ORIGINAL ARTICLE

Reduced leukocyte telomere lengths and sirtuin 1 gene expression in long-term survivors of type 1 diabetes: A Dialong substudy

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ABSTRACT

Aims/Introduction: The shortening of leukocyte telomere length with age has been associated with coronary disease, whereas the association with type 1 diabetes is unclear. We aimed to explore telomere lengths in diabetes patients with regard to coronary artery disease, compared with healthy controls. The longevity factors sirtuin 1 and growth-differentiating factor 11 were investigated accordingly.

Materials and Methods: We carried out a cross-sectional study of 102 participants with long-term type 1 diabetes and 75 controls (mean age 62 and 63 years, respectively), where 88 cases and 60 controls without diagnosed coronary artery disease completed computed tomography coronary angiography. Telomere lengths and gene expression of sirtuin 1 and growth-differentiating factor 11 were quantified in leukocytes.

Results: Telomere lengths and sirtuin 1 were reduced in diabetes patients versus controls, medians (25th to 75th percentiles): 0.97 (0.82–1.15) versus 1.08 (0.85–1.29) and 0.88 (0.65–1.14) vs 1.01 (0.78–1.36), respectively, adjusted $P < 0.05$, both. Previous coronary artery disease in diabetes patients ($n = 15$) was associated with lower sirtuin 1 and growth-differentiating factor 11 messenger ribonucleic acid expression (adjusted $P < 0.03$, both). In the combined diabetes and control group, previous artery coronary disease ($n = 18$) presented with significantly shorter telomeres (adjusted $P = 0.038$). Newly diagnosed obstructive coronary artery disease, defined as >50% stenosis, was not associated with the investigated variables.

Conclusions: Long-term type 1 diabetes presented with reduced telomeres and sirtuin 1 expression, with additional reduction in diabetes patients with previous coronary artery disease, showing their importance for cardiovascular disease development with potential as novel biomarkers in diabetes and coronary artery disease.

INTRODUCTION

Individuals with type 1 diabetes mellitus have increased risk of coronary heart disease (CHD) compared with individuals without diabetes[1]. Underlying mechanisms have so far been related to hyperglycemia, increased blood pressure and triglycerides[2]. Recently, Holte *et al.*[3] reported a high prevalence of undiagnosed obstructive coronary artery disease (CAD) in individuals without established CHD in long-term survivors of type 1

diabetes mellitus, which was related to elevated low-density lipoprotein (LDL)-cholesterol and glycated hemoglobin (HbA1c).

Epidemiological studies have reported an association between cardiovascular disease and telomere lengths. Reduced leukocyte telomere lengths (LTLs) were recently found to be associated with an increased risk of lower-extremity amputation in patients with long-standing type 1 diabetes mellitus[4], and increased risk of stroke and myocardial infarction in type 2 diabetes patients[5–7]. Telomeres are protecting caps at the ends of

chromosomes consisting of repetitive sequences of TTAGGG. Oxidative stress and chronic inflammation can furthermore increase the telomere-shortening rate[8,9]. Cells with critically short telomeres enter cell-senescence, their secretory patterns change, promoting inflammation, apoptosis, aging and age-related diseases, such as CHD[10]. Previous studies on telomeres' importance in type 1 diabetes mellitus have shown an association with progression of severe nephropathy and mortality of the disease. However, limited data exist on the underlying mechanisms, although associations between diabetes duration, hyperglycemia and increased oxidative stress have been suggested[11–14]. Notably, LTLs are thought to reflect telomere lengths in other cells and tissues, including vascular cells[15,16].

We have previously observed associations between LTLs and the longevity factors sirtuin 1 (SIRT1) and growth-differentiating factor 11 (GDF11) in both healthy and CHD patients[17,18]. SIRT1, the most studied of seven sirtuins, is thought to stabilize telomere ends[19], and to play a role in glucose metabolism through induced expression of the insulin gene. SIRT1 is involved in the regulation of insulin sensitivity and autoimmunity[20,21], and was recently shown to correlate inversely with advanced glycation end-products[22]. The main SIRT1-producing cells are leukocytes, with somewhat less expression in the uterus and multiple other tissues.

GDF11 is thought to play a pivotal role in cardiac health and aging[23]. In mouse experiments, GDF11 was involved in maturation of pancreatic β-cells[24], and its replenishment improved cell survival and thereby glucose metabolism. The role of GDF11 in diabetes and cardiovascular disease is disputed[25]. GDF11 is expressed in leukocytes and other tissues, such as the brain, skeletal muscles, pancreas, kidney and retina.

As undiagnosed CAD in long-term type 1 diabetes mellitus patients is common, more knowledge on telomeres and longevity factors in type 1 diabetes mellitus is required. In the current study, we aimed to investigate potential differences in levels of LTL, SIRT1 and GDF11 in long-term survivors of type 1 diabetes mellitus compared with healthy controls of a similar age and according to established CHD or newly diagnosed obstructive CAD. Associations between traditional cardiovascular risk factors and the investigated markers were additionally explored.

METHODS

Study population
The present investigation is a substudy of the cross-sectional controlled Dialong study (type 1 diabetes long-term survivors with a new syndrome of late complications), which consisted of 105 individuals with type 1 diabetes mellitus carried out in 2015 at the Norwegian Diabetics' Center, Oslo, Norway[26]. The inclusion criteria have previously been described. In short, type 1 diabetes mellitus was defined as a medical history characteristic of type 1 diabetes mellitus with glycated hemoglobin (HbA1c) >48 mmol/mol (6.5%) and lack of insulin production

(C-peptide concentration <0.2 pmol/mL). Individuals attending the Norwegian Diabetics' Center in 2015 with type 1 diabetes mellitus diagnosed in 1970 or earlier were included. The mean duration of their diabetes was 49 years. The mean values of HbA1c were calculated based on HbA1c registration since approximately 1980. Of the 105 diabetes patients, 102 were included in the present study. As a control group without diabetes, 75 healthy spouses/friends of the diabetes patients of a similar age were included (exclusion criteria: first-degree relatives or HbA1c >48 mmol/mol [6.5%]).

Clinical characteristics and comorbidity

Previous CHD was defined as either a previous episode of acute coronary syndrome or revascularization. Computed tomography coronary angiography (CTCA) was carried out in participants without diagnosed CHD, to discover the presence of undiagnosed obstructive CAD in type 1 diabetes mellitus patients compared with controls: >50% diameter stenosis in one of the coronary arteries defined the presence of obstructive CAD, whereas no detected plaques in any of the coronary arteries defined its absence. Total CAD represents the combination of either previous CHD or the presence of newly diagnosed obstructive CAD. CTCA was carried out on a dual source computed tomography scanner (Somatom Definition Flash; Siemens, Erlangen, Germany), and image analysis was carried out in SyngoVia® (Siemens) by two independent readers. Hypertension was defined as previously diagnosed. Smoking was categorized as never, current or ex-smoker. Self-reported alcohol intake was categorized into four groups: abstainers, 1–14, 14–21 and >21 units/per week. Physical activity was categorized into four groups based on the number of sessions of minimum 30 min of moderate intensity per week: zero, one or two, three to five, or six or more. The initial study was approved by the Regional Committee of Medical Research Ethics in South-Eastern Norway (project no. 2014/851). The study conformed to the Declaration of Helsinki, and written informed consent was obtained from all participants.

Laboratory methods
Blood samples were collected under fasting conditions between 08.00 and 10.30 hours at inclusion. Routine analyses were carried out by conventional laboratory methods. Serum was prepared by centrifugation within 1 h at 2,500 g for 10 min for the analysis of circulating SIRT1. Ethylenediaminetetraacetic acid blood and PAXGene Blood RNA tubes (Pre-Analytix GmbH, Hombrechtikon, Switzerland) were collected for deoxyribonucleic acid (DNA) extraction and ribonucleic acid (RNA) isolation, respectively. DNA was available from 159 participants (93 in the diabetes group), and RNA was available from 166 participants (98 in the diabetes group). All materials were kept frozen at −80°C until further preparation and analysis. DNA was isolated by the same lot number of the QIAamp DNA Blood Mini Kit throughout the study (Qiagen GmbH, Hilden, Germany). Total RNA was isolated using the PAXGene

Blood RNA kit (Qiagen GmbH for PreAnalytix, Hilden, Germany), with an extra cleaning step (RNeasy MinElute Cleanup kit; Qiagen), both according to the manufacturers' instructions. DNA and RNA purity and quantity were tested on the Nano Drop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Leukocyte telomere length determination

Equal amounts of extracted DNA per experiment (2 ng/µL) were used to measure LTL by singleplex quantitative reverse transcription polymerase chain reaction (PCR)[27]. PCR amplification was carried out on the ViiA™ 7 instrument (Applied Biosystems by Life Technologies, Foster City, CA, USA), using telomere-specific primers (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA; Table S1) and GoTaq®qPCR Master Mix (Promega, Madison, WI, USA). LTLs were relatively quantified to the single-copy gene, SB34 (Invitrogen by Thermo Fisher Scientific; Table S1), and an internal reference sample with an interassay coefficient of variation of 2.51%. The primers for both targets were diluted to a final concentration of 4 pmol/µL. PCR conditions for both targets were as follows: an initial step at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A template negative control was included in each run. All samples were run in triplicates. Individual amplification curves for all samples of both assays were carefully validated. Technical triplicates with a standard deviation exceeding 0.5 Ct were excluded from the analysis, with two remaining valuable parallels and with 16 samples re-analyzed, thus LTLs were successfully analyzed in all available samples ($n = 159$). Samples from the diabetes and control groups were run simultaneously on the same PCR plate.

Gene expression analysis

Equal amounts of isolated total RNA per experiment (100 ng) were reversely transcribed into complementary (c) DNA by use of qScript cDNA SuperMix (Quanta Biosciences Inc., Gaithersburg, MD, USA). The leukocyte expression of GDF11 (Hs00195156_m1) and SIRT1 (Hs01009006-m1) were normalized to β-2-microglobulin (Hs99999907_m1), previously tested as a valid housekeeping gene[28]. The messenger RNA (mRNA) gene expression analyses were measured on the ViiA™ 7 instrument, using TaqMan Universal PCR Master Mix, NoAmpErase UNG and the TaqMan assays, as noted above (Applied Biosystems), as relatively quantified values ($2^{-\Delta\Delta C_t}$ method)[29]. SIRT1 and GDF11 gene expression were successfully analyzed in all available samples, and samples from the diabetes and control groups were run simultaneously on the same PCR plates.

Determination of circulating SIRT1

An enzyme-linked immunosorbent assay (ELISA) kit was used to measure serum SIRT1 levels (Human SIRT1; LSBio LifeSpan BioSciences lnc., Seattle, WA, USA). The inter-assay coefficient of variation was 11.6%, and SIRT1 was successfully analyzed in all available samples, with equal distribution of diabetes and control samples on same ELISA plates.

Statistical analysis

Continuous data are presented as the mean (standard deviation) or median (25th to 75th percentile) for non-parametric variables. Categorical variables are presented as numbers (percentages). Independent Student's t-test and Mann–Whitney U-test were used to compare continuous data with normal and skewed distribution, respectively. The Kruskal–Wallis test was used to compare median values through multiple groups, whereas proportional data were compared using the χ^2-test. Correlations were carried out by Spearman's rho, and multiple comparisons were adjusted for by the Bonferroni correction. To assess whether diabetes was associated with LTLs and SIRT1 gene expression, multivariate linear regression was carried out, with LTLs and SIRT1 relatively quantified values as dependent variables, respectively, in three separate models. Model 1 included demographic and clinical variables: age, sex, body mass index (BMI) and comorbidities (including total CAD, hypertension and physical activity). In model 2, biochemical measurements (LDL cholesterol, HDL cholesterol and triglycerides) were added. In model 3, HbA1c was included. Use of statins, antihypertensive medication and aspirin were not included, as lipid values and the diagnosis of hypertension and CAD were already included. For the association between the markers and previous CHD, a linear regression model only adjusted for age and sex was used, due to low numbers of previous CHD. Values were log-transformed before entering the regression analyses because of skewed distribution. Sample size calculation was carried out for the initial Dialong study[26]. P-values ≤0.05 were considered statistically significant. The statistical analyses were carried out with IBM SPSS statistic software, version 25 (IBM Corp., Armonk, NY, USA).

RESULTS

Baseline characteristics

Clinical characteristics in the type 1 diabetes mellitus and control groups are shown in Table 1. CTCA was obtained in 86 of the cases and in 60 of the controls. The calculated mean HbA1c was higher than the cross-sectional value. The presence of previous CHD, newly diagnosed CAD and hypertension was higher in the diabetes group versus in controls. Age, sex, BMI, smoking and alcohol consumption did not differ between groups, whereas levels of systolic blood pressure, fasting glucose, cross-sectional HbA1c and HDL cholesterol were higher in the diabetes group, and diastolic blood pressure, levels of LDL cholesterol, total cholesterol and triglyceride were lower, the latter probably due to statin treatment. The diabetes group more frequently used angiotensin-converting enzyme inhibitor or angiotensin receptor blockers, aspirin and statins, and controls were more physically active. Cross-sectional HbA1c values were used in further statistical analyses.

**Table 1** | Baseline characteristics of patients with long-term type 1 diabetes mellitus and controls

	Type 1 diabetes mellitus n = 102	Control group n = 75	P-value
Age (years)	61.9 ± 7.1	62.6 ± 7.0	0.52
Sex (male)	51 (50.0)	34 (45.3)	0.54
Previous CHD	15 (15)	3 (4)	0.020*
Newly diagnosed CAD†	20 (23)	6 (10)	0.039*
Total CAD‡	35	9	0.001*
Tobacco smoking			
Never	58 (56,9)	39 (52.0)	0.33
Daily	4 (3.9)	7 (9.3)	
Ex-smoker	40 (39.2)	29 (38.7)	
Hypertension	34 (33.3)	14 (18.7)	0.039*
SBP (mmHg)	146 ± 20	137 ± 19	0.004*
DBP (mmHg)	75 ± 8	81 ± 9	<0.001*
BMI (kg/m²)§	25.8 (23.4, 28.7)	25.5 (22.6, 27.8)	0.45
Waist circumference (cm)	91.5 ± 12.8	90.2 ± 12.7	0.50
HbA1c, mmol/mol (%)	57 ± 8.5 (7.4 ± 0.8)	37 ± 3.5 (5.5 ± 0.3)	<0.001*
Mean HbA1c, mmol/mol (%)	64 ± 9.0 (8.0 ± 0.8)		
Fasting glucose (mmol/L)	8.6 ± 3.5	5.2 ± 0.6	<0.001*
Total cholesterol (mmol/L)	5.0 ± 1.0	5.8 ± 1.2	<0.001*
LDL cholesterol (mmol/L)	2.7 ± 0.8	3.8 ± 1.0	<0.001*
HDL cholesterol (mmol/L)	2.1 ± 0.6	1.7 ± 0.5	<0.001*
Triglycerides (mmol/L)§	0.79 (0.62–0.99)	0.94 (0.71–1.27)	0.001*
Physical activity (exercise/week)			
0	10 (10.0)	1 (1.4)	0.030*
1–2	29 (29.0)	21 (28.4)	
3–5	32 (32.0)	36 (48.6)	
>6	29 (29.0)	16 (21.6)	
Alcohol consumption (units/week)			
0	20 (19.8)	12 (16.0)	0.87
1–14	74 (73.3)	59 (78.7)	
14–21	4 (4.0)	2 (2.7)	
>21	3 (3.0)	2 (2.7)	
Medication			
Statins	54 (54)	9 (14)	<0.001*
ACE-i/ARB	50 (50)	13 (21)	0.004*
Aspirin	33 (33)	8 (13)	<0.001*

Values are mean levels (standard deviation) or proportions (%), if not otherwise stated. *P*-values are independent samples *t*-test, Mann–Whitney *U*-test or Kruskal–Wallis test, as appropriate. *P-values <0.05. ACE-i/ARB, angiotensin-converting enzyme inhibitor or angiotensin receptor blocker; BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; SBP, systolic blood pressure. †Computed tomography coronary angiography was obtained for 86 in the diabetes group and 60 in the control group, numbers in parentheses are valid percentages. ‡Total coronary artery disease: previous coronary heart disease and newly diagnosed coronary artery disease (>50% stenosis on computed tomography coronary angiography). §Median levels (25th to 75th percentiles).

Levels of LTLs, SIRT1 and GDF11 in type 1 diabetes mellitus patients versus controls

LTLs were significantly shorter and SIRT1 gene expression significantly lower in the diabetes versus the control group, medians (25th to 75th percentiles): 0.97 (0.82–1.15) versus 1.08 (0.85–1.29) and 0.88 (0.65–1.14) versus 1.01 (0.78–1.3), respectively, *P* < 0.02 for both (Figure 1a). Genetically expressed GDF11 (Figure 1a) and circulating SIRT1 (pg/mL; Figure 1b) were not associated with the presence of diabetes: 1.03 (0.79–1.36) vs 1.02 (0.81–1.48) and 482 (346–633) versus 461

(347–666), *P* > 0.05 for both. When excluding all participants with coronary disease (previous CHD and newly diagnosed CAD, *n* = 35), the presence of type 1 diabetes mellitus was still significantly associated with LTLs and SIRT1 expression (*P* = 0.041 and *P* = 0.019, respectively). To further explore the associations between diabetes and LTLs and SIRT1 expression, we carried out multivariate linear regression analyses (Table 2). Diabetes was still significantly associated with shorter LTLs when adjusting for covariates in models 1 and 2 (*P* < 0.05, both), but diabetes and LTLs were no longer associated when
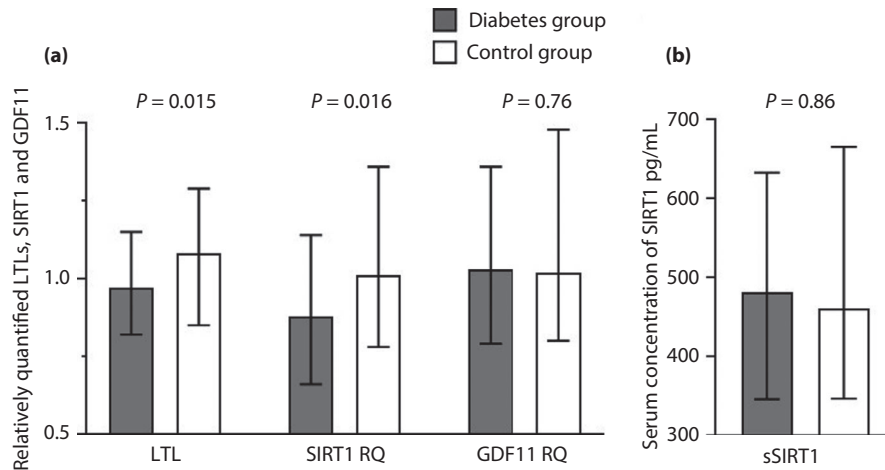
Figure 1 | (a) Leukocyte telomere lengths (LTLs) and genetically expressed sirtuin 1 (SIRT1) and growth-differentiating factor 11 (GDF11) in type 1 diabetes patients versus healthy controls. Levels of relatively quantified LTLs, SIRT1 and GDF11 gene expression; type 1 diabetes (grey bars) and healthy controls (white bars). Error bars indicate the 25th and 75th percentiles. (b) SIRT1 concentrations in type 1 diabetes and healthy controls. Serum levels of SIRT1; type 1 diabetes (grey bars) and healthy controls (white bars). Error bars indicate the 25th and 75th percentiles.

Table 2 | Association between leukocyte telomere lengths and sirtuin 1 expression and type 1 diabetes controlled for relevant covariates by linear regression[†]

	Multivariate model 1			Multivariate model 2			Multivariate model 3		
	Beta	t	P-value	Beta	t	P-value	Beta	t	P-value
Leukocyte telomere lengths									
Diabetes	0.184	2.241	0.027*	0.203	2.057	0.041*	0.254	1.578	0.117
Age	0.019	0.232	0.817	−0.012	−0.146	0.884	−0.028	−0.333	0.739
Sex	−0.064	−0.791	0.430	−0.019	−0.217	0.828	−0.009	−0.097	0.923
BMI	0.168	2.150	0.033*	0.199	2.443	0.016*	0.194	2.353	0.020*
Total CAD	−0.111	−1.315	0.190	−0.080	−0.923	0.358	−0.061	−0.683	0.496
Hypertension	−0.041	−0.491	0.624	−0.016	−0.0185	0.853	−0.019	−0.221	0.826
Physical activity	−0.089	−1.128	0.261	−0.127	−1.501	0.136	−0.117	−1.360	0.176
LDL cholesterol				0.076	0.779	0.437	0.078	0.783	0.435
HDL cholesterol				0.163	1.614	0.109	0.181	1.764	0.080
Triglycerides				0.006	0.062	0.951	0.004	0.041	0.435
HbA1c							0.035	0.234	*0.816*
Sirtuin 1									
Diabetes	0.195	2.375	0.019*	0.197	2.0257	0.041*	0.330	2.121	0.036*
Age	−0.018	−0.216	0.829	−0.024	−0.299	0.766	0.004	0.050	0.960
Sex	0.002	0.024	0.981	−0.002	−0.258	0.797	−0.035	−0.409	0.683
BMI	−0.045	−0.572	0.568	0.005	0.068	0.946	−0.005	−0.058	0.954
Total CAD	<0.000	0.001	0.999	0.007	0.088	0.930	−0.040	−0.458	0.648
Hypertension	−0.001	−0.011	0.991	<0.001	0.006	0.995	0.016	0.195	0.845
Physical activity	0.140	1.787	0.076	0.094	1.154	0.250	0.105	1.262	0.209
LDL cholesterol				0.079	0.823	0.412	0.074	0.763	0.446
HDL cholesterol				−0.071	−0.728	0.468	−0.089	−0.898	0.371
Triglycerides				−0.29	−3.202	0.002*	−0.296	−3.248	0.001*
HbA1c							0.187	1.260	0.209

*P-values <0.05. BMI, body mass index; total CAD; previous coronary heart disease and newly diagnosed coronary artery disease (>50% stenosis on computed tomography coronary angiography). [†]Multivariate model 1 includes demographic and clinical variables, whereas model 2 includes additionally biochemical parameters, and model 3 additionally glycated hemoglobin (HbA1c).

additionally adjusting for HbA1c (model 3; $P = 0.117$). BMI was also a strong predictor of LTL. To further explore any impact of HbA1c on LTLs, HbA1c levels were categorized, and a differential distribution of LTLs through quartiles (Qs) was observed, $P = 0.031$ (Kruskal–Wallis test; Figure S1). When results were dichotomized at the cut-off level between Q1 and Q2–4, significantly longer LTLs were observed in Q1 compared with the three upper Qs combined ($P = 0.003$; Mann-Whitney U-test).

As shown in Table 2, diabetes was also significantly associated with reduced SIRT1 expression when adjusting for the relevant covariates in models 1 and 2 ($P < 0.05$, both), also when adding HbA1c into the model (model 3, $P < 0.05$). Triglycerides were also a strong predictor of SIRT1 expression.

Levels of LTLs, SIRT1 and GDF11 according to previous CHD and newly diagnosed CAD in diabetes patients

Previous CHD ($n = 15$) compared with its absence ($n = 87$) was associated with non-statistically significant shorter LTL ($P = 0.076$), less genetically expressed SIRT1 and GDF11 ($P = 0.036$ and $P = 0.007$, respectively), and elevated circulating SIRT1 levels ($P = 0.009$; Table 3), still significantly associated when adjusting for age and sex ($P = 0.028$; $P < 0.001$) and ($P = 0.015$), respectively. When the diabetes and control groups were combined, previous CHD ($n = 18$) was associated with shorter LTLs (0.92, 0.76–0.99 vs 1.04, 0.83–1.24, $P = 0.011$), remaining statistically significant after adjustment ($P = 0.038$). In those with newly diagnosed CAD ($n = 20$) compared with those without ($n = 67$), no significant differences in levels of the markers were found (Table 3).

Correlations between the investigated markers, metabolic variables and leukocyte count

Table 4 shows the coefficients of correlation in the total population. LTLs were weakly correlated to LDL cholesterol and were inversely correlated to HbA1c. Circulating SIRT1 correlated to BMI and leukocyte count, and inversely to HDL cholesterol. SIRT1 and GDF11 expression were inversely correlated to BMI. After controlling for multiple testing (Bonferroni adjusted: $P = 0.002$ after 24 performed associations), the correlations between circulating SIRT1 and HDL cholesterol and leukocyte count remained statistically significant. Table S2a,b show the coefficients of correlation separately in the diabetes group and controls, respectively. In the diabetes group, circulating SIRT1 correlated inversely to HDL cholesterol and correlated to leukocyte count, the latter being statistically significant after the Bonferroni correction. In the control group, circulating SIRT1 correlated to BMI, triglycerides and leukocyte count. Expression of SIRT1 and GDF11 was inversely correlated to BMI, triglycerides and leukocyte count, and GDF11 expression was additionally inversely correlated to fasting glucose. The correlations between circulating SIRT1 and BMI and leukocyte count, and the inverse correlation between SIRT1 expression and triglycerides remained statistically significant after correction.

Long-term mean values of HbA1c in the diabetes group were neither correlated to LTLs, nor SIRT1 and GDF11 expression or circulating SIRT1 levels (Table S3).

Levels of LTLs, SIRT1 and GDF11 according to traditional cardiovascular risk factors

Results on the influence of sex and selected lifestyle markers on LTL, SIRT1 and GDF11 are solely given for the diabetes group, as results achieved in the control group have previously been published[17]. No significant sex differences were observed ($P = 0.075$). Hypertension was associated with shorter LTLs ($P = 0.006$), but failed to associate when angiotensin-converting enzyme inhibitor or angiotensin receptor blocker use was adjusted for ($P = 0.852$), with no significant difference in levels of circulating SIRT1 and SIRT1 and GDF11 expression. BMI, smoking and physical activity were not associated with levels of the markers, whereas increasing alcohol consumption was associated with numerically higher circulating SIRT1 levels (Table S4).

Table 3 | Leukocyte telomere lengths, sirtuin 1 and growth-differentiating factor 11 levels according to previous coronary heart disease and newly diagnosed coronary artery disease in type 1 diabetes patients

	LTLs RQ	sSIRT1 (pg/mL)	SIRT1 RQ	GDF11 RQ
Previous CHD+ $n = 15$	0.93 (0.74–0.98)	608 (488–843)	0.73 (0.51–0.94)	0.79 (0.50–1.01)
Previous CHD− $n = 87$	1.00 (0.82–1.17)	460 (327–572)	0.92 (0.71–1.17)	1.07 (0.83, 1.38)
	$P = 0.076$	$P = 0.009*$	$P = 0.036*$	$P = 0.007*$
Newly CAD+ $n = 20$†	0.99 (0.80–1.21)	475 (328–736)	1.02 (0.83–1.38)	1.19 (0.94–1.39)
Newly CAD− $n = 67$	1.00 (0.82–1.17)	450 (326–560)	0.89 (0.65–1.14)	1.05 (0.77–1.39)
	$P = 0.814$	$P = 0.396$	$P = 0.084$	$P = 0.17$

Values are median levels (25th to 75th percentiles). Presented P-values are unadjusted and refer to difference in values of markers as related to presence of previous coronary heart disease (CHD) or newly diagnosed coronary artery disease (CAD), Mann–Whitney U-test. *P-values < 0.05. GDF11, growth-differentiating factor 11; LTLs, leukocyte telomere lengths; RQ, relatively quantified; s, SIRT1, serum sirtuin 1. †Defined as >50% diameter stenosis on computed tomography coronary angiography.

Table 4 | Spearman's rho correlations between the investigated markers and biochemical parameters in the total population

	BMI	HDL cholesterol	TGL	LDL cholesterol	F. gluc.	HbA1c	Leukocyte count
LTL	0.034	−0.014	0.100	0.192	−0.017	−0.168	0.088
	0.67	0.86	0.213	0.015	0.83	0.036	0.27
sSIRT1	0.192	−0.267*	0.132	−0.003	0.081	0.032	0.509*
	0.017	0.001*	0.11	0.97	0.32	0.70	<0.001*
SIRT1 RQ	−0.21	−0.036	−0.113	0.095	−0.118	−0.109	−0.075
	0.007	0.64	0.15	0.23	0.14	0.16	0.38
GDF11 RQ	−0.233	0.019	−0.138	0.036	−0.01	0.025	−0.067
	0.003	0.81	0.08	0.64	0.89	0.76	0.39

Spearman's rho values are given in each box together with *P*-values below. BMI, body mass index; F. gluc, fasting glucose; GDF11, growth-differentiating factor 11; HbA1c, glycated hemoglobin; LTLs, leukocyte telomere lengths; RQ, relatively quantified gene expression; sSIRT1, serum sirtuin 1; TGL, triglycerides. *Statistically significant correlation after Bonferroni correction (*P* = 0.002 after 24 performed associations).

DISCUSSION

Long-term survivors of type 1 diabetes mellitus diabetes presented with shorter telomeres and lower SIRT1 mRNA expression compared with healthy individuals of a similar age. Additionally, lower levels were found in diabetes patients with established CHD, also including significantly lower GDF11 mRNA expression and elevated circulating SIRT1.

Reported associations between telomere lengths and diabetes have previously mainly dealt with increased comorbidity and mortality[11–13]. Very high HbA1c levels were recently associated with telomere attrition in a longitudinal study of children[30]. We observed a weak inverse association between LTLs and HbA1c, and the significant association between LTLs and the presence of long-term type 1 diabetes mellitus was not present when HbA1c levels were adjusted for, indicating its possible impact. The coherent result with the longest telomeres at HbA1c levels below the 25th percentile supports this assumption. No association was found for the long-term registered mean HbA1c, with no obvious explanation other than a potential impact of equal sampling time for cross-sectional HbA1c and DNA for LTL analyses. As the adjusted model also showed no impact of cross-sectional measured HbA1c on LTLs, any impacts of hyperglycemia on variables not included in the current study, such as advanced glycation end-products and biomarkers of DNA oxidation, are of interest and should be further explored. However, chronic elevated glucose levels are thought to increase levels of glycated proteins and the production of reactive oxygen species, which in turn leads to subsequent telomere attrition indicating a plausible mechanism[8]. The sustained significant association between LTLs and type 1 diabetes mellitus when CHD was accounted for shows that diabetes or hyperglycemia are involved in telomere shortening.

The impact of BMI on LTL in type 1 diabetes mellitus seems to be protective to a certain extent, as shown in the adjusted models. We have previously reported on longer telomeres in young healthy individuals with BMI >25 kg/m^2, compared with BMI <25 kg/m^2, age 18–39 years, which could merely indicate higher muscle mass in young individuals, and not adiposity[17].
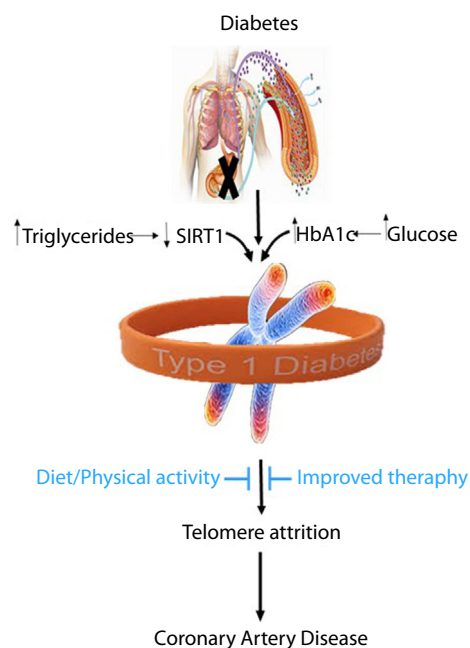
This was contradictory to that reported by others in an age cohort of 20–84 years[31]. Importantly, very low BMI might also be indicative of poor health, such as frailty in the elderly and eating disorders in especially young individuals, partly explaining discrepancy of results.

A more bidirectional causality might be applicable for the observed relationship between diabetes and SIRT1 expression, although only associations have been investigated in the present study. Elevated glucose levels have been shown to reduce levels of SIRT1s' coenzyme NAD$^+$, with subsequent reduced SIRT1 activity, and advanced glycation end-products were shown to downregulate both SIRT1 mRNA and protein levels[32]. Thus, long-term diabetes might potentially have affected transcription of the SIRT1 gene, explaining the observed reduced expression. SIRT1 expression and triglycerides were inversely associated in controls in the current study. Mouse experiments have shown that SIRT1 inhibits the synthesis of triglycerides[33], and is involved in the regulation of multiple proteins and genes related to both lipid and glucose metabolism[34]. As triglycerides have been shown to be a risk factor for nephropathy in type 1 diabetes mellitus patients[35], SIRT1 might potentially be a target of novel therapies in diabetes and other metabolic diseases. Metformin has been reported to activate SIRT1, which might be one of its beneficial effects[36].

Previous CHD was associated with shorter telomeres and lower expression of the SIRT1 and GDF11 genes, whereas newly diagnosed obstructive CAD identified with CTCA was not. This could be explained by the duration of their previous CHD, also being more severe than the definition of >50% diameter stenosis in one of the coronary arteries, without giving any clinical symptoms. Traditional cardiovascular risk factors have been associated with shorter LTL, to some degree shown in the present study, which might indicate senescence also in the endothelial and vascular smooth muscle cells with subsequent dysfunction, accelerating atherosclerosis[37]. SIRT1 has been shown to suppress oxidative stress and inflammation through activation of the forkhead box O transcription factors, and inhibition of nuclear factor kappa B and the Nod-like

receptor pyrin domain containing-3 inflammasome, respectively[38,39]. As leukocytes are the main producer of SIRT1, the observed downregulation in diabetes compared with in the healthy controls might thus have favored inflammation and contributed to progression of their CHD. The contrary elevated serum SIRT1 levels in previous CHD might indicate compensatory mechanisms from other cell-types. GDF11 has also been reported to be preventive of cardiovascular disease by its regenerative effects on cardiomyocytes[40]. The observed lower GDF11 expression might reflect the aging process itself, which might have contributed to their disease state. However, the role of GDF11 expression in leukocytes might be regarded as speculative, unless it reflects GDF11 expression in the heart.

Correlation analyses showed that expression of the SIRT1 and GDF11 genes were negatively influenced by increasing BMI and elevated triglycerides (in controls only), whereas serum SIRT1 was inversely association with HDL cholesterol, which might show that elevated circulating levels of SIRT1

could merely be a sign of metabolic disturbances. The strong correlation between leukocyte count and circulating SIRT1 levels could be discussed as compensatory mechanism in other cell types.

Of the traditional cardiovascular risk factors, only hypertension was associated with shorter telomeres in the diabetes group, probably due to blood pressure-lowering therapy, as lower telomerase activity has been observed with regular antihypertensive treatment[41].

As a summary of the work, potential and hypothetic mechanisms of the main findings are shown in Figure 2.

Although the present study has an exploratory design, one of its limitations was the low number in some subgroups, requiring awareness of potentially false negative results. LTLs as markers of telomere lengths in general are widely used due its availability. However, their validity as a biomarker for CHD risk requires further investigations. Participants in the diabetes group used several medications, which could have influenced the results, as could also the actual absence of disease in the healthy controls. Measuring circulating GDF11 levels would have added to the results, but more sensitive and specific molecular assays are required, as tested ELISA assays resulted in undetectable levels.

Long-term survivors of type 1 diabetes have shorter telomeres and diminished SIRT1 expression in leukocytes, with hyperglycemia and triglycerides as potential contributing factors. The variables' connection to CHD in diabetes patients might potentially reflect both their impact and the aging phenomena in atherosclerotic development, with potential as novel biomarkers in cardiovascular disease and diabetes.

Figure 2 | Summary of results. Long-term type 1 diabetes was associated with reduced telomere lengths and sirtuin 1 (SIRT1) messenger ribonucleic acid expression in circulating leukocytes. Chronic hyperglycemia might lead to extended telomere attrition, probably by an increase in reactive free radicals. Elevated triglycerides, which were associated with reduced SIRT1 expression, might indirectly have affected telomere lengths. By inhibiting these mechanisms, the development of coronary artery disease might be prevented, provided that telomere attrition in leukocytes reflects the shortening of telomeres in the cardiovascular system. Blue colored text is indicative of suggested potential measures, in need of further investigation. HbA1c, glycated hemoglobin.

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Primer sequences for the telomeres and single-copy gene analyses.

Table S2 | Spearman's rho correlations between the investigated markers and lipids, fasting glucose, glycated hemoglobin and leukocyte count separately in the (a) type 1 diabetes group and (b) controls.

Table S3 | Spearman's rho correlations between registered mean glycated hemoglobin values in the type 1 diabetes group and the investigated markers.

Table S4 | Levels of leukocyte telomere lengths, sirtuin 1 and growth-differentiating factor 11 according to selected lifestyle markers in diabetes patients.

Figure S1 | Levels of leukocyte telomere lengths according to glycated hemoglobin quartiles.