Some Serum Cytokines (Adiponectin, Apolipoprotein B, hsCRP, IL-6) in a Cohort of Type 2 Diabetes Mellitus Patients

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ABSTRACT

Diabetes mellitus has over the years become a public health challenge and a complex disease which is characterized by chronic hyperglycemia that results in microvascular and macrovascular complications. The present study was designed to determine the serum IL-6, hsCRP, Adiponectin and apolipoprotein B levels in patients with type 2 diabetes mellitus (T2DM) and controls. A total of 180 subjects comprising 90 type 2 diabetic patients and 90 healthy controls were recruited for the study. Blood samples (7.0ml) were collected from each subject for the analysis of the cytokines, using Enzyme Linked Immunosorbent Assay kit obtained from Sunlong Diagnostic Ltd,China. Data was analysed using T-test, one way analysis of variance and the Turkey post hoc test with level of significance set at p < 0.05.The results revealed increase in hs CRP (10.22 $\pm$ 0.80ng/ml), , Apoliprotein B (300.07 $\pm$ 23.96ng/ml), IL-6 (21.49 $\pm$ 2.86ng/ml) in the T2DM patients compared to the hs CRP (2.80 $\pm$ 1.66ng/ml). Apoliprotein B (261.45 $\pm$ 5.69ng/ml) and IL-6 (4.83 $\pm$ 1.25ng/ml) for the controls. There was a decrease in the level of Adiponectin (6.70 $\pm$ 0.64ng/ml) in the T2DM patients compared to (11.04 $\pm$0.81ng/ml) for the controls. This finding supports the claim for cytokine deregulation in T2DM patients.

Keywords: Cytokines, Adiponectin, Apolipoprotein B, hs CRP, IL-6, Type2 Diabetic Mellitus

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by abnormal carbohydrate metabolism resulting in chronic hyperglycemia caused by defective insulin production, action or both (1,2). Type 2 diabetes mellitus (T2DM) is the most prevalent type of diabetes and accounts for about 90-95% of diabetes cases (3,5). Its global prevalence has increased from 4.7% (108 million) in 1980 to 9.3% (463 million) in 2019, postulated to increase to 10.2% (578 million) by 2020 and 10.9% (700 million) by 2045 (6,7). It is also estimated that 15.5% (9.8-27.8 million)

people have type 2 diabetes mellitus in Sub-Saharan African with Nigeria having the highest burden of cases(8).Chronic (low-grade) inflammation due to dysregulated inflammatory biomarkers has been described as a common feature of type 2 diabetes mellitus(9). Cytokines are important components in inflammatory processes(10). An understanding of the cytokine network creates a new era of understanding of the pathophysiology of type 2 diabetes mellitus and holds a great potential for anticytokine strategies for the treatment of type 2 diabetes(11). There is currently a paucity of scientific data on the

inflammatory cytokine levels in type 2 diabetic patients in the Enugu State University of Science and Technology Teaching Hospital, Enugu State, Nigeria.The present study was therefore designed to determine the serum concentrations of the inflammatory cytokines (Adiponectin, Apolipoprotein B, hs CRP, IL-6) in type 2 diabetic patients compared to controls.

MATERIALS AND METHODS
Study Area
This study was carried out in Enugu State, South East, Nigeria. Enugu State is made up of three senatorial zones namely Enugu East, Enugu West and Enugu North. The senatorial zones are divided into seventeen Local Government Areas comprising 450 communities. The State takes its name from its capital and largest city Enugu. It has an area of $7,161km^2$ with a population of 3,267,837 comprising mainly the Igbo tribe of the south eastern Nigeria; about 50% of which lives in rural areas.It lies between longitudes $6^o 30'$ E and $6^o55'$E and latitudes $5^o 15'$Nand $7^o15'$E.Enugu State University of Science and Technology Teaching Hospital is accessed by people from different socio-economic backgrounds being located at the centre of the city. The hospital serves as referral centre for different primary and secondary healthcare facilities in the State, hence its choice for the present study(12).

Study Design
This was a case-control study in which the type 2 diabetic patients served as the cases while apparently healthy volunteers severed as the controls.

Ethical Considerations
Ethical approval for the study was obtained from the Ethical Review Committee of the Enugu State University of Science and Technology Teaching Hospital, Enugu State, Nigeria(ESUT NP/C-MAC/RA/034/Vol.1/290).The subjects were informed about the purpose of the study and only those that gave their consent were recruited for the study.

Subject Recruitment
Subject's selection was based on a convenient sampling procedure from a population of diabetic patients who gave their consent to participate in the study.

Inclusion Criteria
1. All consenting type 2 diabetic patients on treatment were chosen as cases.
2. All consenting non-diabetic healthy adults were chosen as controls.

Exclusion Criteria
1. Nutritional anemia can be caused by reactive thrombocytosis, therefore male and female patients having mean hemoglobin (Hb) < 12g/dl and < 11g/dl respectively were excluded from the study
2. Non diabetic individuals with any diagnosed malignancy, thrombocytopenia, thrombocytosis or systemic disease were excluded.

Sample Size
The sample size for the study was calculated using the Leslie Kish formal (13)

$$n = \frac{Z^{\alpha 2}PQ}{D^2}$$

Where
n = minimum required sample size when the population is greater than 10,000.

Z^α= the α level of the coefficient interval or the standard normal deviate set at 1.96 corresponding to the 95% confidence interval.

P = the proportion in the target population estimated to have diabetes mellitus 5.8%(14).

D = the width of the confidence interval set at 0.05

Q = (1-P); the proportion of non-occurrence.

Substituting into the formula:

$$n = \frac{1.96 \times 1.96 \times 0.058(1-0.058)}{0.05 \times 0.05}$$

$$= \frac{3.8416 \times 0.054636}{0.0025}$$

$$= 83.95$$

but an estimate of 376 registered type 2 diabetic patients attended the clinic in the

last one year. Since this is less than 10,000, the sample size was adjusted using the formula.

$$nf = \frac{n + (n)}{1 \quad N} = \frac{84}{84}$$

$$\frac{84}{1+84/376} \quad \frac{84}{1.2} = 70$$

Considering a response rate of 90%, the sample size was adjusted to this response rate using the formula

$$Ns = n/r$$

Where

ns = adjusted sample size for response rate

n = calculated sample size

r = the anticipated response rate of 90% (0.9)

Substituting

$$ns = \frac{70}{0.9}$$

$$= 77$$

Therefore, a total of 180 subjects involving type 2 diabetic cases and apparently healthy control were recruited for the study.

Sample Collection

Seven milliliters (7mls) of venous blood sample was collected form each subject by venipuncture using a 10mls sterile disposable syringe. The blood was spun for 5 minutes at 3000 rpm. The serum was separated from the red cells using a dry clean Pasteur pipette into a dry clean plain specimen container. The serum was stored at -20°C for the analysis of the FBS, HbAIC, Adiponectin, hs CRP, IL-6 and Apolipoprotein B.

Glucose Estimation

Glucose oxidase method was used to estimate the level of fasting plasma glucose. This was done with the commercially available kit (Biologo.france, lot no 101205B, Batch no 8009) which has the standard for calibration measured at 500nm using spectrophotometer (SM 23A, England).

Principle

Glucose is oxidized by glucose oxidase (GOD) enzyme to form gluconic acid and hydrogen peroxide. The hydrogen peroxide in conjunction with peroxide dehydrogenase

(POD) reacts with chloro-4-phenol and 4-aminoantipyrine to form a red quinoamine. The absorbance of the colored complex is directly proportional to the glucose concentration in the specimen.

Calculation

Plasma glucose concentration was calculated using the expression.

Glucose concentration = Absorbance of assay/absorbance standard x

Concentration of standard in mmol/L

Reference Range

3.6 – 5.8 (mmol/L)

Glycated Hemoglobin (HbAIC) estimation

Ion exchange chromatographic method was used to estimate the levels of glycated hemoglobin using Fortress Diagnostic kits, England, lot no 102016, Batch no BXCO671A measured at 415nm using spectrophotometer (SMA 23A).

Principle

After preparing the hemolysate where the labile fraction was eliminated hemoglobin were retained by a cationic exchange resin. Hemoglobin AiC is specifically eluted after washing away the Hba 1 + b fraction and quantified by direct photometric reading at 415nm.

Circulation

% HbAIC = HbAIC total x V HbAIC/V Hb total.

The volume of HbAIC (VHbAIC) is 4ml and the volume of Ab total is 12ml.

Therefore, the relation below was deduced.

HbAIC/Hb total x 100/3

The results obtained was converted to international standards. To convent to the US National Slycohemoglobin Standardization Program certified method (NGSP or equivalent to the International Federation of Clinical chemistry Standardised method (IFCC) using the following formulas.

HbAICNGSP = 0.86 x % HbAIC Fortress + 0.24

HbAIC IFCC = 0.94 x % HbAIC Fortress -2.09

Reference Range

(Bcct/ngsp) HbAIC Degree of control

6.0 – 6.5 Target
6.5 – 7.0 Good control
>7.0 Poor control

Estimation of Adiponectin, Apoliprotein B, hs CRP and IL-6

These cytokines were determined using ELISA kit purchased from Sunlong Diagnostics Ltd, China.

Principle

The Enzyme Linked Immunosorbent Assay is a plate-based assay designed to detect analytes by assessing the conjugated enzyme activity through incubation with a substrate to produce a measurable product. Standard solution and sample (100ml each) were added to each well and the blanks left empty and incubated for 90 minutes at 37°C. The solution was removed, 100ml biotinylated detection antibody specific to the analyte was added to it and incubated for 60 minutes at 37°C.The solution was then aspirated, the wells washed thrice and 100ml HRP conjugate was added and left for 30 minutes at 37°C, after which the solution was aspirated and washed five times. Substrate reagent (90ml) was added and incubated for 15 minutes at 37°C, 100 μl of stop solution was added. The optical density (OD) of the blank well was set at zero. The absorbance OD of each well was read at 450nm using a microplate reader.Optical density values were proportional to the concentration of measured parameters.

Statistical Analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS Inc. Chicago) version 23.Statistical significance was defined as $p < 0.05$. Differences between the case and control group was determined using T-test, while differences among the groups were determined using one way analysis of variance and the Turkey Post-hoc test.

RESULTS

The fasting blood sugar (7.25±0.45mmol/L) of T2DM subjects was significantly higher (P = 0.020) compared with the fasting blood sugar of the control subjects (4.25±0.41). The glycated hemoglobin of T2DM subjects (9.68 ± 1.05%) was significantly higher (P = 0.038) compared to the glycated hemoglobin of the control subjects (4.22±0.39%). The hsCRP (10.22 ± 0.80 ng/ml) ofT2DM subjects was significantly higher (P = 0.001) compared with the hsCRP of the control subjects (2.80 ± 1.66 ng/ml). The adiponectin (6.70 ± 0.64ng/ml) of T2DM subjects was significantly lower (P = 0.000) compared to the adiponectin of the controls subjects (11.04±0.81ng/ml). The interleukin -6(21.4 ± 2.86 ng/ml) of T2DM subjects was significantly higher (P = 0.031) compared to the interleukin -6 of the control (4.83 ± 1.25ng/ml). The Apolipoprotein-B (300.07 ± 23.96ng/ml) of T2DM subjects was significantly higher (0.028) compared to the Apolipoprotein-B levels of the control subjects (261.45 ± 5.60ng/ml) (Table 4.1). The post hoc analysis confirmed significant differences in the cytokine levels between the T2DM cases and controls and no significant difference between the male and female cases as well as the male and female controls (Table 4.2).

Table 4.1: Serum cytokine concentrations in the T2DM subjects compared to the control subjects

Parameters	T2DM (n = 90)	Controls (n = 90)	T-test (p-value)
FBS (mmol/L)	7.27 ± 0.45)	4.25 ± 0.41)	20.020*
HBAIC(%)	9.68 ± 1.05)	4.22 ± 0.39	0.038*
HsCRP (ng/ml)	10.11 ± 0.80	2.80 ± 1.66	0.001*
Adiponectin(ng/ml)	6.70 ± 0.64)	11.04 ± 0.81	0.000*
IL-6(ng/ml)	21.49 ± 2.86	4.83 ± 1.25)	0.031*
ApoB (ng/ml)	300.07 ± 23.96	261.45 ± 5.69)	0.028*

Key: FBS = fasting Blood Sugar, HBAIC = glycated hemoglobin, hsCRP = high sensistivity C-reactive protein, IL-6 = Interleukin 6, ApoB = Apolipoproten B< *signifant at P < 0.05, Data expressed as Mean ± SD.

Table 4.2: Posthoc analysis of serum cytokine concentrations of T2DM cases and controls

Group	FBS	HbAIC	hsCRP	Adiponectin	IL-6	Apolipoprotein B
Male cases vs female case	0.210	0.453	0.106	0.229	0.616	0.311
Male cases vs male control	0.010	0.001	0.016	0.001	0.000	0.008
Males cases vs female control	0.003	0.011	0.004	0.000	0.031	0.022
Female cases vs male control	0.010	0.001	0.015	0.006	0.012	0.000
Female cases vs female control	0.001	0.036	0.007	0.001	0.024	0.013
Male control vs female control	0.055	0.903	0.619	0.207	0.461	0.111

Key: FBS = fasting Blood sugar, HBAIC = glycated hemoglobin, hsCRP = high sensitivity C-reactive protein, IL-6 = Interleukin 6, ApoB = Apolipoprotein B,

DISCUSSION
The increase in fasting blood sugar and glycated hemoglobin in the type 2 diabetes mellitus patients reflects the presence of hyperglycemia caused by insulin resistance and poor glycemic control in T2DM(15). Insulun resistance is characterized by the inability of cells of the body to respond adequately to normal levels of insulin(16). The increase in high sensitivity C-reactive protein (hsCRP) level may be due to systemic inflammation secondary to hyperglycemia. This is similar to the findings of Aljack et al (17) and Sauda et al (18) who reported increased high sensitivity C-reactive protein in patients with T2DM.High sensitivity C-reactive protein (hsCRP) has also been suggested to increase the risk of cardiovascular complications and renal dysfunction in T2DM(19). Reduced levels of hsCRP are reported among metformin users, a reduction that may be explained by the reduced insulin levels as well as reduction in insulin resistance(19). It has long been suggested that systemic chronic inflammation mediated through IL-6 is central in the pathophysiology of T2DM. Taking for instance, it is well established that chronic inflammation promotes insulin resistance and blocking IL-6 with tocilizumab improved insulin resistance in persons with T2DM(20). This finding correlates with an increased level of IL-6 recorded in the T2DM patients in the present study. It has been described that adiponectin regulates the sensitivity of peripheral tissues to insulin, glucose and lipid metabolism.In the present study, the levels of serum adiponectin was lower in the T2DM cases than the healthy controls.This is comparable to the findings of those studies which described lower adiponectin

in T2DM patients (21, 22, 23). Conversely, some studies described high adiponectin concentrations in T2DM patients(24). Apolipoprotein B is the principal protein moiety of low density lipoprotein and very low density lipoproteins with its high concentrations associated with increased cardiovascular disease in T2DM. The finding of increased apolipoprotein B concentrations in the T2DM patients in the present study correlates with similar findings in other studies which reported increased levels of apolipoprotein B in T2DM(25),suggesting dyslipidemia in these group of patients.

CONCLUSION
The result of the present study supports the claim for dysregulated cytokine network in patients with T2DM.Thus, the Apoliprotein B, IL-6, hsCRP, and Adiponectin could be applied as an additional diagnostic and prognostic marker for T2DM.

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