamin D) or any medication affecting the red-ox balance during the last three months preceding the recruitment. All participants provided an extra 5 ml sample of blood for research purposes during a fasting blood collection scheduled in their follow-up program. This sample was used to measure circulating markers of oxidative stress and atherosclerosis, as specified in the following paragraphs, as well as current HbA1c, lipids and ALT. Patients underwent a complete medical examination to rule out any current acute illness or inflammation and to take height, weight, and waist circumference, as previously reported [9]. The presence of puberty was assessed by a trained pediatrician by Tanner staging. Blood pressure was measured on the left arm with a digital sphygmomanometer and cuff appropriate for the age and arm circumference, and the average of three measurements was recorded. The total daily insulin dose used by each participant was recorded as units per kilograms of body weight (U/kg). Current estimated insulin sensitivity (eIS) was calculated with the formula: eIS = Exp (4.1075–0.01299*WC – 1.05819*daily insulin dose[unit/kg of body weight] – 0.00354*TG −0.00802*DBP) [18]. For each participant, since the disease onset, there were quarterly collected HbA1c, z-score of the BMI (z-BMI) according to the WHO charts, systolic and diastolic blood pressure (SBP and DBP) and their z-scores according to sex, age, and height. Moreover, there were annually collected lipids, alanine transaminase (ALT), and urinary albumin-creatinine ratio (ACR). All the mentioned variables were averaged over the follow-up period corresponding to the disease duration since diagnosis minus the partial remission period [19–20]. We'll define the follow-up period as the "diabetes duration" throughout the article. Lipids, ALT and ACR were not transformed into z-score because they must be evaluated as such, according to the international recommendations [21].

2.1. Markers of oxidative stress

As markers of global red-ox balance, on the day of recruitment we measured the concentration of serum d-ROMs, TAC and oxLDL in all patients [9,22].

2.2. CGM metrics

CGM data available in the 4-week period preceding the enrollment visit were collected, as previously reported [9]. The following metrics were calculated from CGM data: 1) mean blood glucose (MBG); 2) percentage of time below the range [<70 mg/dL (3.8 mmol/L) (TBR70)]; 3) percentage of time below 54 mg/dL (3.0 mmol/L) (TBR_{54}); 4) percentage of time with glucose between 70 and 180 mg/dl [time in range- TIR; 5) percentage of the time above 180 mg/dL (TAR_{180})]; 6) percentage of the time above 250 mg/dL (TAR_{250}); 7) coefficient of variation (%CV); 8) standard deviation (SD, mmol/l) of mean glucose; 9) continuous overall net glycemic action (CONGA); 10) mean amplitude of glycemic excursions (MAGE). To ensure an adequate amount of data, participants were included in the analysis if at least 80% of expected CGM readings were available.

2.3. Vascular markers

In all participants, we measured current plasma Lp-PLA2 as a marker of atheromatous plaque formation, and we performed vascular tests within a period of three months from the enrollment visit. The carotid intima-media thickness (cIMT) (in mm) was measured as marker of atherosclerosis by ultrasound (LOGIQ P5 pro, GE, Indianapolis, USA) and processed using dedicated hardware (Cardiovascular Suite, Quipu, Pisa, Italy). The cIMT was measured within 1 cm from the bulb and transformed in sex and height-adjusted Z-scores according to the reference values proposed by Doyon et al. [23]. The pulse wave velocity (PWV) was measured (in m/s) as marker of arterial stiffness, using the SphygmoCor XCEL device using a cuff around the femoral artery that captures the femoral waveform and a tonometer that captures the carotid waveform. The length of the arteries was measured using a measuring tape. The velocity is computed by dividing the distance between the carotid and femoral arteries using the pulse transit time. Age and height-adjusted Z-scores were computed for PWV according to the reference proposed for the applanation methods [24]. Central systolic and diastolic blood pressure (cSBP and cDBP) were derived by the SphygmoCor XCEL device; the cuff pulsations were recorded at the brachial artery level, and then a general transfer function was applied to calculate aortic waveform. The measurement was recently validated in the pediatric population also [25], and Z-score was computed for cSBP [26]. Measurements were taken by a single operator who was specifically trained. While Lp-PLA2, cIMT and PWV were assessed as markers

of vascular damage, central blood pressures were assessed as their candidate predictors.

2.4. Laboratory

Cholesterol, HDL, Triglycerides, ALT and urinary albumin and creatinine were determined by standard methods and LDL-cholesterol was calculated with the Friedwald formula (total cholesterol – HDL-cholesterol – triglycerides/5). HbA1c was measured with Cobas b101 (Roche, Switzerland) by immunoturbidimetric assay. D-ROMs concentration was measured with a commercial kit (Diacron, Italy), as previously reported. Total anti-oxidant capacity (TAC) was measured with a commercial kit (Sigma- Aldrich), which gives antioxidant capacity in Trolox equivalents (ranging from 4 to 20 nmole/well). Trolox, a water-soluble vitamin E analog, serves as an antioxidant standard. oxLDL were measured by Oxidized LDL ELISA kit (Mercodia AB, Sweden). Lp-PLA2 activity was measured by the PAF Acetylhydrolase Assay Kit (Cayman Chemical, MI, U.S.).

2.5. Ethical statement

All the parents or guardians of children and adolescents and all adult patients gave written informed consent to participate in the study. The protocol was in accordance with the Declaration of Helsinky for medical research involving human subjects (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013 Nov 27;310(20):2191-4) and was approved by the Ethical Committee of the University Hospital of Verona.

2.6. Statistics

Variables are described by mean(standard deviation) or median [range] and were compared between genders by the Student's *t*-test or Mann Whitney test, as appropriate. To investigate the correlates of early vascular damage we built three general linear models with Lp-PLA2, z-cIMT and z-PWV as independent variables, after verifying their normal distribution in the whole sample as well as by gender, when appropriate. The effect size of each independent variable was described by the B coefficient while the impact on the inter-individual variability of the dependent variable was described by the η^2 coefficient. The dichotomous and continuous variables were tested in a multiple linear model if they were associated with the concerned vascular dependent variable in the univariate analysis (Student *t* test or Pearson/Spearman correlation as appropriate, with a p value ≤ 0.10, respectively). We considered variables with tolerance below 0.1 or with variation inflation factor (VIF) > 10 collinear. In case of collinearity, the variable with the strongest association with the outcome was selected. Variables were retained in the final model if their p-value was ≤ 0.05. Both z-scores of potential risk factors and their corresponding raw values were assessed as candidate correlates of vascular markers. This was done to evaluate the potential effect of both age/gender-adjusted and absolute magnitude of the candidate risk factors on the variance of vascular markers. Central diastolic blood pressure (cDBP) was used to adjust z-PWV while building its model, both because PWV is influenced by contemporary central blood pressure independently of arterial stiffness due to the mechanical laws regulating the relation between vessel diameter and distensibility, and because cDBP showed the highest correlation with z-PWV among the measures of blood pressure taken while assessing PWV, in our cohort [27]. For each model, we verified the absence of biasing outlier observations by comparing each observation Leverage score to the critical value [(2* number of variables)/number of observations].

The sequential goodness-of-fit (SGoF) method was used to take into account multiple testing: all of the associations with a p value < 0.05 were ranked based on their p value, from the largest to the smallest. The number of independent tests performed was assessed defining tests independence as the lack of high correlation (r 0.7) between tests independent variables for a shared dependent variable or vice-versa. Given the number of thirty-one independent tests performed, four associations with the largest p values (one p value $= 0.046$ and the other three ones $= 0.045$) were rejected as being due to chance, whereas those with smaller p values were considered significant. In fact, our p values < 0.05 represent the 95th percentile of the expected number of false positive tests according to a binomial distribution.

All the analyses were performed with SPSS 24.0 statistical package (IBM Statistics).

3. Results

Table 1 describes the characteristics of the study participants according to the gender. Girls had higher dROMs and oxLDL than boys, as well as higher HbA1c, higher current and diabetes duration mean cholesterol, higher current and diabetes duration mean LDL-cholesterol, higher current HDL-cholesterol, higher current z-SBP and higher diabetes duration mean ACR (0.001). Boys had higher 4 week-CGM standard deviation (SD), higher cSBP, higher Lp-PLA2, higher cIMT and z-cIMT and higher PWV than girls, as well as higher current and diabetes duration mean ALT.

In univariate analyses, z-cIMT was associated, with an α- error ≤ 0.10, with male gender, oxLDL, cSBP, z-cSBP and longitudinal mean z-SBP (Table 2 and Table 3). According to multivariate analysis of variance, z-cIMT was associated with male gender, cSBP and oxLDL (Table 4).

In univariate analyses, z-PWV was associated, with an α- error ≤ 0.10, with age, diabetes duration, daily insulin dose, eIS, d-ROMs, cSBP, cDBP, z-cSBP, and the longitudinal means of LDL-cholesterol, HDL-cholesterol, z-SBP, z-DBP and ACR (Table 3). Once adjusted for cDBP in multivariate analysis of variance, z-PWV was associated with diabetes duration, daily insulin dose, longitudinal mean z-SBP and dROMs (Table 5).

In univariate analyses, Lp-PLA2 was associated, with an α- error ≤ 0.10, with male gender, age, diabetes duration, d-ROMs, TAC, oxLDL, cSBP, 1-month glycemic CV, 1-month glycemic TBR_{54}, as well as the longitudinal means of cholesterol, LDL-cholesterol and ACR (Table 2 and Table 3). According to multivariate analysis of variance, Lp-PLA2 was associated with age, oxLDL, longitudinal mean LDL-cholesterol and gender (Table 6).

4. Discussion

The most relevant result of the present study is that oxidative stress and red-ox balance contributed to the inter-individual variability of early vascular damage in young patients with T1D, independently of traditional risk factors collected longitudinally during the whole diabetes duration. In details, oxLDL correlated with both markers of atherosclerosis (z-cIMT and Lp-PLA2), TAC correlated with the Lp-PLA2 activity and dROMs correlated with the vascular stiffness (z-PWV) in 267 children/adolescents with T1D lasted on average eight years. These correlations were independent of age, gender, disease duration, z-BMI, HbA1c, blood lipids, blood pressure, ALT and ACR averaged over the diabetes duration period. Adults with T1D have higher oxLDL than those without T1D and show an association between oxLDL and carotid intima-media thickening and CAD over time [28–32]. Our study extends previous evidence demonstrating that oxLDL might accelerate cIM-thickening in patients with T1D since pre-adolescence. In parallel, the correlation we observed between oxLDL and Lp-PLA2 in patients with T1D is novel and strengthens the hypothesis that oxLDL can contribute to early atherosclerosis in T1D. The association between oxLDL and Lp-PLA2 is biologically plausible, because oxLDL induce the expression of Lp-PLA2 [33]. However, the cross-sectional and observational nature of the observed oxLDL-Lp-PLA2 association, and the fact that Lp-PLA2 can also contribute to LDL oxidation, rule out the possibility to establish a sure causal link between oxLDL and the increase in Lp-PLA2 activity.

Table 1
Physical and biochemical characteristics of patients according to genders.

	Girls (N = 130)	Boys (N = 137)	Total (N = 267)	P value
Age at the time of oxidative stress assessment (years)	16.0 [13.6–18.5]	17.2 [14.0–19.4]	16.5 [13.9–19.0]	0.160
Diabetes duration (years)	8[5–10]	8[6–10]	8[6–10]	0.360
Current treatment (MDI/CSII)	91/39	97/40	188/79	0.890
Puberty [yes (pubertal)/no (pre- or post-pubertal)]	16/114	18/119	34/133	0.900
Height (cm)	162 [157–166]	172 [163–177.4]	165 [159–174]	**0.0001**
Weight (kg)	57.5(11.2)	63.5(16.3)	60.6(14.6)	**0.001**
BMI (kg × m^{-2})	22.0(3.3)	21.9(3.6)	21.9(3.4)	0.880
z-BMI	0.46(0.9)	0.29(0.9)	0.38(0.9)	0.120
HbA1c (mmol/mol)	65(0.11)	62(0.11)	64(0.11)	**0.015**
HbA1c (%)	8.13(0.95)	7.86(0.86)	7.99(0.92)	**0.015**
Daily insulin dose (U × kg^{-1})	0.83 [0.66–1.00]	0.82 [0.69–0.97]	0.82 [0.68–0.98]	0.720
eIS	8.16(2.22)	7.91(2.32)	8.02(2.27)	0.398
Cholesterol (mg × dl^{-1})	156.9(27.5)	146.6(27.4)	151.6(27.9)	**0.002**
LDL-cholesterol (mg × dl^{-1})	83.3(23.6)	76.9(23.1)	80.0(23.5)	**0.021**
HDL-cholesterol (mg × dl^{-1})	60.6(12.3)	56.2(14.0)	58.3(13.4)	**0.005**
Triglycerides (mg × dl^{-1})	60[48–77]	58[46–74]	60[47–76.5]	0.690
ALT (U × L^{-1})	17[14–20]	20[16–25]	18[15–22]	**0.0001**
SBP (mmHg)	110 [100–115]	110 [105–120]	110 [102.5–120]	**0.005**
z-SBP	−0.15 [-0.81–0.52]	−0.44 [-0.99–0.07]	−0.25 [-0.91–0.36]	**0.037**
DBP (mmHg)	70[65–75]	70[65–75]	70[65–75]	**0.013**
z-DBP (mmHg)	0.41 [-0.15–0.87]	0.25 [-0.23–0.71]	0.30 [-0.20–0.77]	0.460
cSBP (mmHg)	101.0 [96.5–107.0]	104.5 [98.5–112.2]	102.0 [97.5–110.0]	**0.011**
z-cSBP	−0.12 [-0.90–0.70]	0.00 [-0.66–1.21]	−0.08 [-0.77–0.84]	0.119
cDBP (mmHg)	69.7(10.1)	68.5(7.7)	69.1(8.9)	0.280
4 week-CGM mean glucose (mg × dL^{-1})	182.0(31.0)	186.3(29.7)	184.1(30.4)	0.330
4 week-CGM SD (mg × dL^{-1})	73.8 [64.0–83.9]	79.7 [67.7–87.3]	76.8 [65.5–85.2]	**0.046**
4 week-CGM CV (%)	40.9(6.4)	42.8(7.7)	41.8(7.1)	0.060
4 week-CGM TBR_{70} (%)	3.9[1.8–7.2]	4.6[2.0–8.3]	4.5[1.9–7.3]	0.570
4 week-CGM TBR_{54} (%)	1.1[0.3–2.4]	1.2[0.2–2.8]	1.1[0.2–2.5]	0.520
4 week-CGM TIR (%)	49.2(14.6)	46.7(12.9)	47.9(13.8)	0.210
4 week-CGM TAR_{180} (%)	45.5(15.9)	47.4(13.9)	46.5(15.0)	0.370
4 week-CGM TAR_{250} (%)	20.8 [10.7–30.0]	18.8 [11.2–30.2]	19.7 [11.2–30.0]	0.780
4 week-CGM MAGE (mg × dL^{-1})	6.6[4.5–9.1]	6.5[4.0–8.4]	6.6[4.1–9.0]	0.440
4 week-CGM CONGA	8.68(1.98)	8.68(1.89)	8.68(1.93)	0.990
Follow-up mean z-BMI	0.50 [-0.10–0.90]	0.50 [-0.10–0.98]	0.50 [-0.10–0.90]	0.760
Follow-up-mean HbA1c (%)	7.96 [7.53–8.29]	7.96 [7.50–8.28]	7.96 [7.52–7.28]	0.700
Follow-up-mean Cholesterol (mg × dl^{-1})	158.6(23.5)	149.0(22.9)	153.6(23.6)	**0.001**
Follow-up-mean LDL-C (mg × dl^{-1})	84.2(20.8)	76.4(19.3)	80.2(20.4)	**0.001**
Follow-up-mean HDLC (mg × dl^{-1})	61.7(12.0)	60.4(12.3)	61.0(12.1)	0.380
Follow-up-mean triglycerides (mg × dl^{-1})	58.5 [49.0–72.0]	55.0 [45.7–65.5]	56.5 [47.0–68.9]	0.057
Follow-up-mean ALT (U × L^{-1})	17.7 [15.6–20.7]	19.7 [16.8–23.0]	18.4 [16.2–22.3]	**0.0001**
Follow-up-mean z-SBP	−0.30 [-0.65–0.10]	−0.40 [-0.60–0.00]	−0.30 [-0.60–0.00]	0.610
Follow-up-mean z-DBP	0.00 [-0.20–0.25]	0.00 [-0.30–0.30]	0.00 [-0.25–0.30]	0.890
Follow-up-mean ACR (mg/mmol)	0.77 [0.54–1.23]	0.52 [0.41–0.85]	0.65 [0.46–0.99]	**0.0001**
d-ROMs (U-Carr)	388.0(59.5)	345.9(63.3)	366.2(64.9)	**$2*10^{-8}$**
TAC (Trolox)	1.03 [0.80–1.16]	1.00 [0.81–1.16]	1.02 [0.81–1.16]	0.946
Ox-LDL	39.7 [33.7–46.4]	37.2 [30.5–43.1]	38.2 [32.0–44.4]	**0.032**

Data are given as mean(standard deviation) or median[interquartile range].
Abbreviations: MDI = multiple daily injections; CSII = continuous subcutaneous insulin infusion; d-ROMs = derivatives of reactive oxygen metabolites; TAC = serum total antioxidant capacity; oxLDL = oxidized LDL-cholesterol; SBP = Systolic blood pressure; DBP = diastolic blood pressure; cSBP = central systolic blood pressure; cDBP = central diastolic blood pressure; Lp-PLA2 = Lipoprotein-associated phospholipase A2; cIMT = carotid intima-media thickness; PWV = pulse wave velocity; BMI = body mass index; eIS = estimated insulin sensitivity; LDL-C = LDL cholesterol; HDL-C = HDL cholesterol; ACR = albunin to creatinine ratio; SD = standard deviation of blood glucose; CV = coefficient of variation; Perc TBR70 = percentage of time below the range with glucose < 70 mg/dl; Perc TBR 70–54 = percentage of time below the range with glucose between 70 and 54 mg/dl; perc hypo54 = percentage of time with glucose < 54 mg/dl; TIR70-180 = percentage of time in range with glucose between 70 and 180 mg/dl; TAR180 = percentage of time above the range with glucose > 180 mg/dl; TAR250 = percentage of time above the range with glucose > 250 mg/dl; MAGE = mean amplitude of glycemic excursions; CONGA = continuous overall net glycemic action.

Type 1 diabetes is characterized by increased oxidative stress [9]. This is the first study, to our knowledge, assessing measures of global oxidative stress and anti-oxidant capacity in relation to early vascular damage in T1D, and demonstrating that an unfavorable red-ox balance is associated with atherosclerosis (TAC) and arterial stiffness (dROMs) since adolescence. Systemic inflammation has been associated with both cIMT and arterial stiffness in adolescents with T1D [8,34] and it can be hypothesized that oxidative stress may be a significant driver of inflammation accelerating atherosclerosis. As for oxLDL, the study design does not allow for inferring a sure causal link between systemic oxidation and atherosclerosis and arterial stiffness in T1D.

The study results do not support the hypothesis that CGM metrics are associated with vascular markers. Prior to this study, measures of glycemic variability over very short periods (1 to 5 days) were tested as potential predictors of surrogate measures of arterial stiffness or atherosclerosis, with conflicting results [35–37]. The present study assesses, for the first time and in a relatively large sample of subjects, measures of glycemic variability issued from a period of CGM longer than 14-day, as recommended by the ATTD to gain accurate estimates of sustained glycemic variability [38]. Even if the study results do not support a role for glycemic variability in triggering vascular damage, further studies assessing CGM metrics longitudinally, i.e., with several time-points measures, are warranted to rule out the hypothesis that glycemic variability contributes significantly to macrovascular injury.

Among the modifiable risk factors, cSBP emerged as being associated with cIMT as we already observed in a previous cross-sectional study on

Table 2
Vascular markers according to gender, puberty and type of therapy.

	Girls	Boys	P	Non pubertal	Pubertal	P	MDI	CSII	P
cIMT (mm)	0.46(0.06)	0.50(0.06)	$4.6 * 10^{-7}$	0.48(0.07)	0.47(0.06)	0.25	0.48(0.07)	0.48(0.06)	0.45
z-cIMT	1.8(1.4)	2.3(1.4)	0.003	2.1(1.4)	2.0(1.2)	0.78	2.1(1.5)	2.0(1.3)	0.70
PWV (m/s)	4.6(0.7)	4.8(0.8)	0.044	4.7(0.7)	4.3(0.3)	0.003	4.8(0.7)	4.4(0.7)	0.001
z-PWV	−0.9(1.1)	−0.7(1.0)	0.36	−0.8(1.0)	−0.9(1.0)	0.51	−0.7(1.0)	−1.0(1.0)	0.014
Lp-PLA2	15.4(2.6)	16.8(3.1)	0.00013	16.3(3.0)	14.9(2.1)	0.013	16.1(3.0)	15.9(2.6)	0.49

Data are given as mean(standard deviation).
Abbreviations: cIMT = carotid intima-media thickness; PWV = pulse wave velocity; Lp-PLA2 = Lipoprotein-associated phospholipase A2.

Table 3
Coefficients of correlation between vascular markers and continuous variables.

	z-cIMT	z-PWV	Lp-PLA2
Age	0.08	0.21*	0.34*
Follow-up duration	0.004	0.19*	0.18*
d-ROMs (U-Carr)	−0.074	0.15*	0.15*
TAC (Trolox)	0.13*	0.017	−0.12*
oxLDL	0.13*	0.05	0.41*
cSBP	0.18*	0.38*	0.19*
z-cSBP	0.17*	0.14*	0.001
cDBP	0.047	0.48*	0.14*
Daily insulin dose (U/kg)	−0.040	0.145*	−0.019
eIS	−0.067	−0.185*	−0.088
Follow-up mean z-BMI	0.073	0.04	0.04
Follow-up-mean HbA1c	0.002	0.07	−0.08
Follow-up-mean Cholesterol	0.003	0.07	0.25*
Follow-up-mean LDL-cholesterol	0.041	0.13*	0.35*
Follow-up-mean HDL-cholesterol	−0.08	−0.14*	−0.10
Follow-up-mean triglycerides	0.07	0.19*	0.04
Follow-up-mean ALT	0.08	−0.10	−0.04
Follow-up-mean z-SBP	0.15*	0.25*	0.01
Follow-up-mean z-DBP	0.10	0.16*	−0.05
Follow-up-mean ACR (mg/mmol)	0.048	0.125*	−0.120*
4 week-CGM mean glucose	0.09	−0.02	0.03
4 week-CGM SD	0.08	0.02	0.10
4 week-CGM CV	0.04	0.06	0.13*
4 week-CGM TBR_{70}	−0.03	0.05	0.11
4 week-CGM TBR_{54}	−0.03	0.03	0.14*
4 week-CGM TIR	−0.07	0.01	−0.09
4 week-CGM TAR_{180}	0.08	−0.03	0.04
4 week-CGM TAR_{250}	0.12	0.002	−0.007
4 week-CGM MAGE	−0.06	−0.008	0.025
4 week-CGM CONGA	0.08	−0.042	−0.013

Abbreviations: cIMT = carotid intima-media thickness; PWV = pulse wave velocity; Lp-PLA2 = Lipoprotein-associated phospholipase A2; d-ROMs = derivatives of reactive oxygen metabolites; TAC = serum total antioxidant capacity; oxLDL = oxidized LDL-cholesterol; SBP = Systolic blood pressure; DBP = diastolic blood pressure; cSBP = central systolic blood pressure; cDBP = central diastolic blood pressure; eIS = estimated insulin sensitivity; BMI = body mass index; LDL-C = LDL cholesterol; HDL-C = HDL cholesterol; ACR = albunin to creatinine ratio; SD = standard deviation of blood glucose; CV = coefficient of variation; Perc TBR70 = percentage of time below the range with glucose < 70 mg/dl; Perc TBR 70–54 = percentage of time below the range with glucose between 70 and 54 mg/dl; perc hypo54 = percentage of time with glucose < 54 mg/dl; TIR70-180 = percentage of time in range with glucose between 70 and 180 mg/dl; TAR180 = percentage of time above the range with glucose > 180 mg/dl; TAR250 = percentage of time above the range with glucose > 250 mg/dl; MAGE = mean amplitude of glycemic excursions; CONGA = continuous overall net glycemic action.

a smaller cohort [39], while longitudinally averaged z-scores of peripheral pressures were not. This is quite intriguing because it would be expected that blood pressure z-scores averaged over time should reflect more accurately the chronic vascular injury associated with blood pressure compared to one current measure. As observed previously, central blood pressure may be superior to peripheral blood pressure in correlating with cIMT, because it is closer to the blood pressure that "challenges" the big arteries [39]. The fact that current cSBP overcomes chronic peripheral pressures as correlate of cIMT, suggests that current cSBP probably reflects well the usual range of cSBP of the patient, and highlights the potential clinical utility of this measure.

Table 4
Predictors of z-cIMT.

	B coefficient	95% C.I. of B coefficient	p value	η^2
Male gender	0.491	0.151–0.832	0.005	0.029
cSBP (mmHg)	0.023	0.006–0.040	0.008	0.026
Ox-LDL (U × L^{-1})	0.022	0.004–0.039	0.014	0.022

Abbreviations: cSBP = central systolic blood pressure, oxLDL = oxidized LDL-cholesterol.

Table 5
Predictors of z-PWV.

	B coefficient	95% C.I. of B coefficient	p value	η^2
Follow-up duration (years)	0.054	0.010–0.099	0.016	0.024
dROMs (U-Carr)	0.002	0.001–0.004	0.004	0.037
Mean z-SBP	0.18	0.050–0.388	0.045	0.018
Daily insulin dose (U × kg^{-1})	0.523	0.019–1.027	0.045	0.018
cDBP (mmHg)	0.062	0.46–0.078	0.0001	0.201

Abbreviations: d-ROMs = derivatives of reactive oxygen metabolites; Mean z-SBP = mean z-score of systolic blood pressure during the whole follow-up; cDBP = central diastolic blood pressure.

Table 6
Predictors of Lp-PLA2.

	B coefficient	95% C.I. of B coefficient	p value	η^2
Age (years)	0.221	0.129–0.312	$3 * 10^{-6}$	0.079
TAC (Trolox)	−1.06	−2.09 to −0.023	0.045	0.015
Ox-LDL (U/L)	0.081	0.038–0.125	0.0002	0.050
Mean LDL (mg × dl^{-1})	0.031	0.010–0.052	0.001	0.043
Male gender	−1.62	−2.22 to −1.04	$1.3 * 10^{-7}$	0.100

Abbreviations: TAC = total anti-oxidant capacity; oxLDL = oxidized LDL-cholesterol; Mean LDL = mean LDL-cholesterol during the whole follow-up.

In our cohort of patients, the average LDL-cholesterol over the diabetes duration period, significantly predicted Lp-PLA2, a known independent risk factor for CAD in T1D [40–41]. The cross-sectional association between LDL and Lp-PLA2 is known and is partly explained by the fact that circulating lipoproteins contain Lp-PLA2 [15]. The prospective association observed in the present study between LDL and Lp-PLA2 extends previous evidence, highlighting that LDL cholesterol accelerates plaque macrophage activation since T1D onset, and high Lp-PLA2 may reflect the sustained elevation of atherogenic lipoproteins.

Among non-modifiable risk factors, age and disease duration were associated with surrogated atherosclerosis and arterial stiffness,

respectively, as expected based on biological plausibility and on previous evidence [1]. Male gender was another non-modifiable risk factor for both atherosclerosis and arterial stiffness (cIMT and PWV), in accordance with previous evidence from another cohort of youth with T1D [42]. Consistently, despite female patients with T1D have a higher excess mortality, in respect to the healthy population, than male patients, the men with T1D have an absolute higher mortality than the women with T1D, like in the general population [43]. This gender difference deserves further investigation which should consider both biological sex specificities and lifestyle gender differences, such as, for example, smoking, physical activity, and diet.

In general, the risk factors highlighted by the present study explained a limited portion of the inter-individual variability of the vascular markers of the study participants (10–30%). Thus, the proportion of "residual risk" to explain is still large. Heritability of cIMT and PWV is high in healthy individuals [44–45]. T1D implies significantly higher cIMT and accelerated vascular stiffening compared to healthy controls, raising the expectation that glycemic control should be a major determinant of vascular damage among patients [1]. However, the glucose dysregulation typical of all patients with T1D might be the principal responsible of the vascular risk burden typical of the disease but may not be the major determinant of the inter-patient risk variability, which could be significantly influenced by genetic factors and by their interaction with glycemic control and other modifiable variables. In accordance with this hypothesis, family history of early cardiovascular disease was associated with cIMT in a small cohort of adolescents with T1D [46].

The principal limitation of this study is that the main nontraditional candidate risk factors for vascular damage, i.e., oxidative markers and CGM metrics, were assessed cross-sectionally instead of longitudinally. Another limitation is that cardiovascular family history and behavioral risk factors like smoking habits and regular sport activity were not analyzed among the covariates. Finally, the recruitment was monocentric, limiting the generalizability of the results. The study has also several strengths: *i.* the size of the studied cohort, which was large enough to permit multiple variable adjustments without compromising statistical power; *ii.* the assessment, for the first time, of several oxidative markers and 4-week CGM metrics in relation to vascular damage; *iii* the longitudinal assessment of several traditional confounders over the entire diabetes duration period.

In conclusion, the present study suggests that oxidative stress can contribute to early vascular damage in T1D, along with male gender, blood pressure, lipids, and insulin dosage.. The study results do not allow to draw definite conclusions or to support any clinical recommendation. Future research, like clinical trials employing antioxidant nutraceuticals, is needed to confirm a causal link between oxidative stress and vascular damage in T1D, and to assess the usefulness of improving the systemic red-ox balance to improve the vascular prognosis of young patients with T1D.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

All datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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