Role of Elevated Serum Sialic Acid in the Progression of both Diabetic Retinopathy and Diabetic Nephropathy

Authors
Soher A. Mohammed Ismail1, Hadeer Bakeer2, Mustafa Abdel Aziz M.3, Iman A. Fahmy4, Mohamed El Hefni5, Shimaa Nabil6, Safyea Mohamed Hussien7, Leqaa A. Moemen8, Mona A. Abdel Hamid9, Margeret A. Aziz10

1,8,9,10 Medical Biochemistry Department Research Institute of Ophthalmology
2Chemistry Department, Faculty of Science. Fayoum University
3Department of Internal Medicine, Faculty of Medicine, October 6 University
4,5 Ophthalmology Department. Research Institute of Ophthalmology
6Center of basic science, Misr University of science and technology
7Clinical Pathology Department Research Institute of Ophthalmology

Abstract
Purpose: The purpose of this study was mainly to determine the relationship between total serum sialic acid and occurrence and progression of diabetic retinopathy and diabetic nephropathy in diabetic patients.

Materials and Methods: The study included 60 subjects, their ages ranged between 45-60 year; most of diabetic patients had controlled blood pressure. These subjects were divided into the following groups:
Control group: involved 15 healthy subjects. Clinical and laboratory investigation were performed for each to exclude the presence of diabetes mellitus or any associated disease.
Group (1): involved 15 diabetic patients without retinopathy
Group (3): involved 15 diabetic patients with proliferative diabetic retinopathy (PDR).

Urine and fasting blood samples were collected. Patients with DR were clinically examined by a specialist. The standard curve of sialic acid was drawn using Ehrlich method. Fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c%), serum total cholesterol, serum HDL-cholesterol, serum triglycerides (STG), serum creatinine, serum urea, microalbuminuria, urine creatinine and serum c-reactive protein were estimated using LABOMED, Inc (spectro uv-vis Double Beam Pce with 8 scanning autocell uv D-3200). Serum LDL-cholesterol and urine albumin/creatinine ratio were calculated.

Results: The study showed statistical significant increase in total serum sialic acid in all diabetic patients but the increase was more prominent in patients with proliferative DR. There were increase in fasting glucose level, glycosylated HB%, total cholesterol, triglycerides, LDL-C, serum urea, serum creatinine and urinary albumin/creatinine ratio with decrease in HDL-C in diabetic retinopathy patients (mainly with proliferative retinopathy), the increase was statistically significant. This increase in these parameters was parallel to this increase in total serum sialic acid but not reach to significant correlation. The statistically significant correlation was found between total serum sialic acid and both age of the patients and duration of diabetes.

Conclusion: Serum sialic acid concentrations were increased in type 2 diabetics with and without complications and this increase was strongly related to the progression of retinopathy and occurrence of nephropathy.

Keywords: sialic acid, diabetic retinopathy, diabetic nephropathy
Introduction
Sialic acid is a component of cell membranes\textsuperscript{[1]} and elevated levels may indicate excessive cell membrane damage, but more specifically to the cells of vascular tissue. If there is damage to vascular tissue, this leads to ischaemia especially in the smallest blood vessels including those of the retina, kidneys, heart and brain. This ischaemia leads to conditions including retinopathy, nephropathy and neuropathy. Patients with either Type 1 or 2 diabetes mellitus have raised serum concentrations of SA \textsuperscript{[2, 3]} and there is evidence that this may be related to the severity of their diabetic complications such as retinopathy.\textsuperscript{[4]}

In addition, sialic acid can be used as a measurement of the acute phase response because many of the proteins of the immune response are actually glycoproteins and these glycoproteins have sialic acid as the terminal sugar on their oligosaccharide chain.\textsuperscript{[5]}

This study tries to determine whether in diabetic retinopathy, there is a higher level of sialic acid, which is a marker of acute phase inflammation and the correlation of this with progression of the disease and its complications.

Patients and methods
Subjects
The study included 60 subjects, their ages ranged between 45-60 years; most of diabetic patients had controlled blood pressure. These subjects were divided into the following groups:

- Control group: involved 15 healthy subjects.
- Clinical and laboratory investigation were performed for each to exclude the presence of diabetes mellitus or any associated disease.
- Group (1): involved 15 diabetic patients without retinopathy.
- Group (3): involved 15 diabetic patients with proliferative diabetic retinopathy (PDR).

Patient diagnosis
Retinopathy was assessed by direct and indirect ophthalmoscopy and documented by color photography and fluorescein angiography. A modified version of early treated diabetic retinopathy study (ETDRS) grading system \textsuperscript{[6]} was used to grade the photographs. Non-proliferative diabetic retinopathy was diagnosed if microaneurisms, dot haemorrhages, exudates or venous changes were present in any field. Proliferative retinopathy was diagnosed if new vessels were present on the disk or elsewhere on the retina.

Sample collection
Blood sample collection
On the day of the study, subjects reported to our laboratory in the morning after an overnight fasting of 6-8 hours. One ml of venous blood was collected from them in bottles containing EDTA for estimation of glycosylated Hemoglobin. Two ml was collected in bottles containing fluoride for glucose estimation. Another sample after an overnight fasting of 12-14 hours was taken, collected in plain tubes, centrifuged at 5000g for 5 minutes and stored at -80 OC till used for the estimation of sialic acid, total cholesterol, HDL-C, triglycerides, creatinine, urea and C-reactive protein.

Urine sample collection:
Random midstream urine samples were collected in clean bottles (preferably plastic disposable containers with cover). The fresh urine samples collected from patients and control groups were used for the estimation of both creatinine and albumin.

Sialic acid determination
TSA analysis for serum samples was carried out according to the method reported by Sydow, G. et al., (1988).\textsuperscript{[7]} Briefly, 400 µl of serum was treated with 3 ml of 5% perchloric acid for 5 min at 100 OC and centrifuged at 1400 g for 4 min. The supernatant (2 ml) was mixed with 400 µl of...
Ehrlich reagent (5 g pdimethylamino-benzaldehyde/50 ml, HCl/50 ml distilled water). After incubation at 100 OC for 15 min, a spectrophotometer was used to read the optical density at 525 nm.

### Preparing for the sialic acid standard curve

1. A stock solution of 90 mg% concentration was prepared by dissolving 9 mg of a solid sialic acid to 10 ml of distilled H2O. (0.009 g to 10 ml of distilled H2O).
2. Different concentrations of standard samples (80,70,60,50,40,30,20,10,5 mg%) were prepared from the stock solution (90 mg%) by serial dilution.
3. The standard samples were then analyzed using the method described by Sydow, G. et al., (1988), and the absorbance of each was determined using LABOMED,Inc (Spectro uv-vis Double Beam Pc with 8 Scanning Autocell UV D-3200).
4. The standard curve was drawn by Microsoft Excell 2003 programme between the sialic acid concentration on the x-axis and the absorbance of the standard sample determined after the analysis on the Y-axis.
5. The following curve was produced with Y=0.0008X+0.0003 equation and R2=0.9259.
6. This equation was used in determining the concentration of test samples as follow:
   \[
   X = (Y - 0.0003) / 0.0008
   \]
   Where, X; concentration of the test samples. 
   Y; absorbance of the test samples.

### Chemicals used in estimation of total serum sialic acid:

1. Sialic Acid or N-(N-acetyl neuraminic acid, 97% (CAS number 131-48-6) with molecular formula C11H19NO9, molecular weight of 309.28 and melting point of 185 °C was purchased from Alfa Aesar Company.
2. 4-Dimethylaminobenzaldehyde, 98% (CAS number 100-10-7) with molecular formula C9H11NO and molecular weight of 149.19 was purchased from Acros Organics Company (New Jersey, USA).
3. Perchloric acid, 70% (product number 20176) with maximum limits of impurities, molecular formula HClO4 and molecular weight of 100.46 was purchased from S.d. FiNE – chem limited, MVMBAI.
4. Hydrochloric acid, 30-34% (product number H0018111) with maximum limits of impurities, molecular formula HCl and molecular weight of 36.46 was purchased from EL-NASR Pharmaceutical Chemicals Company.

### Kits used in biochemical analysis of serum:

1. Kits of glucose, triglycerides, urea and creatinine were purchased from Diamond Diagnostics Company (Cairo, Egypt).
2. Kits of total cholesterol, HDL-C, and C reactive protein were purchased from Vitro Scient (Cairo, Egypt).
3. Kits of glycosylated hemoglobin was purchased from Biosystem (Barcelona, Spain).
4. Kit of microalbumin was purchased from Spectrum "Egyptian Company of Biotechnology (S.A.E)” (Cairo, Egypt).

### Statistical analysis of results:

Data were collected, checked, revised and entered the computer. Data analyzed by SPSS statistical package version 19. Excel computer program was used to tabulate the results, and represent it graphically.

One Way ANOVA used to declare the significant difference between groups at p<0.05. Duncan multiple comparisons test at p<0.05 was used to
declare the significant difference between each two groups. Pearson’s correlation coefficient used to declare the significant correlation between the quantitative parameters within each group at p<0.05.

Results

Fig (2): Comparative presentation of sialic acid values in all studied groups.

Table (1): Levels of all parameters in all studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Total Serum Sialic Acid</td>
<td>13.46±6.04a</td>
</tr>
<tr>
<td>Fasting Serum Glucose (FSG)</td>
<td>93.33±11.94a</td>
</tr>
<tr>
<td>Glycosylated HB%</td>
<td>4.29±1.05a</td>
</tr>
<tr>
<td>Serum Triglycerides (STG)</td>
<td>59.30±9.86a</td>
</tr>
<tr>
<td>Total Serum Cholesterol</td>
<td>147.53±19.49a</td>
</tr>
<tr>
<td>Serum HDL-Cholesterol</td>
<td>62.33±3.52a</td>
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<tr>
<td>Serum LDL-Cholesterol</td>
<td>73.61±22.47a</td>
</tr>
<tr>
<td>Serum Urea</td>
<td>33.33±5.37a</td>
</tr>
<tr>
<td>Serum Creatinine</td>
<td>0.74±0.12a</td>
</tr>
<tr>
<td>Urine Albumin/Creatinine</td>
<td>2.93±1.37a</td>
</tr>
<tr>
<td>Age of Individuals</td>
<td>38.67±2.61a</td>
</tr>
<tr>
<td>Duration of Diabetes</td>
<td>-</td>
</tr>
</tbody>
</table>

*S.D = standard deviation. *P-value < 0.05 is significant.

*Different letters mean that there is a significant difference between the two groups.
Table (1) showed:
Levels of all parameters in all studied groups.
The study showed a significant increase in total serum sialic acid in all diabetic patients but the increase was more prominent in patients with proliferative DR. There was a significant increase in fasting glucose level, glycosylated HB%, total cholesterol, triglycerides, LDL-C, serum urea, serum creatinine and urinary albumin/creatinine ratio with decrease in HDL-C in diabetic retinopathy patients (mainly with proliferative retinopathy).

There was a significant increase in the levels of total serum sialic acid, FSG, glycosylated HB%, serum triglycerides and a significant decrease in the levels of serum HDL-cholesterol in group (1) when compared with the corresponding levels in the control group. As the age of individuals was significantly increased in group (1) compared to the control group (P < 0.0001).

Significant increase in the levels of total serum sialic acid, FSG, glycosylated HB%, serum triglycerides, total serum cholesterol, serum LDL-cholesterol, serum creatinine and urine albumin/creatinine ratio was present with significant decrease in serum HDL-cholesterol in group (2) compared to the corresponding levels in the control group and group (1) (P < 0.0001) as the age of individuals was significantly increased in group (2) compared to the age of diabetics in group (1) and the control group and also as the duration of diabetes was significantly increased in group (2) compared to group (1).

Significant increase in the levels of total serum sialic acid, FSG, glycosylated HB%, serum triglycerides, total serum cholesterol, serum LDL-cholesterol, serum urea, serum creatinine and urine albumin/creatinine ratio was present with significant decrease in serum HDL-cholesterol in group (3) compared to the corresponding levels in the control group and group (2) (P < 0.0001) as the age of diabetics and duration of diabetes were significantly increased in group (3) compared to the age of diabetics and duration of diabetes in group (2). Concerning the correlations between total serum sialic acid and different parameters, the statistically significant correlation was found between total serum sialic acid and both of patients age and diabetes duration. A statistically significant correlation was found between total serum sialic acid and both of patients age and duration of diabetes. The above increase in patients’ parameters was parallel to the increase in total serum sialic acid but did not reach to a significant level.

Discussion
Sialic acid (SA) plays a central role in the biomedical functioning of humans. Increased SA concentrations have been reported during inflammatory processes. [9]

Serum total sialic acid (TSA) not only has been shown to be related to diabetes mellitus (type I and II) without complications [10] but also to diabetic patients with complications as those with diabetic retinopathy [10], diabetic nephropathy [10] or even with neuropathy [10].

This study tries to determine whether in diabetic retinopathy, there is a higher level of sialic acid which is a marker of acute phase inflammation.
and the correlation of this with progression of the disease and its complications mainly with nephropathy.

In this work, a significant increase ($p \leq 0.0001$) was observed in serum sialic acid values in diabetic patients (group 1, 2 and 3) compared with healthy controls. Serum sialic acid level was higher in proliferative retinopathy and the difference between groups was statistically significant ($p \leq 0.0001$).

Results from prospective studies suggest that inflammation involved in the pathogenesis of diabetes\cite{11} and atherosclerosis. \cite{12} Inflammation could be a common antecedent for diabetes. Hyperglycemia and insulin resistance could also promote inflammation, and may be factor linking diabetes to the development of atherosclerosis. Elevated glucose levels could promote inflammation by increased oxidative stress\cite{13}. Another possibility is that inflammatory response is a result of vascular complications following diabetes.

There is an increasing evidence that acute-phase response is closely involved in the pathogenesis of type 2 diabetes and associated complications such as dyslipidemia and atherosclerosis.\cite{5} Elevated circulating inflammatory markers such as C-reactive protein and interleukin-6 predict the development of type 2 diabetes.\cite{5}

In this study, C-reactive protein was positive in only two patients of group 3 and this ensured that the most useful estimate of individual's inflammatory status is the measurement of TSA. The increase of FBS and HbA1c was parallel to the increase in sialic acid in these groups respectively. For the two studied multiple biochemical pathways have been proposed to explain pathogenesis of diabetic retinopathy, all starting initially from hyperglycemia. These mainly include increased polyol pathway, increased advanced glycation end-products (AGEs) formation, activation of protein kinase C (PKC) and increased hexosamine pathway flux.\cite{14}

Sato, K. K., et al (2009)\cite{15} found that combined measurement of FBS and HbA1c is effective for prediction of type 2 diabetes. Also, The International Expert Committee (2009)\cite{16} reported on the role of HbA1c assay in the diagnosis of diabetes as there was a recommendation that an HbA1c value of 6.0 to < 6.5% is associated with diabetes risk and its complications.

The results of lipid profile agreed with Shivananda. N. B. and Geetha. B., (2005)\cite{17} who found a good correlation between sialic acid and each of cholesterol, LDL and TG.

Also, the results represent the Common characteristic features of atherogenic pattern of lipid profile which are the elevation of plasma triglycerides and VLDL-cholesterol, reduced HDL-cholesterol, and an increased number of small dense LDL particles.\cite{18}

The negative correlation which was observed between an acute-phase marker as CRP and HDL-C has been reported by others as well\cite{19, 20} HDL-C, a negative acute-phase reactant, consistently declines during the acute-phase response.\cite{21}

The main risk factors for the development and progression of diabetic retinopathy are the duration of diabetes, glycemic state, blood pressure and co-existing nephropathy.\cite{22}

In order to explore such associations between diabetic retinopathy and diabetic nephropathy, some parameters like serum creatinine, serum urea and urine albumin/creatinine ratio were measured. In all studied groups, the increase in both serum creatinine and urine albumin/creatinine ratio appeared to be parallel to the increase in serum sialic acid.

Only in patients with diabetic retinopathy (group 2 and 3), the increase in serum urea is parallel to the increase in serum sialic acid.

These results could refer to the fact that the increase in serum sialic acid is associated with the incidence of diabetes and with the development and the progression of the disease complications. The results supported the Studies done by El-Wakf, A. M., et al., (2011)\cite{23} who showed the association between the incidence of diabetic
complications (retinopathy, neuropathy and ischemic heart disease) and the development of diabetic renal disease starting by nephropathy.\textsuperscript{[24]} Other workers have presented albuminuria as a powerful predictor of progression of nephropathy in patients with type (2) diabetes.\textsuperscript{[25]}

In type (2) diabetes, microalbuminuria was associated with elevated levels of sialic acid.\textsuperscript{[26]} Albuminuria may reflect underlying renal expression of vascular damage, hypertension, endothelial dysfunction\textsuperscript{[27]}, and inflammation.\textsuperscript{[28]}

This may be explained via elevations of acute-phase proteins and/or inflammatory cytokines that may alter glomerular function.\textsuperscript{[29]} Alteration of glomerular function occurred either by TNF-\(\alpha\) which induces glomerular infiltration by leukocytes\textsuperscript{[30]} or by both TNF-\(\alpha\) and IL-1 which influence the metabolism of glycosaminoglycans\textsuperscript{[31]} which are components of the vascular endothelium and the glomerular basement membrane and are also involved in the etiology of microalbuminuria.\textsuperscript{[32]}

Therefore, it has become clear that albuminuria is not only indicator for diabetic renal disease, but also for progress to more advanced stages of the disease.\textsuperscript{[33]}

Both age and duration of diabetes showed a direct significant correlation with sialic acid in all studied groups i.e. the increase in the data of both parameters is parallel to the increase in total serum sialic acid in all groups.

Generally, it is known that the duration of diabetes is probably the strongest predictor for development and progression of retinopathy.\textsuperscript{[34]}

Because several diseases are known to increase serum sialic acid concentrations\textsuperscript{[35]}, one explanation for the increase of serum sialic acid with age would be a higher frequency of subclinically diseased individuals among the elderly.\textsuperscript{[36]}

Several SA- containing acute-phase reactants also increase with age, e.g., fibrinogen, C-reactive protein, \(\alpha_1\)-acid glycoprotein, \(\alpha_1\)-antichymotrypsin, \(\alpha_1\)-antitrypsin, and haptoglobin.\textsuperscript{[37, 38]}

Another explanation is dependent upon the increase in the production of cytokines from monocytes and macrophages\textsuperscript{[39]} and circulating acute-phase proteins\textsuperscript{[40]} IL-6 and TNF-\(\alpha\)\textsuperscript{[41]} with the increase in age.\textsuperscript{[5]}

**Conclusion**

The study showed statistical significant increase in total serum sialic acid in all diabetic patients but the increase was more prominent in patients with proliferative DR. There were significant increases in fasting glucose level, glycosylated HB\%, total cholesterol, triglycerides, LDL-C, serum urea, serum creatinine and urinary albumin/creatinine ratio in diabetic retinopathy patients ( mainly with proliferative retinopathy ). Serum sialic acid concentrations were increased in type 2 diabetics with and without complications and this increase was strongly related to the progression of retinopathy and occurrence of nephropathy. Further studies of acute phase response markers as indicators or predictors of diabetic Microvascular complications are therefore required.

**References**

patients with atherosclerosis of the coronary and peripheral arteries. Arteriosclerosis, thrombosis, and vascular biology, 19(10), 2355-2363.  


