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Association of serum angiopoietin-like protein 2 with elevated risk of cardiovascular diseases in subjects with type 2 diabetes☆

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ABSTRACT

Aims: Although previous data have suggested ANGPTL2 and ANGPTL8 (betatrophin) to be related to atherosclerosis in humans, little is known whether this applies in patients with type 2 diabetes (T2D). In this work, we investigate association of serum ANGPTL2 and betatrophin with the risk of cardiovascular diseases (CVD) in T2D patients.

Methods: We measured serum levels of ANGPTL2 and betatrophin in 150 T2D patients with and without CVD and in 100 control subjects.

Results: Serum ANGPTL2 was significantly higher in T2D patients than in controls ($p < 0.0001$), and in T2D + CVD patients than T2D only patients ($p = 0.0002$). Serum betatrophin was lower in T2D patients than in controls but with no statistical significance ($p = 0.07$). Elevated serum ANGPTL2 associated with 2.83-fold increased risk of T2D and with 1.18-fold elevated risk of CVD among T2D patients with positive correlations with markers of hyperglycemia, insulin resistance and atherogenic lipid profile. ROC curve indicated ANGPTL2 as risk biomarker for T2D and CVD with sensitivity of 92.2% and 86%; and specificity of 86.7% and 58%; respectively.

Conclusion: We indicate for the first time serum ANGPTL2 as an independent risk biomarker for CVD in T2D patients. Future studies are needed to reveal its role in disease pathogenesis.

1. Introduction

Cardiovascular disease (CVD) is the most common cause of morbidity and mortality among subjects with type 2 diabetes (T2D).[1] In several studies, lipoprotein- related mechanisms have been associated with damage to the cardiovascular system and were linked to elevated risk of CVD in T2D.[2–6]

Angiopoietin-like proteins (ANGPTLs) is a family of proteins structurally similar to angiopoietins and encoded by eight genes, *ANGPTL1–8*. A subset of ANGPTLs has been reported to function in glucose metabolism, and to play major roles in lipid trafficking and metabolism.[7,8] ANGPTL2 is a novel proinflammatory cytokine primarily secreted by adipose tissues, also vascular endothelial cells and monocytes/macrophages secrete this protein.[9,10] ANGPTL2 has been known to regulate angiogenesis similarly to several other ANGPTLs,[11] in addition, ANGPTL2 has the unique capacity to induce an inflammatory response in blood vessels and adipose tissue,[9,10] and several experimental studies have suggested the biological relevance of ANGPTL2 as a regulator of atherosclerosis.[11–13]

There have been conflicting results regarding the role of ANGPTL2 in glucose metabolism. In a previous study on non-obese mice, ANGPTL2 overexpression in adipose tissue was associated with local inflammation and systemic insulin resistance, while ANGPTL2 deletion ameliorated adipose tissue inflammation and improved the insulin sensitivity.[10] However, in another study, replenishment of ANGPTL2 in mice was associated with stimulated insulin sensitivity and improved glucose tolerance.[14] Although previous data support the hypothesis that serum ANGPTL2 is strongly related to atherosclerosis in humans,[9,10] little is known whether this role applies in purely T2D. To our knowledge, no previous studies have studied the association of serum ANGPTL2 with the risk of CVD in T2D.

On the other hand, ANGPTL8 (alternatively referred as betatrophin) is a novel hormone derived from liver and adipose tissue and was found to stimulate beta-cell proliferation in animal models.[15] It also regulates triglycerides (TGs) and total cholesterol (TC) metabolism through the inhibition of lipoprotein lipase (LPL) activity, a key enzyme in lipoprotein lipolysis pathway.[16] Betatrophin has been associated with two functionally important processes in the development of T2D, insulin resistance and lipid metabolism, and has been also reported to regulate the replication of β-cells in response to insulin resistance.[17,18]

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Previous studies have reported a correlation between betatrophin and altered lipid metabolism and a significant association was found between betatrophin levels in serum and the atherogenic lipid profile.[19–22] Moreover, inhibition of ANGPTL8 has been highlighted as a novel therapeutic strategy for reducing plasma lipoprotein levels.[23] Based on the association of T2D with an atherogenic lipid profile and the risk of CVD in these patients, serum concentration of betatrophin in T2D patients has been investigated in several studies, but the results were inconsistent and inconclusive.[24–28]

In this work, we investigate circulating levels of ANGPTL2 and betatrophin in patients with T2D with and without CVD to study potential association with disease risk and correlation with various parameters.

2. Materials and methods

2.1. Subjects

A case-control study included 150 T2D patients with and without CVD and 100 control subjects. All participants were recruited from the Outpatients Clinic of the National Research Center. Data of family and medical history was obtained by questionnaire. Clinical examination including measurement of blood pressure was applied. Anthropometric measurements (weight and height) were collected and used for BMI calculation according to the standard formula BMI = weight (kg) / [height(m)]^2. Hypertension was defined as blood pressure above 140/90 mm Hg or taking antihypertensive drugs. Diagnosis of diabetes based on the criteria of the American Diabetes Association.[29] Study participants were classified into 3 groups:

Control group included 100 healthy subjects with fasting plasma glucose (FPG) <100 mg/dL. Exclusion criteria were diabetes mellitus, CVD, family history of diabetes or any form of CVD, hyperlipidemia, hypertension, systemic diseases, and those under medication.

T2DM patients without CVD included 80 subjects fulfilled diabetes mellitus diagnostic criteria or under diabetes medication (oral and/or insulin) with no history or signs of any form of CVD.

T2DM complicated with CVD included 70 subjects diagnosed to have diabetes and complicated with any CVD e.g. ischemic heart disease (IHD), macroangiopathy and/or cerebrovascular disease. IHD included myocardial infarction, ischemic electrocardiographic (ECG) changes and angina pectoris. Macroangiopathy included peripheral arterial diseases (ankle brachial index ≤ 0.9) and cerebrovascular disease (history of transient ischemic attack, reversible ischemic neurological deficit or stroke caused by cerebral infarction).

Exclusion criteria for diabetic patients included systemic diseases, other metabolic disorders, autoimmune diseases, and those treated with Thiazolidinedione as it affects the expression of ANGPTL2.[10] Informed consent was obtained from all participants and the study protocol was approved by the Ethics Committee of the National Research Center.

2.2. Methods

2.2.1. Assay of biochemical markers

Venous blood samples were collected from all subjects after 12 h of overnight fast, centrifuged within 2 h and assayed for biochemical markers. For ELISA assays, aliquots were frozen at −80 °C till time of assay to avoid erroneous results from repeated freeze/thaw cycles. Fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were assayed on c311 clinical chemistry auto analyzer (Roche Diagnostics, Germany). Glycosylated hemoglobin (HbA1c) was assayed by ion-exchange high-performance liquid chromatographic (HPLC) method using Agilent 1200 series HPLC system (Agilent Technologies, USA) equipped with UV/VIS-Detector 415 nm using the commercially available HbA1c test kit (RECIPE Chemicals and Instruments GmbH, Germany). Serum insulin was quantitatively assayed by commercially available enzyme immunoassay test kit (IMMUNOSPEC, USA), with assay sensitivity of 2.0 μU/ml. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and fasting plasma glucose (FPG) by the following equation: HOMA-IR = fasting insulin (μU/ml) × FPG (mg/dl) / 405.

2.2.2. Assay of ANGPTL2 and betatrophin in serum

Serum aliquots were thawed and centrifuged just prior to assay. ANGPTL2 was assayed using human ANGPTL2 ELISA kit MBS704691 (MyBioSource, Inc., USA) with detection range from 1.56 ng/ml to 100 ng/ml and sensitivity of 0.39 ng/ml, with no significant cross-reactivity or interference with other analogues. Betatrophin was assayed using a commercially available human ANGPTL8 ELISA kit MBS2515814 (MyBioSource, Inc., USA). The detection range is from 125 pg/ml to 8000 pg/ml, and sensitivity of 75 pg/ml. The intra-assay and inter-assay precision coefficients of variation (CV%) were <10%.

2.3. Statistical analysis

Analysis of data was performed using the IBM SPSS version 20.0 software. Data were expressed as mean ± standard deviation (SD) for continuous variables and as frequency (%) for categorical variables. Normally distributed data were compared using Student's *t*-test for two groups and ANOVA test for more than two groups followed by post hoc Bonferroni multiple comparison test. For categorical clinical variables, differences between groups were evaluated by chi-square test. The correlations between serum ANGPTL2 and betatrophin with other variables were tested using Pearson correlation coefficient (r) and correlations were analyzed using Spearman's correlation coefficient. Logistic regression analyses were used to assess association of serum ANGPTL2 and betatrophin with the incidence of T2D and CVD with unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals (CIs). A receiver operating characteristics (ROC) analysis was used to calculate the area under the curve (AUC) to find the best cutoff value of serum ANGPTL2 providing the highest diagnostic specificity followed by the best sensitivity to differentiate between control and T2D patients and between T2D patients with CVD from those without CVD. p value < 0.05 was considered significant.

3. Results

3.1. General characteristics and biochemical variables of the studied participants

The study included 250 subjects classified into 3 groups: T2D (n = 80), T2D + CVD (n = 70) and control group (n = 100). Age ranged from 49 to 68 years. The CVD in our patients were: 40 patients with IHD (57%), 14 patients with cerebrovascular disease (20%), 7 patients with macroangiopathy (10%), 5 patients with combined IHD and cerebrovascular disease (7%) and 4 patients with combined macroangiopathy and cerebrovascular disease (6%).

Patients and control groups were age and gender matched (p > 0.05). T2D + CVD patients had significantly higher incidence of hypertension, smoking, and longer mean duration of diabetes. Significantly higher levels of FPG, HBA1C, fasting insulin, HOMA-IR, TC, TG and LDL-C and lower levels of HDL-C were demonstrated in patients compared to controls and in T2D + CVD patients compared to T2D only patients. General characteristics and biochemical data of the studied subjects are summarized in Table 1.

3.2. Circulating ANGPTL2 and betatrophin in patients and control

Serum ANGPTL2 concentration was significantly higher in total T2D patients (with and without CVD) than in control subjects (7.19 ± 1.97 ng/ml vs. 4.36 ± 1.27 ng/ml, p < 0.0001), and in CVD patients

Table 1
General characteristics and biochemical data of the studied subjects.

Variable	Controls (n = 100)	T2D (n = 80)	T2D + CVD (n = 70)
Age (years)	49.5 ± 3.5	50.7 ± 4.8	57.3 ± 7.8
Gender (male/female)	55/45	42/38	44/26
Smoking status (%)	5	7	12**,†
Diabetes duration (years)	–	5.5 ± 4.4	12 ± 5.3†
Hypertension (%)	–	21	62†
CVD [n (%)]			
IHD	–	–	40 (57%)
Cerebrovascular	–	–	14 (20)
Macroangiopathy	–	–	7 (10)
IHD + cerebrovascular disease	–	–	5 (7)
Macroangiopathy + cerebrovascular disease	–	–	4 (6)
BMI (kg/m^2)	23.6 ± 1.7	24.5 ± 1.8	24.3 ± 2.2
FPG (mg/dl)	87 ± 8.5	120 ± 40*	155 ± 36**,†
HbA1c (%)	5.1 ± 0.8	6.1 ± 1.9*	6.8 ± 2.1**,†
TC (mg/dl)	180 ± 22	190 ± 45*	210 ± 33**,†
TG (mg/dl)	145 ± 31	150 ± 40*	179 ± 59**,†
HDL-C (mg/dl)	51 ± 14	49 ± 13*	43 ± 9**,†
LDL-C (mg/dl)	102 ± 12	115 ± 20*	125 ± 35**,†
Insulin (μU/ml)	6.8 ± 0.6	10.5 ± 5*	12.1 ± 4.7**,†
HOMA-IR (%)	1.4 ± 0.4	2.9 ± 1.5*	4.2 ± 2.4**,†
Serum betatrophin (pg/ml)	2301.84 ± 938.44	1849.25 ± 931.38	2121 ± 892.39
Serum ANGPTL2 (ng/ml)	4.36 ± 1.27	6.53 ± 1.61*	7.71 ± 2.16**,†

Bonferroni multiple comparison test was applied.
CVD: cardiovascular disease; IHD: ischemic heart disease; BMI: body mass index; FPG: fasting plasma glucose; HbA1c: hemoglobin A1C; TC: total cholesterol; TG: triglyceride; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment of insulin resistance.
* Significant p in comparison between controls and T2D.
** Significant p in comparison between controls and T2D + CVD.
† Significant p in comparison between T2D and T2D + CVD.

compared to control subjects (7.71 ± 2.16 ng/ml vs. 4.36 ± 1.27 ng/ml, $p < 0.0001$). T2D + CVD patients had significantly higher levels of ANGPTL2 than T2D only patients (7.71 ± 2.16 ng/ml vs. 6.53 ± 1.61 ng/ml, $p = 0.0002$). Serum level of betatrophin was lower in total T2D patients than in controls but of no statistical significance (1778.6 ± 877.6 pg/ml vs.2301.84 ± 938.44 pg/ml, $p = 0.07$). Patients with T2D + CVD had higher circulating levels of betatrophin compared to T2D only patients, yet of no statistical significance (2121 ± 892.39 pg/ml vs. 1849.25 ± 931.38 pg/ml, $p = 0.35$) (Table 1).

3.3. Association of ANGPTL2 and betatrophin with T2D and CVD

Univariate analysis to study the association of circulating levels of ANGPTL2 with the risk of T2D and CVD as compared to healthy controls showed that elevated circulating levels of ANGPTL2 was associated with 2.83 times increased risk to develop T2D ($p < 0.0001$, OR = 2.83, 95% CI = 1.75–3.90) and with 3.35 times elevated risk for CVD ($p < 0.0001$, OR = 3.35, 95% CI = 2.11–4.58). Association studies of the CVD risk among T2D patients showed that elevated circulating levels of ANGPTL2 in T2D patients was associated with 1.18 elevated risk for CVD ($p = 0.0002$, OR = 1.18, 95% CI = 0.55–1.79). All associations remained significant after adjustment for other covariates: age, gender, BMI, hypertension, disease duration, lipid parameters and smoking status. On the other hand, circulating levels of betatrophin was not significantly associated with T2D as compared to control subjects ($p = 0.07$) or with CVD among T2D patients ($p = 0.35$) (Tables 2 & 3).

3.4. Relationship between serum ANGPTL2 and betatrophin and various parameters

Correlation analyses to investigate the relationship of circulating ANGPTL2 and betatrophin levels with various parameters showed positive correlations between serum ANGPTL2 level and FPG, HbA1c, HOMA-IR, TC, TGs and LDL. On the other hand, serum level of betatrophin correlated positively with age, duration of diabetes, HOMA-IR, TC and TGs. All correlations remained significant after adjustment for other covariates (Table 4).

3.5. ROC analysis

Studying ANGPTL2 as risk biomarker for T2D and CVD, receiver-operating characteristic (ROC) curve analysis indicated serum ANGPTL2 as a risk biomarker for T2D with an area under the curve (AUC) of 0.9 ($p = 0.0000$) and a cut-off value of ≥5.6 ng/ml with sensitivity of 92.2%, specificity of 86.7%, positive predictive value (PPV) of 96% and negative predictive value (NPV) of 76.5% (Fig. 1). As compared to controls, ANGPTL2 was a risk biomarker for CVD with AUC of 0.87 ($p = 0.0000$) and a cut-off value of ≥4.4 ng/ml with sensitivity of 100%, specificity of 60%, PPV of 76% and NPV of 100% (Fig. 2). ANGPTL2 was also identified as a risk biomarker for CVD in T2D patients with AUC of 0.7 ($p = 0.006$) and a cut-off value of ≥6.4 ng/ml with sensitivity of 86%, specificity of 58%, PPV of 75% and NPV of 73% (Fig. 3).

Table 2
Association of serum levels of ANGPTL2 and betatrophin with T2D and CVD compared to controls.

	Control (n = 100)	Total T2D (n = 150)	p*	T2D + CVD (n = 70)	p**
ANGPTL2 (ng/ml)	4.36 ± 1.27	7.19 ± 1.97	<0.0001	7.70 ± 2.16	<0.0001
Betatrophin (pg/ml)	2301.8 ± 938.4	1778.6 ± 877.6	0.07	2121 ± 892.39	0.2

Data presented as mean ± SD.
* Bonferroni corrected p value of total T2D compared to controls.
** Bonferroni corrected p value of T2D + CVD compared to controls.

Table 3
Association of serum levels of ANGPTL2 and betatrophin with CVD risk in T2D patients.

	T2D (N = 80)	T2D + CVD (N = 70)	p-Value*
ANGPTL2 (ng/ml)	6.53 ± 1.61	7.70 ± 2.16	0.0002
Betatrophin (pg/ml)	1849.25 ± 931.38	2121 ± 892.39	0.35

Data presented as mean ± SD.
* Bonferroni corrected p value.

4. Discussion

In this work, we studied the association of serum ANGPTL2 and betatrophin with the risk of CVD in T2D patients. We have demonstrated for the first time that circulating level of ANGPTL2 was significantly associated with elevated risk of T2D and CVD. We also indicate ANGPTL2 as an independent risk biomarker for CVD in T2D patients.

Previous studies on mice have reported ANGPTL2 to promote atherogenesis,[13] and elevated serum ANGPTL2 was associated with coronary heart disease (CHD)[10] and was abundantly expressed in atheromatous plaques.[9] Although these previous data support the hypothesis that serum ANGPTL2 might strongly related to atherosclerosis in humans, to date, there have been a very few studies that have measured the serum ANGPTL2 concentrations in human and little is known whether this role of ANGPTL2 applies in purely T2D.

In our study, we found significant positive correlations between serum ANGPTL2 level and variables of hyperglycemia (FPG and HbA1c), insulin resistance (HOMA-IR) and atherogenic lipid profile (TC, TGs and LDL). Our findings support the previous reports that circulating ANGPTL2 correlates with the levels of systemic insulin resistance.[30,31] In agreement with our results, HbA1c was previously reported as an independent contributor to the elevated serum ANGPTL2 level in T2D.[31] Although further studies are needed to reveal the role of ANGPTL2 in the pathogenesis of T2D and CVD, our present findings may suggest ANGPTL2 as a potential key mediator that link T2D and CVD.

On the other hand, we demonstrated lower serum levels of betatrophin in T2D patients than in control subjects, but the difference was statistical insignificant. Previous reports gave inconsistent results, while reduced betatrophin levels were reported in youth-onset T2D[25] and in T2D,[20,27] significantly elevated levels of serum betatrophin were reported in prediabetes and T2D.[26,32,33] Discrepancies were attributed to ethnic variation, difference in sample sizes, oral hypoglycemic agents (it is not clear yet whether oral hypoglycemic agents affect serum betatrophin level or not), and different disease duration of T2D. In a meta-analysis included 490 cases and 508 controls investigating the relationship between circulating betatrophin and T2D, a significant association was found, however in subgroup analysis, no statistical significance association within Caucasian population was observed[34]

Table 4
Correlation analysis of serum ANGPTL2 and betatrophin with various parameters in T2D patients.

Variable	ANGPTL2		Betatrophin	
	r	p*	r	p*
Age	0.304	0.21	0.359	0.02
Duration of diabetes	0.291	0.19	0.362	0.03
BMI	0.344	0.07	0.305	0.12
FPG	0.356	0.04	0.291	0.19
HbA1c	0.345	0.04	0.267	0.55
TC	0.335	0.04	0.355	0.04
TG	0.347	0.03	0.353	0.02
HDL-C	0.284	0.12	0.302	0.12
LDL-C	0.344	0.03	0.033	1.0
Insulin	0.288	0.51	0.284	0.21
HOMA-IR	0.366	0.04	0.399	0.02

* Bonferroni corrected P value after adjustment for gender, sex, hypertension, and smoking.

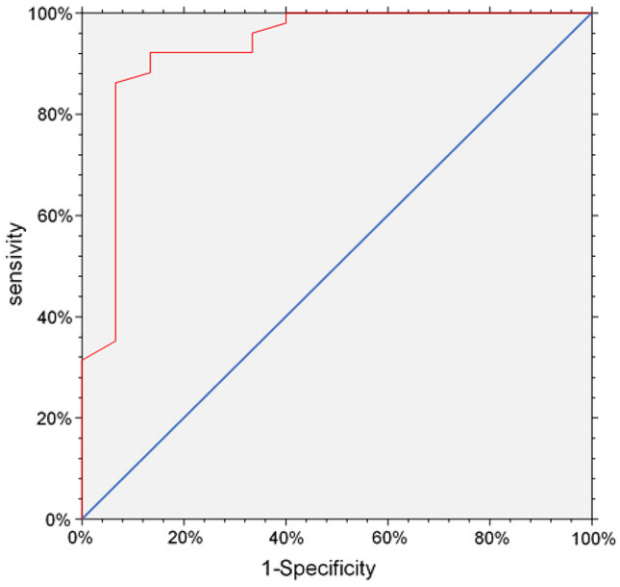

Fig. 1. Receiver operating characteristic (ROC) curve analysis of serum level of ANGPTL2 for prediction of T2D.

suggesting an ethnic variation in the interpretation of circulating level of betatrophin in T2D.

In our study, circulating betatrophin showed a significant positive correlation with age. Despite the reported decline in beta-cell replication rate with age in both mouse and humans,[35,36] in a study on167 non-diabetic subjects aged 20 to 102 years, the calculated beta-cell mass remained constant, however, the mean beta-cell nuclear diameter increased with age and no change in the proportion of apoptotic beta-cells was observed[36]; therefore, increased betatrophin levels with age has been attributed to a compensatory response to aging.[26] In our patients, circulating betatrophin positively correlated with duration of diabetes and hepatic insulin resistance (HOMA-IR). In a previous study, Yi et al[15] reported that hepatic expression of betatrophin was upregulated in animal models of insulin resistance in which beta-cell replication is increased, while Hu et al[26] speculated that the increase in serum betatrophin in T2D might be attributable to a defensive response,

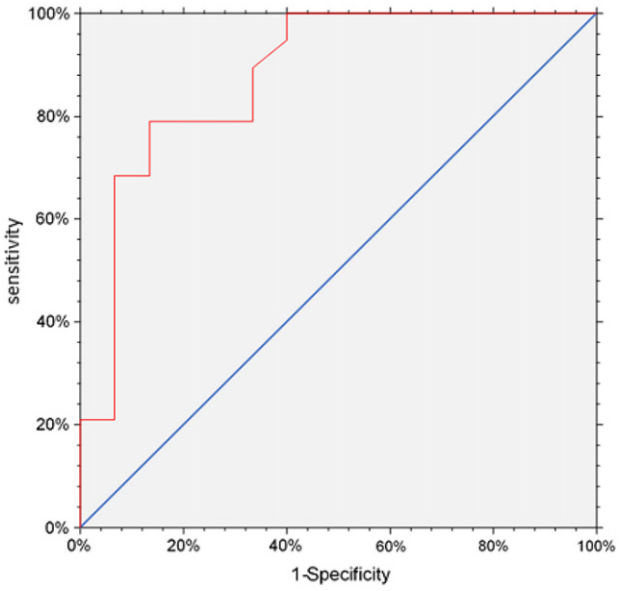

Fig. 2. Receiver operating characteristic (ROC) curve analysis of serum level of ANGPTL2 for prediction of CVD.

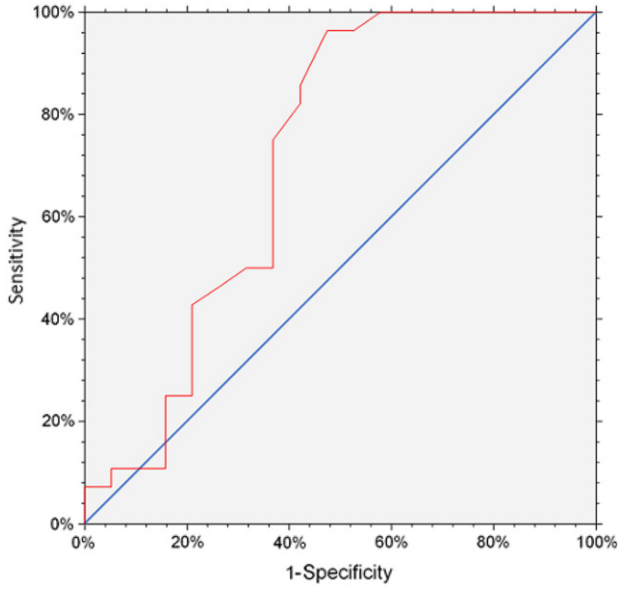

Fig. 3. Receiver operating characteristic (ROC) curve analysis of serum level of ANGPTL2 for prediction of CVD in T2D.

which may represent an ability to adapt to hepatic insulin resistance by increasing beta-cell proliferation and insulin secretion. All of the above can explain the demonstrated higher levels of betatrophin in our CVD patients (who were older in age, had longer duration of diabetes and higher IR) than in T2D only patients.

There is a rising evidence from animal-based studies suggests that betatrophin plays a key role in lipid metabolism. Betatrophin affects TG metabolism by inhibiting LPL activity and thereby increasing the TG level.[21] Betatrophin knockout mice had a 70% reduction in plasma triglyceride levels compared with the wild type.[37] Also, hepatic overexpression of betatrophin increased plasma triglyceride levels more than five-fold in mice.[21] Based on the association of T2D with an atherogenic lipid profile and the risk of cardiovascular complications, we studied the relationship between serum betatrophin and lipid profile parameters. We found circulating betatrophin was positively correlated with plasma TC and TG levels. Our findings are in agreement with Fenzl et al who reported significant association between serum betatrophin and TC, LDL, and apolipoprotein B in patients with long duration of T2D.[20] Also, Ghasemi et al[33] found serum betatrophin was positively correlated with pathologic lipid profile in T2D patients and suggested betatrophin as a novel therapeutic target. In contrast, absence of any correlation between betatrophin and TG in newly diagnosed T2D has also been reported.[26] On the other hand, a study conducted on patients with T1D and T2D showed a significant association between circulating betatrophin and plasma TG level.[38] A similar positive correlation was also found in subjects with insulin resistance.[20] Previous results with our present findings suggest betatrophin plays a role in the pathogenesis of CVD in T2D.

One of the main limitations of our study is its cross-sectional design, which did not allow us to establish the causality and the role that ANGPTL2 may play in the pathogenesis of T2D and/or CVD development. Yet, its hyperglycemic and atherogenic roles can be presumed from our findings. Another limitation, our analyses are based on single measurements of betatrophin which may not reflect betatrophin levels over time, serial changes in serum betatrophin need to be measured at different stages of T2D to be able to clarify its role in the pathogenesis of T2D and cardiovascular complications.

5. Conclusions

We indicate for the first time the association of serum ANGPTL2 with elevated risk of T2D and CVD in humans, we also indicate serum ANGPTL2 as an independent risk biomarker for CVD in T2D patients. Our findings suggest ANGPTL2 as a potential key mediator linking T2D and CVD. Further prospective studies to reveal its role in the pathogenesis of T2D and CVD are needed.

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