Decreased Plasma Adropin: A Possible Risk Factor for Metabolic Syndrome in Type 2 Diabetic Patients in South-West, Nigeria

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ABSTRACT

Objective: This study was designed to evaluate the relationship between plasma adropin, glucose, lipids and lipoproteins as well as anthropometric indices in T2DM and control participants.

Materials and Methods: A total of 130 patients (37 males, 93 females) 42-70 years diagnosed as T2DM, were recruited. Forty-three apparently healthy volunteers (16 males, 27 females) 30-55 years were included as controls. Fasting blood samples were collected from all participants and dispensed in appropriate anticoagulant bottles for biochemical assays. The anthropometric indices were determined using standard procedures. Plasma adropin was determined by ELISA technique. Plasma glucose, lipids, lipoproteins were determined using standard methods.

Results: The results showed significantly higher values in waist circumference, hip circumference, body fat percentage, pulse rate ($p = 0.001$), waist to hip ratio ($p = 0.006$) and systolic blood pressure ($p = 0.05$) compared with the controls. There were remarkable significantly lower values in high density lipoprotein cholesterol (HDLc) and adropin ($p = 0.001$) compared with the controls. Significantly higher values were obtained in plasma glucose, total cholesterol (TC) triglyceride, low density lipoprotein cholesterol/HDLc and TC/HDLc ratios ($p = 0.001$) compared with the controls. **Conclusion:** The results provide evidence that lower levels of adropin, HDLc coupled with higher levels of fasting plasma glucose, TC, triglyceride, low density lipoprotein cholesterol/HDLc, TC/HDLc ratios are associated with metabolic syndrome in T2DM; and are possible predisposing factors to early risk of CVD event. Future treatment of T2DM patients with synthetic adropin may be beneficial and worthwhile. Further study is warranted.

Keywords: Adropin, diabetes mellitus, dyslipidaemia, hyperglycaemia

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INTRODUCTION

Diabetes mellitus is a group of metabolic disorders of carbohydrate metabolism in which glucose is underutilised, overproduced or both, leading to hyperglycaemia (1). It is characterised by increase in the plasma glucose along with alterations in fat and protein metabolism, associated with defects in insulin secretion and or insulin action or both (2,3,4). This metabolic disease is one of the most common endocrine disorders affecting almost 8.5 per cent of the world's population (5).

It is one of the killer diseases waging war against the survival, growth, and development of human beings globally (6). It has assumed epidemic proportion both in developed and developing nations of the world (7). It has become one of the world's most important public health problems (8). Available report has shown that Nigeria has the highest burden of DM in Africa with 3.95 million cases (9). The high burden of DM in Nigeria is largely attributable to cardiovascular diseases which account for 50 per cent of all DM deaths (10).

The dynamics of the T2DM epidemic are changing rapidly. Once a disease of the West, it has now spread to every country in the world. Once a "disease of affluence", it is now increasingly common among the poor (11). Type 2 diabetes mellitus is characterised by hyperglycaemia, insulin resistance and relative impairment in insulin secretion (12).

Adropin is a peptides hormone that has been reported to improve dyslipidaemia, hyperglycaemia and hepatic steatosis associated with T2DM through maintenance of metabolic homeostasis (13). Adropin is predominantly produced by the liver and hypothalamus region of the brain. It is composed of 76 highly conserved amino acid residues peptide with a molecular weight of 4.5 kDalton (14). It has been suggested to act as an endocrine factor that plays important roles in metabolic regulation, insulin sensitivity and endothelial functions (15) *vis a vis* regulating glucose and fatty acid metabolism (16).

Recently, adropin was reported to promote preferential use of glucose over fat in fuel selection (17). Use of synthetic adropin in laboratory animals with obesity and T2DM revealed improve glucose homeostasis and dyslipidaemia in these animals (18). Conversely, reduced plasma level has been linked with hyperglycaemia and dyslipidaemia in T2DM animals. Since this peptide is an important hormone in the metabolism of glucose, will alteration in adropin level affect plasma glucose concentration in T2DM patients in this environment? This study was designed to evaluate the relationship between plasma adropin, glucose and lipids as well as lipoproteins in Nigerians T2DM and control participants.

MATERIALS AND METHODS

Study Population:

The subjects consisted of 130 patients (37 males, 93 females) with type 2 diabetes mellitus as diagnosed by the attendant Consultant Endocrinologist based on clinical and laboratory findings. The patients were attending Endocrinology Clinic at Lagos State University Teaching Hospital, Ikeja, Lagos State, Nigeria. Ethical approval was obtained from the Health Research and Ethics Committee of the Lagos State University Teaching Hospital, Ikeja (Reference Number:

LREC/10/06/545/2015). Informed consent was obtained from each participant.

Inclusion Criteria of Participants: All confirmed T2DM participants within the ages of 30-70 years, all confirmed T2DM participants on treatment with hypoglycaemic drugs**,** participants within the ages of 30 and 70 years without T2DM as controls.

Exclusion Criteria of Participants: Participants on herbal mixtures, outside the allowable ages of 30-70 years, participants with underlying disease like chronic liver and renal disease, malignant diseases and those on lipid lowering drugs, participants on immune suppressive drugs, steroids, participants with medical history of stroke, pregnant women and participants who did not consent to be enrolled in the study.

Anthropometric Measurement:
Heights were measured to the nearest centimetres with subjects in standing positions, without foot wears using a non-stretchable measuring tape. The participants were made to stand against the wall with their heels touching the wall while their heights were directly read off a graduated tape rule. The OMRON BF-400 body fat monitoring/scale was used for the determination of body weight while height in centimetres, age in years and gender were imputed into the OMRONBF-400 body fat monitor/scale to determine body fat percentage and BMI.
Waist circumference in centimetres was measured at the midpoint between the lower border of the rib cage and the iliac crest of the hip bones, measured in the horizontal plane with subjects standing during inspired expiration using a non-stretchable tape. Hip circumference in centimetres was taken by placing non-stretchable measuring tape around the point with the maximum circumference over the buttocks. The non-stretchable measuring tape was held firmly to ensure its horizontal position. The pulses per minute, systolic and diastolic blood pressures in millimetres of mercury were measured two times using OMRON electronic blood pressure monitor and the average taken.

Blood Sample Collection:
An overnight fasting (10-12 hours) blood samples were collected from each participant and dispensed into Sodium Fluoride Oxalate bottles (2mg/ml), Potassium-Ethylene-diamine-tetra-acetic acid (kEDTA)-1.5mg/ml (19). The blood samples were centrifuged at 1,000 rpm for 15 minutes. The plasma was carefully separated from cells within 30 minutes of blood collection and aliquot stored at -20ºC until analysed.

Methods of Analysis:
The plasma glucose concentration was estimated using the Glucose Oxidase method (20). Total cholesterol was estimated based method of Allain *et al*. (21), high density lipoprotein cholesterol was estimated after precipitating out other lipoproteins with Phosphotungstic acid method and triglyceride was estimated using enzymatic-colorimetric method (22). Low density lipoprotein cholesterol was calculated using Friedewald *et al*. formula (23). The plasma adropin concentration was estimated using the Enzyme-Linked Immunosorbent Assay method (24). Commercial Quality control samples were included in every batch of assay to check for coefficient of variations.

Statistical Analysis:

The data obtained were subjected to statistical analysis using Statistical Package for Social Sciences (SPSS) software version 20. The results were expressed as Mean ± SEM. Student t-test was used to compare statistical differences between means and Correlation between quantitative variables were assessed using Pearson's correlation coefficient. The difference was regarded as significant at $p \leq 0.05$. The results are presented in tables.

RESULTS:

Table 1: Shows the biophysical parameters of all subjects. There were significantly higher values in body fat percentage, waist circumference, hip circumference, pulse (p = 0.001), waist to hip ratio (p = 0.006) and systolic blood pressure (p = 0.05) compared with the control values. T2DM participants were shorter than controls. Diastolic blood pressure was significantly lower in T2DM (p = 0.004) compared with the control value. There were no significant differences in body weight and body mass index compared with the control values.

Table 1: Biophysical Parameters of all Subjects (Mean ± SEM)

Variable	T2DM (n = 130)	Controls (n = 43)	t-value	p-value
Weight (kg)	72.93 ± 1.23	70.68 ± 1.52	0.98	0.329
Height (cm)	160.57 ± 0.84	165.17 ± 1.17	-2.86	0.005*
BF%	37.71 ± 0.73	32.10 ± 1.47	3.69	0.001*
BMI (kg/m^2)	29.63 ± 1.29	26.02 ± 0.62	1.59	0.113
WC (cm)	100.08 ± 0.92	82.51 ± 1.32	9.90	0.001*
HC (cm)	107.75 ± 0.90	94.14 ± 1.81	7.27	0.001*
WHR	0.93 ± 0.01	0.90 ± 0.01	2.80	0.006*
SBP (mmHg)	148.25 ± 7.90	121.05 ± 1.05	1.97	0.050*
DBP (mmHg)	73.64 ± 0.92	78.47 ± 0.90	-2.88	0.004*
Pulse (second)	82.35 ± 1.16	73.33 ± 0.71	4.38	0.001*

p = Level of significance * = Significant
BF% = Body Fat Percentage T2DM = Type 2 Diabetes Mellitus
BMI = Body Mass Index WC = Waist Circumference
DBP = Diastolic Blood Pressure WHR = Waist to Hip Ratio
HC = Hip Circumference SEM = Standard Error of the Mean SBP = Systolic Blood Pressure

Table 2: Shows the biochemical parameters of all subjects. There were significantly higher values in fasting plasma glucose, total cholesterol, triglyceride, LDLc/HDLc ratio and TC/HDLc ratio compared with corresponding controls. There were significantly lower values in plasma HDLc ($p = 0.001$) and adropin ($p = 0.001$) compared with the controls. There was no significant difference in plasma LDLc compared with the control value.

Table 2: Biochemical Parameters of all Subjects (Mean ± SEM)

Variable	T2DM (n = 130)	Controls (n = 43)	t-value	p-value
FPG (mmol/L)	7.24 ± 0.25	4.88 ± 0.07	5.48	0.001*
TC (mmol/L)	4.07 ± 0.08	3.61 ± 0.11	2.87	0.001*
TG (mmol/L)	1.16 ± 0.07	0.66 ± 0.03	4.38	0.001*
HDLc (mmol/L)	1.15 ± 0.03	1.61 ± 0.08	-6.58	0.001*
LDLc (mmol/L)	2.38 ± 0.07	2.17 ± 0.09	1.53	0.127
LDLc/HDLc	2.26 ± 0.09	1.56 ± 0.12	4.06	0.001*
TC/HDLc	3.73 ± 0.10	2.47 ± 0.13	6.90	0.001*
ADROPIN (pg/ml)	598.24 ± 23.93	827.22 ± 34.57	-4.96	0.001*

p = Level of significant
* = Significantly Different
TC = Total Cholesterol
FPG = Fasting Plasma Glucose
HDLc = High Density Lipoprotein Cholesterol
LDLc = Low Density Lipoprotein Cholesterol
SEM = Standard Error of the Mean
TG = Triglyceride
T2DM = Type 2 Diabetes Mellitus

Table 3: Shows Pearson's correlation coefficients of the biophysical and biochemical parameters in type 2 diabetes mellitus patients.
Weight was correlated with diastolic blood pressure (r = 0.174, p = 0.048), waist circumference (r =0.813, p = 0.001), hip circumference (r = 0.790, p = 0.001), waist to hip ratio (r = 0.216, p = 0.014). Body mass index was correlated with pulse (r = 0.220, p = 0.012) and inversely correlated with adropin (r = -0.190, p = 0.031). Waist circumference was correlated with hip circumference (r = 0.792, p = 0.001) and waist to hip ratio (r = 0.483, p = 0.001). Hip circumference was positively correlated with diastolic blood pressure (r = 0.195, p = 0.027). Fasting plasma glucose was negatively correlated with adropin (r = -0.176, p = 0.045). Total cholesterol was correlated with triglyceride (r = 0.312, p = 0.001), high density lipoprotein cholesterol (r = 0.374, p = 0.001) and low density lipoprotein cholesterol (r = 0.611, p = 0.001).

Table 3: Pearson's correlation coefficients of the biophysical and biochemical parameters in T2DM patients.

Variable	r	p-value
Weight (kg) – DBP (mmHg)	0.174	0.048*
Weight (kg) – WC (cm)	0.813	0.001**
Weight (kg) – HC (cm)	0.790	0.001**
Weight (kg) – WHR	0.216	0.014*
BMI (kg/m^2) – Pulse (second)	0.220	0.012*
BMI (kg/m^2) – Adropin (ng/ml)	-0.190	0.031*
WC (cm) – HC (cm)	0.792	0.001**
WC (cm) – WHR	0.483	0.001*
HC (cm) – DBP (mmHg)	0.195	0.027*
FPG (mmol/L) – Adropin (ng/ml)	-0.176	0.045*
TC (mmol/L) – TG (mmol/L)	0.312	0.001**
TC (mmol/L) – HDLc (mmol/L)	0.374	0.001**
TC (mmol/L) – LDLc (mmol/L)	0.611	0.001**

****** Correlation is significant at the 0.01 level-2 tailed p = Level of Significance
* Correlation is significant at the 0.05 level-2 tailed, T2DM = Type 2 Diabetes Mellitus
FPG = Fasting Plasma Glucose BMI = Body Mass Index
DBP = Diastolic Blood Pressure HC = Hip Circumference
LDLc = Low Density Lipoprotein Cholesterol HDLc = High Density Lipoprotein Cholesterol
TC = Total Cholesterol WC = Waist Circumference WHR = Waist to Hip Ratio

DISCUSSION

The participants studied were diagnosed as having T2DM based on clinical and laboratory investigations. They were placed on treatment based on the outcome of laboratory investigations. In this study, the waist circumference, hip circumference and waist to hip ratio measurements were significantly higher in T2DM participants than the controls. An earlier study in this environment on type 2DM showed raised anthropometric indices in these patients, similar to the current finding. According to Global Burden of Disease Risk Factors Collaborators (24), higher waist circumference is associated with increased cardiovascular disease risk in T2DM; however, this relationship is thought to vary in different populations (25). The T2DM patients that participated in the present study showed statistically significant higher systolic blood pressure and pulse values even though they were on medications; these are possible indices of cardiovascular disease risks associated with metabolic syndrome.

Notable change was obtained in fasting plasma glucose irrespective of the fact that all the T2DM patients that participated in this study were on at least one hypoglycaemic drug; it is either the drugs were not achieving the desired effects or the patients were noncompliance. From the record, all the T2DM patients that participated in this study were regular attendees at the Endocrinology Clinic. The preponderance of fasting plasma glucose could predispose the T2DM subjects to glucotoxicity; which is one of the metabolic risk factor in diabetes mellitus. It could be speculated from the result of this study that the primary purpose for administering drugs to these T2DM may not have been achieved because glucose homeostasis was not maintained. Earlier studies (26,27) noted that complications in T2DM patients are mainly due to chronic hyperglycaemia that exerts its effects on the health of people living with the disease through several mechanisms such as dyslipidaemia, platelet activation, and altered endothelial metabolism.

The results of lipids and lipoproteins showed remarkable changes compared with the controls. There were significantly higher values in plasma total cholesterol, triglyceride and notable lower value in high density lipoprotein cholesterol in T2DM subjects when compared with the control values. Dyslipidaemia as a metabolic abnormality is frequently associated with T2DM, and these abnormalities in lipid metabolism were reported in T2DM patients with metabolic syndrome; which are possible likelihood of early event of cardiovascular disease (28).

Risk Index-I (CRI-I) which is calculated as (TC/HDL-C) and Castelli Risk Index-II (CRI-II) as (LDL-C/HDL-C) as reported by Ridker *et al*. (29) were also used to assess the cardiovascular risk of the subjects recruited for this study. As a result of significantly higher values obtained in Castelli Risk Indices compared with the controls, coupled with significantly higher values in total cholesterol, triglyceride and notable lower value in high density lipoprotein cholesterol, there is high degree of probability that most of the T2DM subjects that participated in this study could be predisposed to developing premature coronary artery disease as these risk indices are strong indicators associated with metabolic syndrome.

The significantly lower value in adropin found among the T2DM subjects in this study could be responsible for the hyperglycaemia and dyslipidaemia. Butler *et al.* (16) reported that lower level of adropin may be an indication of risk for insulin resistance in obesity and consequently, an increased risk for metabolic diseases including T2DM. In this study, plasma adropin was inversely correlated with fasting plasma glucose. Recently, adropin was reported to promote preferential use of glucose over fat in fuel selection (17). Use of synthetic adropin in laboratory animals with obesity and T2DM showed improve glucose homeostasis and decreased lipid abnormities in these animals (18). It could be inferred therefore, that the lower level of plasma adropin in T2DM patients in the present study, could in part be responsible for the hyperglycaemia and dyslipidaemia. These changes are predisposing factors to metabolic imbalance as a result of hyperglycaemia *vis-a-vis* low level of adropin. Interestingly**,** adropin was inversely correlated with BMI suggesting an imbalance in homeostasis and thus enhancing body weight accumulation. There is a strong indication that adropin plays a role in glucose homeostasis and invariably in metabolic syndrome associated with diabetic mellitus. Alteration in adropin level could have affected plasma glucose concentration in T2DM patients in this study.

CONCLUSION

In conclusion, the findings in this study provide evidence that low level of adropin associated with T2DM patients who participated in this research, could be a predisposing factor in part, to hyperglycaemia, dyslipidaemia, metabolic imbalance and metabolic syndrome; which could expose them to possible increased cardiometabolic risk.

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REFERENCES

1. David B Sacks. Diabetes Mellitus. In: Rifai N, Horvath AR, Wittwer C (Eds), Tietz textbook of clinical chemistry and molecular diagnostics (6th edition). Elsevier Inc: Missouri, 2018; pp 1160-1200.

2. Wu Y, Ding Y, Tanaka Y, Zhang W. Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. Int J Med Sci, 2014; 11(11): 1185-1200.

3. Lebovitz HE. Oral anti-diabetic agents. In: Kahn CR, Weir GC. (Eds), Josin's Diabetic Mellitus, 13th ed., vol. 29. Lee and Febiger: Philadelphia, 1994; pp 508-524.

4. Andreoli TE, Carpenter CCJ, Plum F, Smith LH. Diabetes mellitus. In: Dyson J (Ed), Cecil Essential of Medicine, 2nd edition, vol. 1. WB Saunders: Philadelphia, 1990; pp 496-505.

5. World Health Organisation. Global report on diabetes. Geneva, Switzerland, 2016.

6. Harande YI. Exploring the literature of diabetes in Nigeria: a bibliometric study.

African Jour of Diabetes Med, 2011; 19(2): 1-4.

7. Thévenod Frank. Pathophysiology of Diabetes Mellitus Type 2: Roles of Obesity, Insulin Resistance and β-Cell Dysfunction in:Masur K, Thévenod F, Zänker KS. (Eds), Diabetes and Cancer: Epidemiological Evidence and Molecular Links, Karger: Basel, 2008; pp 1-18

8. Bhargavi SK, Maruthi-Prasad BV, Vishwanath HL. Serum leptin as a risk factor for diabetes mellitus. International Jour of Pharm and Biol. Scs, 2013; 3(3): 252-259.

9. Oputa RN, Chinenye S. Diabetes in Nigeria- A translational medicine approach. African Journal of Diabetes Medicine, 2015; 23(1): 7-10.

10. Ojobi JE, Odoh G, Aniekwensi E, Dunga J. Mortality among type 2 diabetic in-patients in a Nigerian tertiary hospital. African Journal of Diabetes Medicine, 2016; 24(2): 17-20.

11. Frank B Hu. Globalisation of diabetes: The role of diet, lifestyle, and genes. Diabetes Care, 2011; 34: 1249-1257.

12. Harris MI. Impaired glucose tolerance in the United States population. Diabetes Care, 1989; 12: 464.

13. Kumar KG, Zhang JG, Su RJ, McGuinness OP, Halem HH, Culler MD *et al*. Adropin Deficiency Is Associated With Increased Adiposity and Insulin Resistance. Obesity, 2012; 20(7): 1394–1402.

14. Kumar KG, Trevaskis JL, Lam DD, Sutton GM, Koza RA, Chouljenko VN *et al*. Identification of adropin as a secreted factor linking dietary macronutrient intake with energy homeostasis and lipid metabolism. Cell Metab, 2008; 8: 468-481.

15. Wong C, Wang Y, Lee JTH, Huang Z, Wu D, Xu A, Lam KSL. Adropin is a brain membrane-bound protein regulating physical activity via NB-3/Notch signalling pathway in mice. J. Biol. Chem, 2014; 289(37): 25976-25986.

16. Butler AA, Tam SC, Stanhope LK, Wolfe MB, Ali MR, O'Keeffe M *et al*. Low Circulating Adropin Concentrations with Obesity and Aging Correlate with Risk Factors for Metabolic Disease and Increase after Gastric Bypass Surgery in Humans. Journal of Clinical Endocrinology and Metabolism, 2012; 97(10): 3783–3791.

17. Gao S, McMillan RP, Zhu Q, Lopaschuk GD, Hulver MW, Butler AA. Induced obese mice with insulin resistance. Molecular Metabolism, 2015; 4: 310-324.

18. Lovren F, Pan Y, Quan A, Singh KK, Shukla PC, Gupta M *et al*. Adropin is a novel regulator of endothelial function. Circulation, 2010; 122: S185–S192.

19. Bolarin Adebayo M. Bolarin's Aids to Chemical Pathology. O & A Publications: Ibadan, 2012; pp 10-12.

20. Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J. Clin Pathol, 1969; 22(2): 158-161.

21. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic

determination of total serum cholesterol. Clin Chem, 1974; 20(4): 470-475.

22. Bucolo G, David H. Quantitative determination of serum triglycerides by the use of enzymes. Clin Chem, 1973; 19(5): 476-482.

23. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem, 1972; 18(6): 499-502.

24. Global Burden of Disease Risk Factors Collaborators. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet, 2015; 386(10010): 2287–2323.

25. Vazquez G, Duval S, Jacobs DR, Jr, Silventoinen K. Comparison of body mass index, waist circumference and waist/hip ratio in predicting incident diabetes: a meta-analysis. Epidemiologic Reviews, 2007; 29: 115–128.

26. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature, 2001; 414(6865): 813–820.

27. Taskinen MR. Diabetic dyslipidaemia: from basic research to clinical practice. Diabetologia, 2003; 46(6): 733–749.

28. Krauss RM. Lipids and lipoproteins in patients with type 2 diabetes. Diabetes Care, 2004; 27(6): 1496–1504.

29. Ridker PM, Stampfer MJ, Rifai N. Novel risk factors for systemic atherosclerosis: a comparison of C- reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. JAMA, 2001; 285: 2481–2485.