Pro-coagulant activity in children and adolescents on intensive insulin therapy

Running title: Pro-coagulant activity in childhood diabetes

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Contribution statement

VB, HDM, MH, SS, HA, KDJ and IS were all involved in the conception and design of the study. VB, HDM and MH managed the data acquisition and VB was responsible for analyses of data. All contributed to the interpretation of the data. VB and IS drafted the manuscript which were revised critically by all co-authors. All authors approved the final version and accepted to be responsible for all aspects of the work.

Abstract

Background: Type-1 diabetes is associated with atherothrombosis, but limited data exist on pro-coagulant activity in the young. We investigated pro-coagulant activity in children/adolescents with type-1 diabetes using intensified insulin-treatment compared with controls in a five-year follow-up study, and further any associations with cardiovascular risk-factors.

Methods: The study included 314 diabetes children/adolescents and 120 healthy controls. Prothrombin fragment 1+2 (F1+2), D-dimer, tissue-factor-procoagulant-activity (TF-PCA) and tissue-factor-pathway-inhibitor (TFPI) were analysed with ELISAs.

Results: F1+2, D-dimer and TF-PCA did not differ between the groups or correlate to HbA_{1c} in the diabetes group at either time-points. TFPI was significantly higher in the diabetes group compared with controls both at inclusion and follow-up (both $p< 0.001$). In the diabetes group TFPI correlated significantly to HbA_{1c} at both time-points ($r= 0.221$ and $r= 0.304$, both $p< 0.001$). At follow-up females using oral contraceptives had significantly elevated F1+2, D-dimer and TF-PCA and lower TFPI compared to no-users (all $p< 0.005$), and females had lower TFPI ($p= 0.017$) and higher F1+2 compared with males ($p= 0.052$), also after adjusting for the use of oral contraceptives.

Conclusions: The current results show similar pro-coagulant activity in children/adolescents with type-1 diabetes compared with controls over a five-year period, indicating that these children using modern intensified insulin-treatment are not at high thrombotic risk at younger age. The elevated levels of TFPI in the diabetes group, related to hyperglycaemia, are probably reflecting increased endothelial activation. These findings highlight the significance of optimal blood glucose-control in children/adolescents with type-1 diabetes, to maintain a healthy endothelium.

Keywords

Childhood diabetes, pro-coagulant activity, hyperglycaemia, endothelial dysfunction, tissue-factor-pathway-inhibitor

Introduction

In Norway, approximately three hundred children and adolescents below 15 years of age are annually diagnosed with type 1 diabetes [1]. In addition to microvascular complications in type 1 diabetes, cardiovascular disease (CVD) is the main reason for morbidity and mortality due to accelerated progression of atherosclerosis and increased thrombotic risk [2]. In adults with type 1 diabetes approximately 80% die from CVD and early onset of disease have impact on survival, especially in women, [3] who have twice the risk of vascular complications compared to men with type 1 diabetes [4].

High prevalence of cardiovascular risk factors in children and adolescents with type 1 diabetes has previously been reported [5]. Hyperglycemia along with other contributors to vascular disease like hypertension and hyperlipidemia, trigger a cascade of biochemical alterations including increased oxidative stress and reduced bioavailability of the vasodilator nitric oxide. Disrupted vascular homeostasis with development of endothelial dysfunction results in a thrombogenic and pro-inflammatory milieu [6].

Intensive blood glucose control at an early stage reduces cardiovascular events [7]. Glucose control is mainly assessed by HbA_{1c} which estimates control for the last couple of months. In addition, glycemic burden, calculated as cumulative glycemic exposure from the time of diagnosis (A_1 months) or the area under the curve can be estimated [8,9]. Hyperglycemia per se seems to have a pro-thrombotic impact on the endothelium and the coagulation system by mechanisms like accelerated transcription and release of coagulation factors and also direct glycation of coagulation factors resulting in changed activity [10,11]. Tissue factor (TF) is a key molecule in the initiation of the coagulation cascade. It is a membrane

glycoprotein present in the adventitia of normal blood vessels, in atherosclerotic plaques and a minor fraction is circulating in the blood [12]. In vascular diseases increased amounts of TF are present, also on circulating monocytes and microvesicles, contributing to accelerated thrombosis [13]. When prothrombin is converted to thrombin, prothrombin fragments 1+2 (F1+2) are released, and D-dimer is released after lysis of cross-linked fibrin as the final step in coagulation. Both these fragments are widely utilized as markers of thrombin generation.

TF- pathway inhibitor (TFPI) is the primary regulator of TF initiated coagulation and is mainly synthesized by endothelial cells and megakaryocytes. TFPI is secreted or associated with the endothelial layer via heparan sulfate proteoglycans or a glycosyl phosphatidyl inositol anchor [14]. In plasma approximately 80% is bound to lipoproteins although a minor fraction is free. Both forms, in addition to full-length and truncated molecules are measurable in plasma and in particular total TFPI antigen is thought to be a marker of endothelial damage in arterial diseases [15].

Pro-coagulant activity in the young with type 1 diabetes has received little attention. The aims of the present study were to 1) compare pro-coagulant activity between children and adolescents with type 1 diabetes and healthy control subjects, at inclusion and five years after and 2) determine its association with selected glucose related variables and cardiovascular risk factors. Our hypothesis was increased pro-coagulant activity in children and adolescents with type 1 diabetes, in association with glucose related variables and cardiovascular risk factors.

Materials & Methods

Study population

The total study cohort (n= 434) consisted of subjects with type 1 diabetes (n=314) and healthy controls (n= 120), aged 8-18 years from the Norwegian Atherosclerosis and Childhood Diabetes (ACD) Study [16]. An invitation letter was sent to the subjects with type 1 diabetes registered in the Norwegian Childhood Diabetes Registry (NCDR) in the South East health region of Norway (n= 800) with a response rate of

40%. The type 1 diabetes diagnosis was set according to the American Diabetes Association and International Society of Pediatric and Adolescent Diabetes criteria and the cohort is highly representative for all children with type 1 diabetes in Norway [16]. Approximately all of the diabetic subjects (97%) were on intensive insulin treatment from the time of diagnosis, using either insulin pumps or basal-bolus regimens with > four daily insulin injections. None had micro- or macrovascular complications at inclusion. The healthy controls were primarily classmates and in a few cases relatives of the subjects with type 1 diabetes. The cohort has previously been described in detail [16]. All participants, and parents of those below 18 years of age, signed written informed consent to participate. The National Committee for Research Ethics in Norway and the Norwegian Social Science Data Services approved the study protocols and the trial was conducted according to the Declaration of Helsinki. The participants were enrolled at the pediatric department, Oslo University Hospital, Ullevaal between 2006- 2008 and encouraged to a follow-up after five years (2011-2013) with a total attendance of 346 subjects (type 1 diabetes (n= 249) and controls (n= 97)).

Laboratory measurements

Fasting blood samples were drawn between 07.30 and 10.00 am at inclusion and at the five-year follow-up. Tubes containing 3.8% sodium citrate were placed on ice and separated by centrifugation at 2500 x g for 20 min at 4° C and stored at - 80° C until analysis. Tissue factor procoagulant activity (TF-PCA) was assessed by a chromogenic assay (Actichrome® TF, Ref 843, Sekisui Diagnostics, Stamford, CT, USA). Total TFPI, recognizing full-length, truncated and TFPI bound to lipoproteins and D-dimer were analyzed by enzyme linked immunosorbent assays (ELISA) (Asserachrom® TOTAL TFPI and D-DI, Diagnostica Stago, Asniéres, France), respectively). Prothrombin fragment 1+2 (F1+2) was assessed by Enzygnost® F1+2 (monoclonal) (Siemens, Marburg, Germany). Inter-assay coefficients of variation (CVs) in our laboratory were 17.9%, 6.2%, 6.7% and 15.6%, respectively. Routine laboratory analyses were determined by conventional methods and arterial blood pressure was performed according to the National High Blood Pressure Educational Program Working Group on High Blood Pressure in Children and

Adolescents [17]. HbA_{1c} was assessed at a Diabetes Control and Complications Trial- standardized laboratory by high pressure liquid chromatography (Bio-Rad, Richmond, CA, USA), CV < 3%. The NCDR provided annual HbA_{1c} values for almost all patients and glycemic burden was calculated by a modified version of cumulative glycemic exposure (A_1 months) [16] first described by Orchard et al [8]. Briefly, HbA_{1c} units above the upper normal reference value (46 mmol/mol (6.4%) were multiplied by the number of months from the type 1 diabetes diagnosis until the first reported HbA_{1c} value and added to the number of months between the first and second reported values, multiplied by HbA_{1c} units above the upper normal reference value of the second reported value, and so further. Urinary albumin-creatinine ratio (U-ACR) was performed in spot urine.

Statistical methods

The statistical calculations were performed by IBM© SPSS© statistics for windows, v 25.0 (IBM Corp., New York, NY, USA). Continuous data were predominantly skewed and reported as median values (25^{th}, 75^{th} percentiles) if not otherwise stated. Categorical data are presented as number or proportions. Bivariate correlations were performed by Spearman's rho. Group comparisons were determined by Mann-Whitney U Test, Independent Student T Test and Chi-square, as appropriate. Kruskal Wallis test was used to compare more than two groups. To calculate changes within groups at the five-year follow-up, the Wilcoxon Signed Rank Test was applied. Correlation analyses were adjusted for covariates with partial correlation using transformed values (natural logarithm) for non-parametric variables. Multivariate linear regression models were applied to adjust group differences for covariates and to explore if any baseline factors could predict changes over time in our variables of interest. The STROBE guidelines were followed [18]. A p-value ≤ 0.05 was considered statistically significant.

Results

Baseline characteristics of the diabetes and healthy control groups are shown in **Table 1** and have also previously been published. [16] At inclusion, the median duration of disease in the type 1 diabetic

population was five years and they had significantly higher levels of HbA1c, anthropometric measures, lipid levels and CRP, compared to the control group.

At the five-year follow-up the participation rate was 80%. Some of the participants had moved away to work or for educational reasons and others were weary of their disease and did not want to attend the follow-up. Similar differences were present, however, triglycerides and U-ACR were significantly increased in the type 1 diabetes group, while systolic blood pressure and high density lipoprotein cholesterol did not differ (**Table 2)**.

Thrombotic markers in type 1 diabetes versus controls (Table 3)

Children and adolescents with type 1 diabetes had significantly higher levels of TFPI compared with controls, both at inclusion and at the five-year follow-up ($p<0.001$, both). Adjustments for differences in clinical characteristics did not change the results. F1+2, D-dimer and TF-PCA did not differ between the groups at any time point.

During the five-year follow-up TFPI decreased within the controls ($p< 0.001$) and the change (delta) was significantly different from the type 1 diabetic children ($p= 0.004$) who remained unchanged. TF-PCA increased within both groups ($p <0.001$, both) and D-dimer decreased within the diabetes group ($p<0.001$), however, delta values were not significantly different from controls ($p= 0.271$).

In the type 1 diabetes group, correlation analyses between changes in (delta values of) traditional cardiovascular risk factors and pro-coagulant markers showed change in CRP to be significantly correlated to changes in F1+2 and D-dimer ($r= 0.199$ and $r= 0.267$, respectively, $p< 0.005$, both). Changes in total and LDL cholesterol were furthermore significantly correlated to change in TFPI ($r= 0.373$ and $r= 0.339$, respectively, $p< 0.0005$, both). None of the clinical characteristics at inclusion (BMI, LDL, SBP, CRP and HbA1c) predicted any changes over time in our measured markers (data not shown). The number of missing cases analysed was similar in both groups at both time points (3-5%) and was due to restricted amount of citrated plasma.

Correlations between thrombotic markers and glucose related variables in the type 1 diabetes group (Table 4)

Levels of TFPI correlated significantly to HbA_{1c} both at inclusion and at the five-year follow-up (r= 0.221 and r= 0.304, p< 0.001, both), even after adjusting for covariates (BMI, LDL, SBP and CRP) (p< 0.005) **(Supplemental Figure 1a and b).** With HbA_{1c} divided into quartiles, significant differences across quartiles at both time points was revealed (p= 0.004 and p< 0.001, respectively) and subjects with type 1 diabetes with HbA_{1c} in the upper quartile (>75 mmol/mol (9%) at inclusion / 84 mmol/mol (9.8%) at five years) had an odds ratio (OR) of 2.1 (95% confidence interval (CI) 1.2, 3.8), p= 0.018 and 5.4 (95% CI 2.8, 10.2), p< 0.001, respectively, for having higher TFPI levels compared to the three lower quartiles (Q1-Q3) (**Figure 1.)**. TFPI also had a weak correlation to U-ACR at the five-year follow-up.

TF-PCA and F1+2 showed no relationship to either HbA_{1c}, A_1months, years of diabetes or U-ACR at any time point, whereas D-dimer correlated weakly to years of diabetes at inclusion, however, that correlation was not present at the five-year follow-up.

Relationship between thrombotic markers and cardiovascular risk factors in the type 1 diabetes group (Table 5)

TFPI was significantly lower in females compared to males at both time points, also after adjusting for relevant covariates (use of OC, CRP, BMI and LDL) (p= 0.018 and p= 0.010, respectively). F1+2, D-dimer and TF-PCA did not differ between sexes at inclusion, but at the five-year follow-up females had higher levels of F1+2 (p= 0.052). As previous data from the same cohort demonstrated elevated levels of CRP in females, regardless of OC use, [19] the given p- value was adjusted for use of OC and CRP. A similar pattern was observed in the total population and in the control group alone (data not shown).

Females with type 1 diabetes using OC had significantly higher F1+2 levels compared to females not using OC at inclusion (p= 0.002), these findings were still significant at the five-year follow-up (p< 0.001). After five years TF-PCA and D-dimer were also elevated (p< 0.001 and p= 0.004, respectively), while TFPI levels were lower (p<0.001). SBP below the median (< 100 mmHg at inclusion and < 110 mmHg at follow-up) was significantly associated with higher levels of D-dimer at both time points (p= 0.045 and p= 0.001, respectively). Body mass index (BMI) above the median (> 20.2 kg/m^2 at inclusion / > 23.2 kg/m^2 at follow-up) was significantly associated with higher F1+2, although only at inclusion (p= 0.043). Low density lipoprotein cholesterol (LDL) above the mean (> 2.48 mmol/L at inclusion/ > 2.69 mmol/L at follow-up) was associated with higher levels of F1+2 (p= 0.034) and TFPI (p< 0.001) at inclusion, the latter was also significantly associated after five years (p<0.001). It should be emphasized that LDL and TFPI was significantly inter-correlated (r= 0.372 and 0.375, respectively, p< 0.001, both).

Discussion

Children and adolescents with five years duration of type 1 diabetes have signs of hyperlipidaemia and inflammation as well as higher BMI and suboptimal glycemic control, compared with healthy control subjects. The differences persisted during the five-year follow-up, in which also a slight rise in U-ACR in the children and adolescents with type 1 diabetes was noted.

The main findings of the present study were comparable levels of F1+2, D-dimer and TF-PCA between the study groups and significantly higher TFPI levels in the children and adolescents with type 1 diabetes at both time points. TFPI associated with glycemic control, and hyperglycemia significantly increased the risk for having higher TFPI levels. Furthermore, females with type 1 diabetes had a more hypercoagulable profile than men, also when taking the use of OC and inflammatory status into consideration.

Cardiovascular risk factors were present in our cohort of children and adolescents with type 1 diabetes as previously mentioned [5], and the change in CRP during the follow-up was positively related to changes in F1+2 and D-dimer. CRP is an inflammatory mediator and a well-known predictor of CVD and might

represent progression of atherosclerosis with subsequent activation of the coagulation system [20]. The observed proportional relationship between increasing lipid levels and TFPI (total) is well in line with accepted knowledge [21].

Despite the established association between vascular diseases and hypercoagulability in adults with type 1 diabetes, markers of thrombin generation and TF-PCA did not differ between the type 1 diabetes individuals and healthy controls in the present cohort. In accordance with our results, a case-control study (n= 150) investigating haemostasis in children and adolescents with type 1 diabetes vs controls, showed similar levels of F1+2 in both groups, and lower levels of thrombin-antithrombin complex in the diabetes group, supporting no ongoing thrombin generation [22]. The same conclusion, with no differences in clotting parameters was also drawn in a study comparing children with diabetes with healthy controls (n= 70) [23]. In contrast, hypercoagulability was observed in diabetic children (n= 73) by shorter clot formation time and stronger clot firmness by a ROTEM assay. However, no differences in fibrinogen and D-dimer were observed [24].

In people with type 1 diabetes, blood cells and the endothelial layer are exposed to chronic hyperglycaemia, which leads to a pro-inflammatory state with hyper-reactive platelets, glycation of proteins and accelerated progression from endothelial dysfunction to atherosclerosis [25]. The expression of TF has shown variable results regarding response to high glucose [26,27], and we found neither TF-PCA nor markers of thrombin generation to be associated with long-term glycaemic control. However, whether daily glucose fluctuations influence pro-coagulant activity we cannot answer. It has previously been published from this cohort, that the degree of atherosclerosis (carotid intima media thickness, cIMT) was only slightly increased in the diabetes group, [28] which may explain the current results. Ten years of disease duration is relatively short, and at this low age children and adolescents are free from longstanding exposure from other vascular risk factors, demonstrated in our cohort by the absence of microvascular complications.

We found the diabetes children to have significantly elevated TFPI levels, which related to hyperglycaemia. The relationship was further confirmed by the increased risk of having high TFPI levels with poorly controlled disease. Optimisation of blood glucose control has previously been shown to normalize TFPI activity in young adults with type 1 diabetes [29]. Elevated levels of TFPI in adults with type 1 diabetes with microalbuminuria was described already back in 1997, and vascular damage with changes in glycosaminoglycans was proposed to explain the results [30]. Since then, several reports have shown hyperglycaemia to degrade the protective endothelial glycocalyx, and in particular heparan sulfate which is a prominent binding site for TFPI [31-33]. In concordance with our results, a cross-sectional study on children with type 1 diabetes (n= 155) demonstrated an association between dysglycaemia and levels of von Willebrand Factor (vWF), another important regulator of coagulation and marker of endothelial dysfunction [34]. In contrast, an Italian study demonstrated endothelial perturbation (tissue-plasminogen activator and vWF) to be correlated to markers of inflammation rather than HbA1c, which might be explained by the short duration of type 1 diabetes and small number in that study. However, the authors observed a reversion of endothelial perturbation in almost half of the patients after one year [35].

Hyperglycaemia has downstream effects like generation of reactive oxygen species, advanced glycation end products and protein kinase C activation, inducing vascular inflammation and endothelial dysfunction, accelerating the risk for micro- and macrovascular disease [36]. Although the children and adolescents with type 1 diabetes in our cohort were free from moderate albuminuria, the U-ACR was significantly elevated and correlated to TFPI in the diabetes children after ten years of disease, potentially an early sign of angiopathy. Elevated levels of TFPI in the present study may thus be a consequence of endothelial activation/dysfunction rather than a compensatory action following TF-induced coagulation. In similar, an association between total TFPI, markers of endothelial activation and subclinical atherosclerosis has also been shown in an adult population, free of CVD [15].

Several reports have demonstrated increased relative risk of vascular mortality in women with diabetes compared with men, although the precise mechanism is not known [4,37]. Our data, with lower levels of TFPI

accompanied by increased F1+2 in females with type 1 diabetes supports a pro-thrombotic milieu, which might lead to vascular disease. Type 1 diabetes affects both glucose- and lipid-metabolism [38] and an atherogenic profile dominates in females [39]. These findings were also confirmed in our study with significantly higher levels of LDL, BMI and CRP in the females with type 1 diabetes compared with men (data not shown).

Use of OC has been associated with increased risk of both arterial and venous thrombosis [40]. In the present study, females with diabetes using OC had signs of in vivo thrombin generation with higher levels of F1+2 already at a mean age of 13.7 years compared to females not using OC. Five years later our female subjects using OC still had significantly higher F1+2, in addition to elevated D-dimer and TF-PCA and lower levels of TFPI, compared to non-users. In subjects using OC in general, reduction in TFPI and Protein C levels have been shown in several studies and might be the most important mechanism of accelerated thrombosis in these women [40-42].

The higher levels of D-dimer with SBP below median in the diabetes cohort are not easily explainable. It should nevertheless be noted that both SBP and D-dimer levels were within a normal range. It has previously been reported on a positive relationship between blood pressure and D-dimer, however, in a cohort of patients with diabetic microvascular complications [43], not present in our diabetes cohort.

The metabolic changes and chronic inflammation present in obese patients have been shown to associate with a pro-thrombotic shift and accelerated risk of CVD [44]. In accordance, we could demonstrate F1+2 to be slightly elevated at inclusion, in the diabetes children with BMI above the median. Although BMI increased during the follow-up period, no differences in pro-coagulant activity were, however, observed five years later.

In our cohort with type 1 diabetes LDL levels above mean were associated with higher levels of TFPI and F1+2, somewhat in line with Morange et al who found total TFPI to be related to conventional CVD risk factors [45]. Oxidized LDL is an excellent trigger of vascular inflammation and atherothrombosis [46] and is

shown to have pro-coagulatory effects on vascular cells like platelets and endothelial cells [47]. The rise in TFPI might be explained as a compensatory mechanism to the pro-coagulant state and/or the thrombin generation, but may also be due to the fact that TFPI in the circulation is mainly bound to LDL, also reflected in the correlation shown between TFPI and LDL levels. As, previously discussed, children and adolescents with type 1 diabetes are susceptible to endothelial dysfunction with damages to the proteoglycans harboring coagulation factors like TFPI.

The strengths of the current research is the prospective design and large cohort of children and adolescents with type 1 diabetes, representative for all children and adolescents with type 1 diabetes in Norway, together with healthy control subjects at similar age. To the best of our knowledge, this is the largest study investigating pro-coagulant activity in children and adolescents with type 1 diabetes.

The study has some limitations. The relative short follow-up period might be insufficient in these young subjects for development of pro-thrombotic activity and implementation of additional biomarkers reflecting endothelial perturbation and/or platelet activity, might have strengthened the study. SolubleTF assays are debated especially regarding specificity. We utilized the ACTICHROME®, a chromogenic activity assay to quantitate TF procoagulant activity, which has been criticized for falsely elevated results [48]. Further, activity- compared to antigen assays, are hampered with larger variation, also seen in our coefficient of variation. We also lack information regarding types of OC used.

In conclusion, the current study shows similar pro-coagulant activity in children and adolescents with type 1 diabetes compared with healthy controls at similar age over a five-year period, indicating that type 1 diabetes children using intensified insulin treatment with either multiple (> four) daily insulin injections or insulin pumps, are not at high thrombotic risk at this younger age. However, females with diabetes showed accelerated pro-thrombotic activity compared to males, also when taking the use of OC into consideration. The higher plasma levels of TFPI in the diabetes children and adolescents compared with controls, which also related to hyperglycemia, are probably reflecting increased endothelial activation.

Our findings underscore the significance of children and adolescents with type 1 diabetes to aspire optimal blood glucose control to maintain a healthy and functional endothelium and the burden of CV risk factors needs attendance also with regard to pro-coagulant activity, especially in female subjects.

Data availability

Data supporting the present results are available on request.

Duality of interest

None declared.

References

1. Skrivarhaug T, Stene LC, Drivvoll AK, Strom H, Joner G. Incidence of type 1 diabetes in Norway among children aged 0-14 years between 1989 and 2012: has the incidence stopped rising? Results from the Norwegian Childhood Diabetes Registry. *Diabetologia.* 2014;57(1):57-62.
2. Grant PJ. Diabetes mellitus as a prothrombotic condition. *J Intern Med.* 2007;262(2):157-172.
3. Rawshani A, Sattar N, Franzen S, et al. Excess mortality and cardiovascular disease in young adults with type 1 diabetes in relation to age at onset: a nationwide, register-based cohort study. *Lancet.* 2018;392(10146):477-486.
4. Huxley RR, Peters SA, Mishra GD, Woodward M. Risk of all-cause mortality and vascular events in women versus men with type 1 diabetes: a systematic review and meta-analysis. *Lancet Diabetes Endocrinol.* 2015;3(3):198-206.
5. Margeirsdottir HD, Larsen JR, Brunborg C, Overby NC, Dahl-Jorgensen K. High prevalence of cardiovascular risk factors in children and adolescents with type 1 diabetes: a population-based study. *Diabetologia.* 2008;51(4):554-561.
6. Hamilton SJ, Watts GF, Hamilton SJ. Endothelial dysfunction in diabetes: pathogenesis, significance, and treatment. *Rev Diabet Stud.* 2013:133-156.
7. Gubitose-Klug RA LJ, Backlund J-YC, et al. Intensive Diabetes Treatment and Cardiovascular Outcomes in Type 1 Diabetes: The DCCT/EDIC Study 30-Year Follow-up. *Diabetes Care.* 2016;39(5):686-693.
8. Orchard TJ, Forrest KY, Ellis D, Becker DJ. Cumulative glycemic exposure and microvascular complications in insulin-dependent diabetes mellitus. The glycemic threshold revisited. *Arch Intern Med.* 1997;157(16):1851-1856.
9. Brown JB, Nichols GA, Perry A. The burden of treatment failure in type 2 diabetes. *Diabetes Care.* 2004;27(7):1535-1540.
10. Ceriello A. Hyperglycaemia and the vessel wall: the pathophysiological aspects on the atherosclerotic burden in patients with diabetes. *Eur J Cardiovasc Prev Rehabil.* 2010:s15-s19.
11. Lemkes BA, Hermanides J, Devries JH, Holleman F, Meijers JC, Hoekstra JB. Hyperglycemia: a prothrombotic factor? *J Thromb Haemost.* 2010;8(8):1663-1669.

12. Grover PS, Mackman PN. Tissue Factor: An Essential Mediator of Hemostasis and Trigger of Thrombosis. *Arterioscler Thromb Vasc Biol.* 2018;38(4):709-725.
13. Tatsumi K, Mackman N. Tissue Factor and Atherothrombosis. *J Atheroscler Thromb.* 2015;22(6):543-549.
14. Wood JP, Ellery PE, Maroney SA, Mast AE. Biology of tissue factor pathway inhibitor. *Blood.* 2014;123(19):2934-2943.
15. Mitchell CT, Kamineni A, Palmas W, Cushman M. Tissue factor pathway inhibitor, vascular risk factors and subclinical atherosclerosis: the Multi-Ethnic Study of Atherosclerosis. *Atherosclerosis.* 2009;207(1):277-283.
16. Margeirsdottir HD, Stensaeth KH, Larsen JR, Brunborg C, Dahl-Jorgensen K. Early signs of atherosclerosis in diabetic children on intensive insulin treatment: a population-based study. *Diabetes Care.* 2010;33(9):2043-2048.
17. Libby P, Nathan DM, Abraham K, et al. Report of the National Heart, Lung, and Blood Institute-National Institute of Diabetes and Digestive and Kidney Diseases Working Group on Cardiovascular Complications of Type 1 Diabetes Mellitus. *Circulation.* 2005;111(25):3489-3493.
18. von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies. *Int J Surg.* 2014;12(12):1495-1499.
19. Heier M, Margeirsdottir HD, Brunborg C, Hanssen KF, Dahl-Jorgensen K, Seljeflot I. Inflammation in childhood type 1 diabetes; influence of glycemic control. *Atherosclerosis.* 2015;238(1):33-37.
20. Verma S, Szmitko PE, Ridker PM. C-reactive protein comes of age. *Nat Clin Pract Cardiovasc Med.* 2005;2(1):29-36; quiz 58.
21. Hansen JB, Huseby KR, Huseby NE, Sandset PM, Hanssen TA, Nordoy A. Effect of cholesterol lowering on intravascular pools of TFPI and its anticoagulant potential in type II hyperlipoproteinemia. *Arterioscler Thromb Vasc Biol.* 1995;15(7):879-885.
22. Cimenti C, Schlagenhauf A, Leschnik B, et al. Only minor changes in thrombin generation of children and adolescents with type 1 diabetes mellitus - A case-control study. *Thromb Res.* 2016;148:45-49.
23. Zeitler P, Thiede A, Muller HL. Prospective study on plasma clotting parameters in diabetic children--no evidence for specific changes in coagulation system. *Exp Clin Endocrinol Diabetes.* 2001;109(3):146-150.
24. Binay C, Bozkurt Turhan A, Simsek E, Bor O, Akay OM. Evaluation of Coagulation Profile in Children with Type 1 Diabetes Mellitus Using Rotational Thromboelastometry. *Indian J Hematol Blood Transfus.* 2017;33(4):574-580.
25. Sena CM, Pereira AM, Seica R. Endothelial dysfunction - a major mediator of diabetic vascular disease. *Biochim Biophys Acta.* 2013;1832(12):2216-2231.
26. Singh A, Boden G, Rao AK. Tissue factor and Toll-like receptor (TLR)4 in hyperglycaemia-hyperinsulinaemia. Effects in healthy subjects, and type 1 and type 2 diabetes mellitus. *Thromb Haemost.* 2015;113(4):750-758.
27. Singh A, Boden G, Homko C, Gunawardana J, Rao AK. Whole-blood tissue factor procoagulant activity is elevated in type 1 diabetes: effects of hyperglycemia and hyperinsulinemia. *Diabetes Care.* 2012;35(6):1322-1327.
28. Heier M, Margeirsdottir HD, Gaarder M, et al. Soluble RAGE and atherosclerosis in youth with type 1 diabetes: a 5-year follow-up study. *Cardiovasc Diabetol.* 2015;14:126.
29. Rigla M, Mateo J, Fontcuberta J, Souto JC, de Leiva A, Perez A. Normalisation of tissue factor pathway inhibitor activity after glycaemic control optimisation in type 1 diabetic patients. *Thromb Haemost.* 2000;84(2):223-227.

30. Leurs PB, van Oerle R, Wolffenbuttel BH, Hamulyak K. Increased tissue factor pathway inhibitor (TFPI) and coagulation in patients with insulin-dependent diabetes mellitus. *Thromb Haemost.* 1997;77(3):472-476.
31. Nathan DM, Lachin J, Cleary P, et al. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. *N Engl J Med.* 2003;348(23):2294-2303.
32. Nieuwdorp M, van Haeften TW, Gouverneur MC, et al. Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo. *Diabetes.* 2006;55(2):480-486.
33. Kato H. Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: basic and clinical aspects. *Arterioscler Thromb Vasc Biol.* 2002;22(4):539-548.
34. Kyrgios I, Maggana I, Giza S, et al. Suboptimal glycaemic control enhances the risk of impaired prothrombotic state in youths with type 1 diabetes mellitus. *Diab Vasc Dis Res.* 2014;11(3):208-216.
35. Romano M, Pomilio M, Vigneri S, et al. Endothelial perturbation in children and adolescents with type 1 diabetes: association with markers of the inflammatory reaction. *Diabetes Care.* 2001;24(9):1674-1678.
36. Pechlivani N, Ajjan RA. Thrombosis and Vascular Inflammation in Diabetes: Mechanisms and Potential Therapeutic Targets. *Front Cardiovasc Med.* 2018;5:1.
37. Gnatiuc L, Herrington W, Halsey J, et al. Sex-specific relevance of diabetes to occlusive vascular and other mortality: a collaborative meta-analysis of individual data from 980 793 adults from 68 prospective studies. *Lancet Diabetes Endocrinol.* 2018;6(7):538-546.
38. Mostofizadeh N, Hashemipour M, Roostazadeh M, Hashemi-Dehkordi E, Shahsanai A, Reisi M. The impact of poor glycemic control on lipid profile variables in children with type 1 diabetes mellitus. *J Educ Health Promot.* 2019;8:6.
39. Collier A, Ghosh S, Hair M, Waugh N. Gender differences and patterns of cardiovascular risk factors in Type 1 and Type 2 diabetes: a population-based analysis from a Scottish region. *Diabet Med.* 2015;32(1):42-46.
40. Sandset PM. Mechanisms of hormonal therapy related thrombosis. *Thromb Res.* 2013;131 Suppl 1:S4-7.
41. Raps M, Helmerhorst FM, Fleischer K, et al. The effect of different hormonal contraceptives on plasma levels of free protein S and free TFPI. *Thromb Haemost.* 2013;109(4):606-613.
42. van Vliet HA, Bertina RM, Dahm AE, et al. Different effects of oral contraceptives containing different progestogens on protein S and tissue factor pathway inhibitor. *J Thromb Haemost.* 2008;6(2):346-351.
43. El Asrar MA, Adly AA, El Hadidy ES, Abdelwahab MA. D-dimer levels in type 1 and type 2 diabetic children and adolescents; Relation to microvascular complications and dyslipidemia "own data and review". *Pediatr Endocrinol Rev.* 2012;9(3):657-668.
44. Blokhin IO, Lentz SR. Mechanisms of thrombosis in obesity. *Curr Opin Hematol.* 2013;20(5):437-444.
45. Morange PE, Renucci JF, Charles MA, et al. Plasma levels of free and total TFPI, relationship with cardiovascular risk factors and endothelial cell markers. *Thromb Haemost.* 2001;85(6):999-1003.
46. Obermayer G, Afonyushkin T, Binder CJ. Oxidized low-density lipoprotein in inflammation-driven thrombosis. *J Thromb Haemost.* 2018;16(3):418-428.
47. Ohkura N, Hiraishi S, Itabe H, et al. Oxidized phospholipids in oxidized low-density lipoprotein reduce the activity of tissue factor pathway inhibitor through association with its carboxy-terminal region. *Antioxid Redox Signal.* 2004;6(4):705-712.

48. Bogdanov VY, Cimmino G, Tardos JG, Tunstead JR, Badimon JJ. Assessment of plasma tissue factor activity in patients presenting with coronary artery disease: limitations of a commercial assay. *J Thromb Haemost.* 2009;7(5):894-897.

Table 1. Baseline characteristics according to the study groups (median (25, 75percentiles))

	Type 1 diabetes (n= 314)	**Controls (n= 120)**	**p**
Age (years)†	13.7 (8, 20)	13.2 (9, 20)	0.092
Females, n (%)	158 (50.3)	68 (56.7)	0.282
Years of Diabetes	5.0 (2.8, 7.7)		
HbA1c, mmol/mol	67 (60, 75)	34 (32, 37)	< 0.001
%	8.3 (7.6, 9.0)	5.3 (5.1, 5.5)	< 0.001
Insulin pump, n (%)	144 (53.1)		
Smokers, n (%)	9 (2.9)	2 (1.7)	0.712
Oral contraceptives, n (%)	16 (15.1)	7 (17.5)	0.919
BMI, kg/m^2	20.2 (18.0, 22.9)	18.8 (17.1, 20.9)	< 0.001
Weight (kg) †	54.6 (81.3)	47.7 (68.4)	< 0.001
Waist circumference (cm)	69.5 (64.5, 77.0)	65.6 (61.5, 70.8)	< 0.001
Height (cm)	162 (151, 170)	159 (145, 165)	0.012
SBP (mmHg)	100 (95, 110)	97 (92, 105)	0.005
DBP (mmHg)	60 (55, 65)	60 (54, 64)	0.026
Total cholesterol (mmol/L) †	4.61 (5.40)	4.32 (4.10)	0.001
LDL (mmol/L) †	2.48 (5.54)	2.31 (4.26)	0.027
HDL (mmol/L) †	1.78 (2.75)	1.69 (1.89)	0.047
TG (mmol/L)	0.66 (0.52, 0.89)	0.66 (0.45, 0.88)	0.357
S-creatinine (µmol/L)	52 (46, 59)	54 (46, 61)	0.150
U-ACR (mg/mmol)	0.80 (0.50, 1.40)	0.61 (0.36, 1.31)	0.157
CRP (mg/L)	0.51 (0.27, 1.83)	0.31 (0.19, 0.67)	<0.001

†Mean (range or SD)

BMI Body Mass Index, SBP Systolic Blood Pressure, DBP Diastolic Blood Pressure, LDL Low Density Lipoprotein Cholesterol, HDL High Density Lipoprotein Cholesterol, TG Triglycerides, U-ACR Urinary Albumin-Creatinine Ratio, CRP C-Reactive Protein, SD Standard Deviation

Table 2. Characteristics according to the study groups at five years follow-up (median (25, 75percentiles))

	Type 1 diabetes (n= 249)	**Controls (n= 97)**	**p**
Age† (years)	18.6 (13, 24)	18.1 (14, 25)	0.155
Females, n (%)	132 (53.0)	54 (55.7)	0.745
Years of Diabetes	9.7 (7.5, 12.8)		
HbA1c, mmol/mol	74 (65, 84)	33 (31, 36)	<0.001
%	8.9 (8.1, 9.8)	5.2 (5.0, 5.4)	<0.001
Insulin pump, n (%)	138 (61.1)		
Smokers, n (%)	8 (3.2)	2 (2.1)	0.828
Oral contraceptives, n (%)	51 (40.2)	25 (49.0)	0.361
BMI, kg/m^2	23.2 (21.3, 26.5)	22.1 (19.9, 23.9)	<0.001
Weight (kg) †	71.0 (14.9)	65.6 (12.0)	0.002
Waist (cm)	77 (73, 84)	74 (69, 79)	<0.001
Height (cm)	171 (164, 177)	172 (164, 177)	0.735
SBP (mmHg)	110 (105, 120)	110 (100, 115)	0.102
DBP (mmHg)	70 (65, 75)	65 (60, 70)	0.001
Total cholesterol (mmol/L) †	4.76 (0.99)	4.34 (0.94)	0.001
LDL (mmol/L) †	2.69 (0.79)	2.47 (0.79)	0.024
HDL (mmol/L) †	1.62 (0.46)	1.53 (0.37)	0.113
TG (mmol/L)	0.88 (0.64, 1.31)	0.80 (0.68, 0.99)	0.039
S-creatinine (µmol/L)	67 (59,75)	71 (64, 80)	0.001
U- ACR (mg/mmol)	0.60 (0.30, 1.50)	0.30 (0.20, 1.10)	0.003
CRP (mg/L)	1.17 (0.43, 3.67)	0.69 (0.28, 2.08)	0.006

†Mean (range or SD)

BMI Body Mass Index, SBP Systolic Blood Pressure, DBP Diastolic Blood Pressure, LDL Low Density Lipoprotein Cholesterol, HDL High Density Lipoprotein Cholesterol, TG Triglycerides, U-ACR Urinary Albumin-Creatinine ratio, CRP C-Reactive Protein, SD Standard Deviation

Table 3. Thrombotic markers in type 1 diabetes versus controls at inclusion and five years follow-up (median (25, 75percentiles))

	Inclusion			5 years follow-up			
	T1D (n= 314)	Controls (n= 120)	p_1	T1D (n= 249)	Controls (n= 97)	p_2	Δ p
F1+2 (pmol/L)	166 (129, 208)	151 (132, 206)	0.559	157 (123, 198)	155 (124, 207)	0.803	0.561
D-dimer (ng/ml)	230 (168, 302)	227 (175, 287)	0.815	199† (131, 286)	209 (152, 299)	0.161	0.271
TF-PCA (pM)	0.82 (0.41, 3.05)	0.72 (0.41, 2.13)	0.345	4.92† (2.31, 9.50)	4.08† (2.02, 10.12)	0.299	0.951
TFPI total (ng/ml)	78.5 (70.3, 92.8)	71.2 (60.3, 79.5)	< 0.001‡	78.9 (68.6, 90.3)	61.8† (54.6, 74.2)	< 0.001‡	0.004

p_1 refers to differences between groups at inclusion. p_2 refers to differences between groups at five years follow-up.

Δ p denotes differences in changes from inclusion to 5 year follow-up between the groups.

† denotes within group changes over the five year period

‡ p< 0.001 (adjusted for ln BMI, LDL, ln SBP and ln CRP)

T1D Type 1 Diabetes, F1+2 Prothrombin Fragment 1+2, TF-PCA Tissue Factor Procoagulant Activity, TFPI Tissue Factor Pathway Inhibitor, ln natural logarithm, BMI Body Mass Index, LDL Low Density Lipoprotein cholesterol, SBP Systolic Blood Pressure, CRP C-Reactive Protein

Table 4. Correlations between thrombotic markers and glucose related variables in the type 1 diabetes group

		Inclusion				5 years follow-up			
		TF-PCA	TFPI (total)	F1+2	D-dimer	TF-PCA	TFPI (total)	F1+2	D-dimer
HbA_{1c}	**r**	0.066	0.221	0.059	- 0.058	- 0.061	0.304	0.074	0.072
	p	0.253	< 0.001‡	0.309	0.321	0.351	< 0.001‡	0.256	0.268
A_1 Months†	**r**	0.057	0.081	0.028	-0.065	-	-	-	-
	p	0.341	0.179	0.637	0.283	-	-	-	-
Years of Diabetes	**r**	0.004	0.039	- 0.027	- 0.116	0.021	- 0.029	0.030	- 0.072
	p	0.952	0.501	0.639	0.044§	0.742	0.650	0.645	0.272
U-ACR	**r**	-0.077	0.040	0.036	0.082	0.077	0.128	0.015	0.047
	p	0.306	0.590	0.634	0.268	0.243	0.050§	0.822	0.479

r denotes coefficient of correlation (Spearman rho)

p denotes the significance level of the correlation

† Calculated only at inclusion

‡ p< 0.005 (adjusted for ln BMI, LDL, ln SBP and ln CRP)

§ p< 0.05 (adjusted for ln BMI, LDL, ln SBP and ln CRP)

TF-PCA Tissue Factor Procoagulant Activity, TFPI Tissue Factor Pathway Inhibitor, F1+2 Prothrombin Fragment 1+2, U-ACR Urinary Albumin-Creatinine ratio, BMI Body Mass Index, LDL Low Density Lipoprotein cholesterol, SBP Systolic Blood Pressure, CRP C-Reactive Protein

Table 5. Thrombotic markers as related to selected cardiovascular risk factors in the type 1 diabetes group (median (25, 75percentiles))

		Inclusion (n= 314)				**5 years follow-up (n= 249)**			
		TF-PCA (pM)	TFPI (total) (ng/ml)	F1+2 (pmol/L)	D-dimer (ng/ml)	TF-PCA (pM)	TFPI (total) (ng/ml)	F1+2 (pmol/L)	D-dimer (ng/ml)
Sex	Male	0.74 (0.36, 3.29)	78.9 (71.4, 94.6)	156 (127, 201)	225 (171, 290)	3.83 (1.32, 7.90)	79.6 (70.6, 92.4)	146 (120, 165)	167 (113, 256)
	Female	0.84 (0.46, 2.88)	77.9 (68.7, 91.8)	171 (131, 226)	242 (167, 308)	6.38 (2.98, 11.36)	77.5 (65.4, 89.8)	171 (130, 229)	209 (163, 301)
	p	*0.146*	*0.033*	*0.121*	*0.319*	*0.003*	*0.052*	*< 0.001*	*< 0.001*
	adj.	-	*0.018*†	-	-	*0.254* ‡	*0.010*†	*0.052* ‡	*0.189* ‡
OC	No	0.84 (0.42, 2.26)	77.2 (67.3, 91.7)	165 (122, 211)	225 (162, 287)	4.88 (2.80, 8.73)	81.9 (70.0, 90.3)	146 (117, 188)	198 (151, 272)
	Yes	1.41 (0.50, 7.29)	74.9 (63.9, 87.4)	277 (157, 319)	225 (163, 309)	9.88 (3.84, 14.13)	68.5 (62.0, 78.5)	208 (173, 305)	260 (176, 370)
	p	*0.104*	*0.413*	*0.002*	*0.606*	*0.004*	*< 0.001*	*<0.001*	*0.004*
SBP§	< median	0.92 (0.39, 3.38)	79.3 (69.8, 93.6)	167 (127, 203)	244 (171, 309)	5.98 (2.44, 10.09)	78.9 (69.0, 89.3)	161 (130, 209)	221 (163, 299)
	> median	0.75 (0.43, 2.56)	78.5 (70.2, 94.7)	166 (130, 217)	214 (163, 289)	4.69 (2.31, 8.35)	78.9 (68.2, 92.8)	154 (121, 186)	167 (116, 260)
	p	*0.470*	*0.767*	*0.412*	*0.045*	*0.386*	*0.988*	*0.126*	*0.001*
BMI§	< median	0.78 (0.40, 3.24)	79.6 (70.8, 94.4)	157 (125, 199)	244 (178, 299)	5.26 (2.41, 8.87)	79.5 (70.6, 90.2)	150 (122, 186)	199 (126, 285)
	>	0.82 (0.41, 2.63)	78.4 (69.0, 93.9)	171 (132, 230)	213 (160, 306)	5.01 (2.29, 9.71)	77.0 (66.6, 90.8)	163 (125, 210)	198 (133, 292)
	p	*0.911*	*0.439*	*0.043*	*0.075*	*0.986*	*0.322*	*0.109*	*0.731*
	< mean	0.76 (0.40, 2.26)	75.5 (67.0, 87.6)	156 (128, 196)	235 (171, 308)	5.21 (2.10, 8.94)	74 (64, 84)	156 (122, 187)	191 (127, 287)

LDL[§]	>	0.98 (0.42, 4.24)	86.8 (75.0, 102.4)	173 (130, 217)	225 (166, 301)	4.96 (2.81, 10.16)	84 (71, 96)	163 (124, 206)	205 (137, 289)
	p	*0.170*	*<0.001*	*0.034*	*0.516*	*0.212*	*<0.001*	*0.222*	*0.625*

p refer to differences between groups

† adjusted for use of OC, ln CRP, ln BMI and LDL

ǂ adjusted for use of OC and ln CRP

§ median SBP at inclusion: 100 mmHg and median SBP at five years: 110mmHg, median BMI at inclusion: 20.2 kg/m^2 and median BMI at five years: 23.2 kg/m^2 and mean LDL at inclusion: 2.48 mmol/L and mean LDL at five years: 2.69 mmol/L

OC Oral Contraceptives, SBP Systolic Blood Pressure, BMI Body Mass Index, LDL Low Density Lipoprotein Cholesterol, TF-PCA Tissue Factor Procoagulant Activity, TFPI Tissue Factor Pathway Inhibitor, F1+2 Prothrombin Fragment 1+2, adj. adjusted, ln natural logarithm, CRP C-Reactive Protein

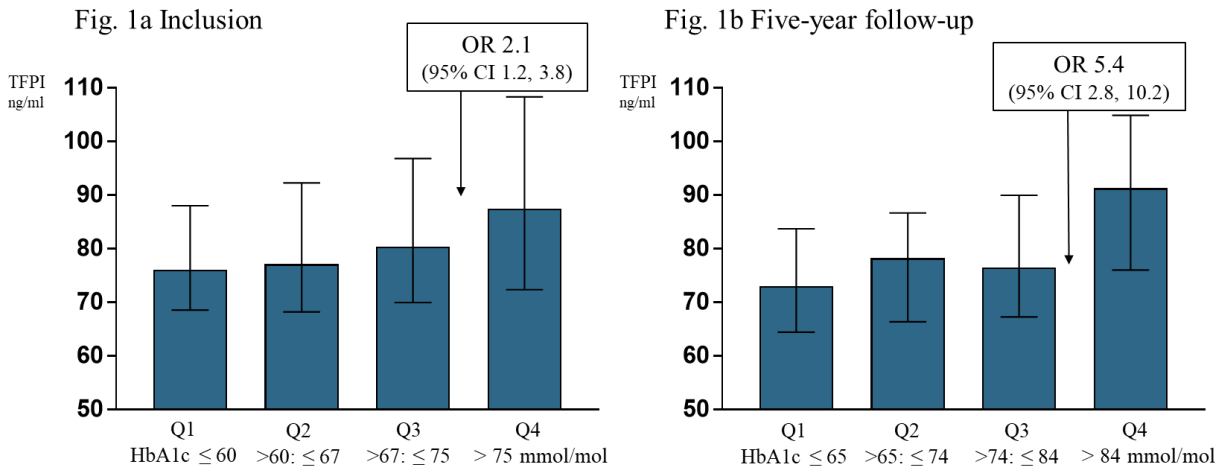
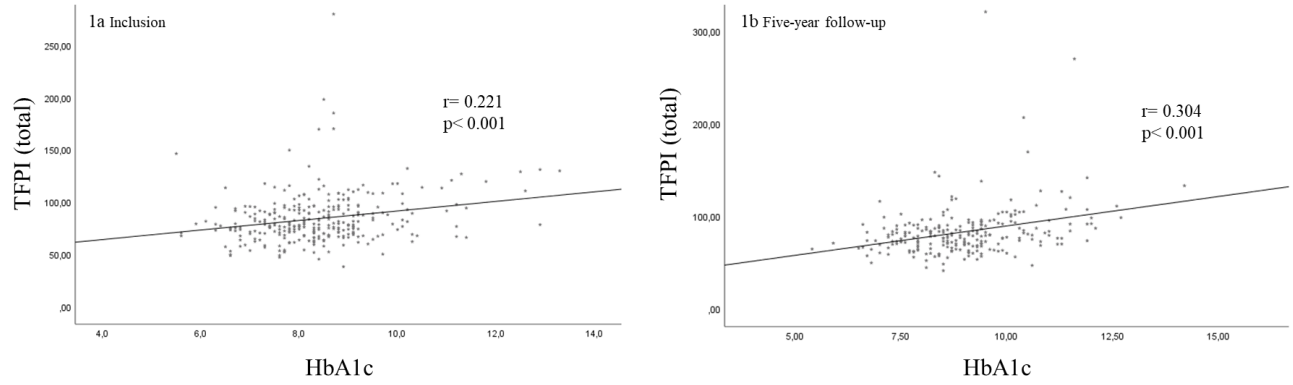


Figure 1. Levels of tissue factor pathway inhibitor (TFPI) in quartiles of HbA_{1c} in the type 1 diabetes group, at inclusion (Fig. 1a) and at the five-year follow-up (Fig. 1b). Type 1 diabetes children and adolescents with HbA_{1c} in quartile 4 had significantly higher levels of TFPI than quartile1-3, p= 0.005 and p< 0.001 and their odds ratio (OR) for having higher TFPI levels are 2.1 with a 95% confidence interval (CI) (1.2, 3.8), p= 0.018 and 5.4 CI (2.8, 10.2), p< 0.001.



Supplemental Figure 1a and b. Scatterplots for HbA1c and TFPI (total) at inclusion and five-year follow-up in the type 1 diabetes group

r denotes the coefficient of correlation (Spearman)

p indicates the significance level

Abbreviation: TFPI Tissue Factor Pathway Inhibitor