Correlation of long-term glycemic control as measured by glycated hemoglobin with serum angiopoietin-like 6 protein levels in type 2 diabetes mellitus patients

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Abstract:
AIMS: Angiopoietin-like growth factors (ANGPTLs) regulate glucose, lipid homeostasis, and insulin sensitivity. This study aimed to find whether long-term glycemic control (glycated hemoglobin [HbA1c]) has any correlation with serum ANGPTL6 levels in patients of type 2 diabetes mellitus.

MATERIALS AND METHODS: It was an open-label, observational, prospective clinical study. Sixty-five participants (41 diabetic patients receiving daily dose of oral metformin for a minimum of 3 months and 24 matched controls) completed the study. A single venous blood sample was taken from each participant to determine serum HbA1c and serum ANGPTL6 levels. Comparison of serum ANGPTL6 levels according to the HbA1c levels, in groups A, B, and C ranging from 6.5%–8%, 8.1%–9.5%, and >9.5%, respectively, was done using Kruskal–Wallis H-test followed by pairwise comparisons.

RESULTS: Serum HbA1c and serum ANGPTL6 levels were raised significantly (P < 0.05) in diabetic patients when compared with control participants. A positive correlation was observed between serum HbA1c and serum ANGPTL6 levels (r = 0.88, 95% confidence interval 0.81, 0.92). Mean ANGPTL6 level for Group A (n = 20) was 394.3 pg/ml, for Group B (n = 8) 692.8 pg/ml, and for Group C (n = 13) 896.2 pg/ml.

CONCLUSIONS: Serum ANGPTL6 levels were significantly higher in type 2 diabetic patients in comparison with healthy controls. Poor glycemic control in diabetes mellitus as reflected by higher serum HbA1c levels is associated with raised serum ANGPTL6 levels.

Keywords: Endocrine signal, glucose, insulin resistance

Introduction
Type 2 diabetes mellitus develops owing to insulin resistance, a condition in which insulin is improperly utilized by the cells, while sometimes, it is accompanied by reduced insulin production. Glycated hemoglobin (HbA1c) has been used as a diagnostic test for diabetes, and it reflects average plasma glucose over the previous 8–12 weeks. Recent studies have proposed that adipose tissue in obese case develops persistent low-grade activation of pro-inflammatory pathways, thereby promoting systemic insulin resistance. Effective preventive and therapeutic approaches to insulin resistance require better understanding of the molecular mechanisms causing inflammation.
in adipose tissue. Circulating levels of human angiopoietin-like 6 protein (ANGPTL6) are elevated in obesity or diabetes mellitus. The ANGPTLs are produced in the liver and mediate endocrine signaling in the various peripheral tissues. ANGPTL6 plays an important role in glucose and lipid metabolism and insulin sensitivity. It may directly antagonize obesity and related insulin resistance, therefore assuming significance as a potential target for developing newer pharmacological interventions against metabolic diseases such as obesity and diabetes mellitus. Data regarding serum ANGPTL6 levels in healthy and diabetic population are scarce. The aim of our study was to assess whether long-term glycemic control as reflected by serum HbA1c has any correlation with serum ANGPTL6 levels in patients of type 2 diabetes mellitus.

**Materials and Methods**

This study was conducted in the Departments of Pharmacology and Medicine in Lady Hardinge Medical College and Smt. Sucheta Kriplani Hospital, New Delhi, after clearance from the Institutional Ethics Committee for Human Research. It was an open-label, observational, prospective clinical study. Diabetic patients, who attended the medicine outpatient diabetes clinic and receiving daily dose of oral metformin for a minimum of 3 months from January 2013 to March 2014, were screened for eligibility. Patients <18 years of age, type 1 diabetes patients, newly diagnosed (<3 months) type 2 diabetes mellitus, and those on insulin or any other oral hypoglycemic drugs except metformin were excluded from the study. Fifty patients came out to be eligible. Out of 50, 41 patients gave written consent and were analyzed. Twenty-four age- and sex-matched healthy volunteers were also included in the study.

Diagnosis of diabetes was made according to the American Diabetes Association definition of type 2 diabetes mellitus.

A single venous blood sample (3–5 ml) was taken from each participant under aseptic conditions to determine serum ANGPTL6 and serum HbA1c levels. All the samples were first centrifuged, and serum was separated and stored at −20°C, avoiding repeated freeze-thaw cycles till their analysis. These serum samples were batch analyzed for ANGPTL6 levels using serum ANGPTL6 enzyme-linked immunosorbent assay kits (Bmassay, Beijing, China). HbA1c levels were estimated by latex agglutination inhibition method using Beckman Coulter AU480® autoanalyzer.

**Steps for estimation of serum ANGPTL6**

The human serum ANGPTL6 polyclonal antibodies were precoated onto 96-well plates. Add 100 µl prepared serum samples or standards and incubate the plate at 37°C for 90 min, wash plate twice with 300 µl Tris-buffered saline (TBS). Add 100 µl biotinylated antibodies and incubate the plate at 37°C for 60 min, wash plate 3 times with TBS. Add 100 µl avidin-biotin-peroxidase complex working solution and incubate the plate at 37°C for 30 min, wash plate 5 times with TBS. Add 90 µl 3,3′,5,5′-Tetramethylbenzidine color developing agent and incubate at 37°C. Add 100 µl stop solution. Optical density absorbance at 450 nm is read within 30 min. The intensity of the color is proportional to the amount of human ANGPTL6 bound in samples which is calculated by plotting graph between concentrations and corresponding absorbencies of standards.

Primary outcome of this study was correlation of long-term glycemic control as reflected by serum HbA1c with serum ANGPTL6 levels in type 2 diabetes mellitus patients.

Likely potential sources of confounding factors were adjusted. To adjust for treatment with various hypoglycemic medications, diabetes patients taking oral metformin in various doses were only included in the study. However, differing doses of metformin among diabetic patients might be a potential confounder. The use of concomitant medications for treating related diseases such as hypertension and dyslipidemia had not been adjusted. Any biochemical analytical bias was adjusted by keeping the analyst blind. The laboratory personnel had no knowledge of case or control status of the samples.

The statistical analysis was performed using Microsoft® Excel and IBM SPSS®, version 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, version 20.0., IBM Corp., Armonk, NY, USA). Serum HbA1c and serum ANGPTL6 levels in the control and diabetic patients were compared by Mann–Whitney U-test. To establish the correlation of serum HbA1c with serum ANGPTL6 levels, Pearson’s correlation coefficient was calculated. Comparison of serum ANGPTL6 levels according to the HbA1c levels, in groups A, B, and C ranging from 6.5%–8%, 8.1%–9.5%, and >9.5%, respectively, was done using Kruskal–Wallis H-test followed by pairwise comparisons. The statistical significance was set at P < 0.05.

**Results**

Table 1 shows baseline demographic and biochemical parameters of control and diabetic patients. Serum HbA1c and serum ANGPTL6 levels were raised significantly (P < 0.05) in the diabetic patients compared with control participants which was expected [Table 1].
A positive correlation was observed between serum HbA₁c and serum ANGPTL6 levels \((r = 0.88, 95\%\text{ confidence interval [CI] 0.81, 0.92})\) [Figure 1]. A weaker correlation existed between fasting plasma glucose and serum ANGPTL6 levels \((r = 0.59, 95\%\text{ CI 0.41, 0.75})\). Kruskal–Wallis H-test showed that there was a statistically significant difference in serum ANGPTL6 levels between the different subgroups, \(\chi^2 (2) =27.1, P < 0.01\), with a mean ANGPTL6 levels of 394.3 (pg/ml) for Group A \((n = 20)\), 692.8 (pg/ml) for Group B \((n = 8)\), and 896.2 (pg/ml) for Group C \((n = 13)\). Pairwise comparison showed that serum ANGPTL6 levels were significantly different between Group A and Group B, Group A and Group C, but not between Group B and Group C.

## Discussion

This study aimed to find whether poor long-term glycemic control in patients with type 2 diabetes mellitus being treated with metformin has any association with the serum ANGPTL6 levels. We observed that serum ANGPTL6 levels were lower in healthy controls when compared with type 2 diabetes mellitus patients. A previous study done by Ebert et al. showed that obese or diabetic patients had elevated serum ANGPTL6 levels.\(^4\) Further, our results show that levels of serum ANGPTL6 are positively correlated with the corresponding serum HbA₁c levels.

No difference was observed in mean serum ANGPTL6 levels in males and females. However, an earlier study showed that levels of serum ANGPTL6 are raised in females compared with males.\(^3\) As expected, mean fasting plasma glucose levels in our study are much higher in diabetic patients in comparison with nondiabetic controls. However, correlation between fasting plasma glucose and serum ANGPTL6 levels was not as strong as correlation between serum HbA₁c and serum ANGPTL6 levels. This shows that chronic mechanism is involved in raising serum ANGPTL6 levels. This is in accordance with previous reports shown by Kitazawa et al. where the serum ANGPTL6 levels correlated positively with fasting serum glucose levels.\(^7\)

Our study did not find whether the positive correlation between ANGPTL6 and poor glycemic control \((r = 0.88)\) is contributory to the pathophysiological events in type 2 diabetes mellitus, or it is a beneficial phenomenon where there is a compensatory increase in serum ANGPTL6 levels. Kitazawa et al. have shown that in mice, deletion of the Angptl6 gene led the development of hyperinsulinemia and glucose intolerance.\(^\text{[7]}\) Other investigators like Shimomura et al. have proposed that ANGPTL6 might be involved in counteracting obesity and related insulin resistance.\(^\text{[8]}\) However, a recent study conducted by Kadomatsu indicated that circulating levels of human ANGPTL6 are elevated in obesity or diabetes and does not reverse obesity.\(^\text{[9]}\) Thus, it was suggested that raised ANGPTL6 levels could be related either to improved metabolic profile in the disease groups by ANGPTL6 or to decreased sensitivity to ANGPTL6 in these participants leading to ANGPTL6 resistance.\(^\text{[9]}\) The underlying mechanism causing this phenomenon would be understood once the function of human ANGPTL6 is better defined. However, as yet, such experimental data are not available. So far, ANGPTL6 remains an orphan ligand; thus, membrane targets for ANGPTL6 will need

### Table 1: Comparison of baseline parameters in control and diabetic patients

| Characteristic          | Controls \((n=24)\) | Diabetic patients \((n=41)\) |
|-------------------------|---------------------|-----------------------------|
| Age (years)             | Mean: 49.2          | 48.5                        |
|                         | Range: 21-73        | 19-72                       |
| Sex                     | Male/female: 15/9   | 20/21                       |
| HbA₁c (%)               | Mean: 5.44±0.50     | 8.85±2.04*                  |
|                         | Range: 4.4-6.3      | 6.5-13.1                    |
| Fasting glucose (mg/dl) | Mean: 108.30±24.66  | 199.10±75.30*               |
|                         | Range: 75-177       | 85-387                      |
| Postprandial glucose    | Mean: 158.83±53.13  | 266.20±80.57*               |
|                         | Range: 71-313       | 123-447                     |
| ANGPTL6 (pg/ml)         | Mean: 144.42±83.56  | 618.65±280.87*              |
|                         | Range: 4.09-345.59  | 31.06-117.64                |

*\(P<0.05\) significant in comparison with control. ANGPTL6=Angiopoietin-like 6 protein, HbA₁c=Glycated hemoglobin
to be identified to prove the hypothesis of ANGPTL6 resistance.

Taking these results into consideration, upregulation of ANGPTL6 production in type 2 diabetes mellitus might be a compensatory mechanism that may attenuate hyperglycemia in humans. Our results show that ANGPTL6 levels were not significantly different in the Subgroups B and C. These results support the earlier evidence that ANGPTL6 counteracts insulin resistance before the development of type 2 diabetes mellitus but is not an effective antagonist after the disease is manifested.\(^\text{[9]}\)

Some limitations of the present study may be pointed out: First, cross-sectional data are presented, and therefore, temporal association between glycemic control and ANGPTL6 levels cannot be established. Second, the sample size is relatively small (\(n = 65\)).

**Conclusions**

This study shows that serum ANGPTL6 levels were significantly higher in type 2 diabetic patients in comparison with healthy controls. Poor glycemic control in diabetes mellitus as reflected by higher serum HbA\(_1c\) levels is associated with raised serum ANGPTL6 levels.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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