ISPAD-JDRF FELLOWSHIP AWARD PROGRESS REPORT

Title: Early markers of long-term diabetes complications in children and youths with Type 1 Diabetes: pathophysiologic role of glycemic control, glucose variability and oxidative stress.

Claudia Piona, MD,Research Fellow

Pediatric Diabetes and Metabolic Disorders Unit ,Regional Center for Pediatric Diabetes, University City Hospital of Verona, Italy

Primary mentor: Prof. Claudio Maffeis

Full Professor, Center Director

Pediatric Diabetes and Metabolic Disorders Unit, Regional Center for Pediatric Diabetes, University City Hospital of Verona, Italy

Co-mentor: Prof. Anita Morandi

Associate Professor

Pediatric Diabetes and Metabolic Disorders Unit,Regional Center for Pediatric Diabetes, University City Hospital of Verona, Italy

BACKGROUND

Long-term complications of type 1 diabetes (T1D) include macrovascular disease and microvascular complications (retinopathy, neuropathy, nephropathy)[1]. Despite the important advances in the treatment of T1D of the last decades, these complications represent the leading cause of morbidity and mortality in patients affected by diabetes[2].

These complications are characterized by an insidious onset, with overt signs and symptoms manifesting only when damage to retina, nerves, kidneys and big vessels of the cardiovascular system are at an advanced stage[3-4]. Consistent evidence is available on the occurrence of structural and functional alterations of retina, nerves, kidney and large arteries in the first years after the onset of diabetes[1]. For this reason, in recent years research in the field of diabetes complications progressively focused on two major topics, which are particularly challenging for the improvement of their diagnosis and treatment since childhood.

The first topic is related to the identification of new biomarkers and diagnostic methods able to identify early signs of complications. In fact, conventional diagnostic tools for diabetic neuropathy and retinopathy recommended by current guidelines can only identify the presence of gross signs of damages to the neuronal and vascular components of the nervous and ocular systems[1]. In recent years, two new ophthalmologic

imaging technique, Spectral domain-optical coherence tomography (SD-OCT) and In vivo confocal microscopy (IVCM), emerged for their ability of identify early neurodegenerative changes in the retina and the cornea of subjects with diabetes[5]. We recently demonstrated that a significant impairment of the minimum neuroretinal rim width (MRW) of the optic nerve head (ONH) measured with SD-OCT and of the sub basal nerve plexus (SBP) of the cornea is present in children and adolescents with T1D compared to age and gender-matched controls[6-7]. Regarding diabetic kidney disease (DKD), microalbuminuria is conventionally defined as the first marker in clinical practice. However, a multicenter study conducted on a large cohort of Italian children with T1D identify two main phenotypes of alterations of renal biomarkers related to initial stage of DKD. The first is characterized by albuminuria with normal estimated glomerular filtration rate (eGFR), the second one, more prevalent in this study population, by non-albuminuric mildly reduced estimated glomerular filtration rate (MRGFR)[8] Moreover, a worse cardiovascular risk factors profile has been found in youths with T1D with microalbuminuria and in their parents[9]. Finally, as regards diabetes macrovascular complications, several studies have demonstrated that adult patients with T1D have an accelerated atherosclerosis and increased arterial stiffness[4]. In a recent study evaluating subclinical indices of atherosclerosis, we found that a high proportion of early vascular damage, especially an increased carotid intima-media thickness (cIMT), is present in children and youths with T1D[10]

The second research topic in the field of diabetes complications is related to the identification of specific risk factors and pathogenetic processes responsible for the development of these complications. Long term glycemic control, assessed by HbA1c measurements, is acknowledge as one the most important risk factor and predictor of diabetes complications[1]. Although HbA1c remains the gold-standard assay for assessing long-term glycemic control, in the last decade growing evidence outlined that it is not able to comprehensively reflect the patients' glycemic status and to explain the risk of diabetes complications related to glycemic control[11]. To the best of our knowledge, to date limited studies investigated whether short-term glycemic control and glucose variability measured by Continuous Glucose Monitoring (CGM), besides HbA1c levels, can contribute to the development of diabetes complications. These studies have produced inconsistent results and mainly involved adult subjects with T1D.

From the pathophysiologic point of view, in the last decade, diabetes-associated oxidative stress (OS) has been advocated as one of the main possible pathogenetic pathway involved in the processes leading to diabetes complications. Hyperglycemia can cause an increased synthesis of ROS in the mitochondria and depletion of nitric oxide (NO)[12]. Advanced glycated end products (AGEs), which make up the metabolic memory of patients with T1D, induce NAD(P)H oxidase, an important producer of ROS, and up-regulate the inflammatory cascade and the sorbitol pathway, further fuelling oxidative stress[13]. In parallel, antioxidant response is impaired by chronic hyperglycemia[14]. Nuclear factor erythroid 2–related factor 2 (Nrf2, encoded by the NFE2L2 gene), is a master regulator of redox balance triggering antioxidant response element (ARE) responsive genes [heme oxygenase-1 (HO-1), NADPH dehydrogenase [quinone] 1 (NQO1), superoxide dismutase (SOD), and catalase (CAT)]. In a recent study, we demonstrated that oxidative stress is increased in youths with T1D and maintenance of physiologic glucose concentration, measured through time in range (TIR), rather than the absence of glucose fluctuations, together with male sex and appropriate BMI are protective predictors of oxidative stress[15]. To date limited studies have been conducted to comprehensively evaluate the pathway of oxidative stress and the genetic predisposition to it in relation to diabetes complications.

AIMS:

Specific aim 1: To evaluate the associations between early signs of diabetic complications and metrics

measuring short term glycemic control and glucose variability in children and youths with T1D;

Hypothesis 1.1: Children and youths with T1D with worse glycemic control (i.e., higher time in hyperglycemic range >180 mg/dL [10 mmol/L] and 250 mg/dL [13.9 mmol/L] and lower TIR) and/or higher glucose variability (coefficient of variation ≥36%) are at higher risk of presenting early signs of diabetic complications;

Hypothesis 1.2: Children and youths with T1D with worse glycemic control (i.e., higher time in hyperglycemic range >180 mg/dL [10 mmol/L] and 250 mg/dL [13.9 mmol/L] and lower TIR) and/or higher glucose variability (coefficient of variation ≥36%) are at higher risk of presenting early signs of diabetic complications.

Specific aim 2: To evaluate the associations between early signs of diabetic complications, oxidative stress and its genetic predisposition in children and youths with T1D;

Hypothesis 1.1: Children and youths with T1D with increased OS (higher level of serum derivatives of reactive oxygen metabolites (d-ROMs) are at higher risk of presenting early signs of diabetic complications;

Hypothesis 1.2: Children and youths with T1D with specific genotype of single nucleotide polymorphisms (SNPs) predisposing to increased oxidative stress are at higher risk of presenting early signs of diabetic complications.

BRIEF OVERVIEW METHODS AND RESEARCH DESIGN

We conducted a cross-sectional study at the Regional Center for Pediatric Diabetes, University Hospital of Verona (Italy).

Study Population:

Inclusion criteria: patients with T1D with at least 2 years of diabetes duration, aged between 10 to 25 years, in intensive insulin treatment (either multiple daily insulin doses – MDI, or continuous subcutaneous insulin infusion – CSII) and using continuous glucose monitoring (either intermittently scanned CGM - isCGM or real time CGM – rt CGM) for at least the last 6 months

Exclusion criteria: duration of diabetes less than 2 years; presence in the previous six months of ketoacidosis; history of hypertension, therapy with ACE inhibitors or angiotensin-receptor blockers (ARBs) or statins; diagnosis of glaucoma, corneal and lens opacities, major refractive errors (>+1,5 and <-3 diopters sphere) or other ophthalmological disorders; untreated celiac disease and/or thyroid disease; chronic infection and/or chronic inflammatory diseases; other significant systemic chronic diseases other than T1D likely to interfere with the study procedure; pregnancy; unwillingness to participate in the study.

Laboratory and clinical data collected**:**

- medical history, including age at onset and diabetes duration
- auxological parameters
- laboratory parameters (lipids, creatinine, urinary albumin-creatinine ratio, uric acid)
- metrics of long-term glycemic control (HbA1c values measured for the entire duration of diabetes) and CGM metrics of short-term glycemic control (mean glucose, TIR, time in hyperglycemic range >180 mg/dL [10 mmol/L] and 250 mg/dL [13.9 mmol/L], time in hypoglycemic range < 54 mg/dL [3.0 mmol/L] and 70 mg/dL [3.9 mmol/L]) and glucose variability (coefficient of variation , SD of mean glucose) measured in the 4-week period preceding the study procedures visit and the previous outpatients visits (up to 3 years of retrospective CGM data collection)
- serum derivatives of reactive oxygen metabolites (d-ROMs), serum total antioxidant capacity (TAC) and oxidized LDL-cholesterol (oxLDL)

- genotypes of eNOS, Nrf2 and Nfr2 enhanced genes
- early retinal and corneal neurodegenerative markers measured using Spectral domain-optical coherence tomography (SD-OCT) and In vivo confocal microscopy (IVCM), respectively
- early biomarkers of CKD defined by the presence of microalbuminuria and the measurement of estimated glomerular filtration rate – eGFR - calculated using bedside Schwartz's equation
- early signs of subclinical atherosclerosis (carotid intima-media thickness- cIMT, distensibility coefficient – cDC and carotid-femoral pulse wave velocity - PWV).

SUMMARY OF PROJECT ACCOMPLISHMENTS:

We recruited 267 children/adolescents with T1D (130 girls, age 9.1 – 23.0 years). All the study subjects completed the study procedures except for early retinal and corneal neurodegenerative markers measurement as to date 150 subjects underwent Spectral domain-optical coherence tomography (SD-OCT) and In vivo confocal microscopy (IVCM).

To date, a complete data set was created and analysed focusing on early signs of subclinical atherosclerosis measurements, oxidative stress and CGM metrics collected in the 4-week period preceding the enrolment visit in order to assess whether, besides "traditional" risk factors, overall oxidative stress, oxidized lipoproteins, and glycemic variability are associated with early macro-vascular damage in children and youths with T1D.

PRELIMINARY RESULTS:

Physical and biochemical characteristics of enrolled subject are shown in table 1.

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.

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 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).

In univariate analyses, z-cIMT was associated with male gender, oxLDL, cSBP, z-cSBP and longitudinal mean z-SBP. According to multivariate analysis of variance, z-cIMT was associated with male gender, cSBP and oxLDL.

In univariate analyses, z-PWV was associated 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. 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.

In univariate analyses, Lp-PLA2 was associated with male gender , age , diabetes duration , d-ROMs , TAC , oxLDL , cSBP , 1-month glycemic CV , 1-month glycemic TBR54 , as well as the longitudinal means of cholesterol, LDL-cholesterol and ACR . According to multivariate analysis of variance, Lp-PLA2 was associated with age, oxLDL , longitudinal mean LDL-cholesterol and gender.

According to these results, we concluded that oxidative stress, male gender, insulin dose, diabetes duration and longitudinal lipids and blood pressure, but not CGM metrics of glycemic control and variability, contributed to the variance of early vascular damage in young patients with T1D.

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 x 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 x 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 x dl^{-1})	156.9(27.5)	146.6(27.4)	151.6(27.9)	**0.002**
LDL-cholesterol (mg x dl^{-1})	83.3(23.6)	76.9(23.1)	80.0(23.5)	**0.021**
HDL-cholesterol (mg x dl^{-1})	60.6(12.3)	56.2(14.0)	58.3(13.4)	**0.005**
Triglycerides (mg x dl^{-1})	60[48-77]	58[46-74]	60[47-76.5]	0.690
ALT (U x 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 x dL^{-1})	182.0(31.0)	186.3(29.7)	184.1(30.4)	0.330
4 week-CGM SD (mg x 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 x 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 x dl^{-1})	158.6(23.5)	149.0(22.9)	153.6(23.6)	**0.001**
Follow-up-mean LDL-C (mg x dl^{-1})	84.2(20.8)	76.4(19.3)	80.2(20.4)	**0.001**
Follow-up-mean HDLC (mg x dl^{-1})	61.7(12.0)	60.4(12.3)	61.0(12.1)	0.380
Follow-up-mean triglycerides (mg x 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 x 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.

CURRENT WORK PRODUCTS:

- These results were presented at the ISPAD's 48th Annual Conference in October 2022 in a poster presentation.
- A manuscript titled "RISK FACTORS FOR PRE-CLINICAL ATHEROSCLEROSIS IN ADOLESCENTS WITH TYPE 1 DIABETES" has been submitted in a Q1 journal of Endocrinology and Metabolism and it is currently under revision.

NEXT STEP AND FUTURE WORK PRODUCTS:

The next step will be:

- implement the current data set adding data regarding early retinal and corneal neurodegenerative markers measurement, estimated glomerular filtration rate – eGFR and CGM metrics calculated from CGM data retrospectively collected in the 3-year period preceding the study procedures visit;
- conduct further statistical analysis focusing on early signs of ocular neurodegeneration, nephropathy and the impact of longitudinal exposure to worse glycemic control, as measured by CGM metrics (low TIR and/or high glucose variability).

The results from these analyses will be submitted for presentation at a scientific meeting in the next six month and another manuscript detailing the primary findings of this part of the study protocol will be prepared for publication within the next year. The ISPAD-JDRF Research Fellowship will be appropriately acknowledged in the submission for publication and whenever findings associated with this study are disseminated.

FUTURE PLAN

Following the end of the first cross-sectional phase of the study protocol, a longitudinal study has been planned with the collection of laboratory and clinical data 24 months after the enrolment. This longitudinal study will explore the potential predicting role of glycemic control, glucose variability and oxidative stress in the development and worsening of early signs of diabetes complications.

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