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Estimation of pancreatic enzymes, zinc transporter 8, zinc, and selenium could improve the diagnosis of type 2 DM

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***Abstract*---**Diabetes mellitus (DM) is a chronic metabolic disorder which could lead to debilitating conditions due to deficiency or lack of insulin. The study aimed to evaluate the levels of glucose, amylase, glutamic acid decarboxylase, zinc, selenium and zinc transporter 8 in type 2 DM. A total of 90 subjects were investigated comprising 45 type 2 DM subjects and 45 apparently healthy individuals age-matched. Fasting plasma glucose and amylase were determined colorimetrically; glutamic acid carboxylase and zinc transporter 8 were determined

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using ELISA technique while zinc and selenium levels were estimated using AAS. The results showed significant increase in the levels of glucose, GAD and zinc in type 2 DM compared with control ($p < 0.000$, 0.037 and 0.004 respectively) whereas the levels of amylase and zinc transporter 8 showed significant decrease ($p = 0.001$ and 0.043 respectively). There was no significant difference in the parameters based on gender. There was significant positive correlation ($p=0.031$) between selenium and zinc/selenium ratio. The study concluded that assessing the levels of these parameters especially zinc transporter 8, GAD, zinc, and selenium could provide more insight to the diagnosis of type 2 DM and also be targeted in developing therapeutic option for type 2 DM.

***Keywords*---**glutamic acid carboxylase, zinc transporter 8, zinc, type 2 DM

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia as a result of deficiency or lack of insulin, or both (Assis and Nobrega, 2022). The aetiology of type 2 diabetes mellitus (T2DM) is highly complex and multifaceted but in its simplest form, is caused by insulin resistance (IR), hyperglycaemia, pancreatic dysfunction and beta-cell failure (Pajvani and Accili, 2015; Adulcikas *et al.,*2019). Diabetes mellitus is one of the main causes of cardiovascular dysfunction, visual impairment, kidney failure, and removal of lower limbs (Akalu and Birhan, 2020). Type 2 diabetes mellitus, mainly appears in adulthood and it is the result of insulin resistance and / or relative insulin deficiency (Galicia-Garcia *et al.,*2020).

Several biochemical changes may occur before the development of type 2 DM. Amylase is an exocrine enzyme that is produced by pancreatic acinar cells, and low serum amylase levels may be associated with endocrine diseases, such as metabolic syndrome and diabetes (Zhuang *et al.,*2016). It has been hypothesized that low serum amylase levels may be associated with impaired islet β cell function in type 2 diabetes (Kabadi, 2021; Cinti *et al.*, 2021). Zinc transporter 8 (ZnT8) out of the family of Zinc transporters is the most strongly expressed Zn^{2+} transporter in the islet of Langerhan. ZnT8, which is highly and selectively expressed on the secretory granule of pancreatic islet β and α cells, has been implicated in the risk of developing Type 2 Diabetes (Yi *et al.,*2016). ZnT8 is the major protein in human pancreatic islet cells, responsible for both intracellular zinc buildup in insulin-containing vesicles and insulin secretion control. Insulin, is stored as a hexamer containing two zinc ions in the β-cells of the pancreas and released into the portal venous system at the time of degranulation. It has been shown that there is the association between type 2 diabetes and mutations in the SLC30A8 zinc transporter (ZnT8), which transports zinc into secretory granules (Yi *et al*, 2016; Fukunaka & Fujitani, 2018). In response to hyperglycemic stimuli, ZnT8 down regulated cells had lower insulin content and lower insulin release. However, it was stated that the lack of ZnT8 expression, on the other hand, had no effect on insulin biosynthesis, insulin content, or glucose metabolism, but it

did help to improve the packaging efficiency of stored insulin (Shan *et al.,*2014). Glutamic acid decarboxylase (GAD) is an enzyme which is present in the pancreas and the nervous system, and its roles include relaxing the muscles and helping the pancreas function, among other processes. GAD can also trigger the immune system to produce autoantibodies against healthy cells (Jun *et al.*, 2000; Mendivil *et al.,* 2017). Moreso, Zinc transporter 8 (ZnT8) autoantibodies (ZnT8A), along with GAD65 autoantibodies (GADA), islet antigen-2 autoantibodies (IA-2A), and insulin autoantibodies (IAA), were identified as one of the four major islet autoantibodies (Williams and Long, 2019).

Zinc accumulates in glucagon-producing α cells of the pancreas under low and high glucose circumstances via both Ca^{2+} channels and other Zinc transporting pathways, inhibiting glucagon release. Furthermore, during hypoglycaemia, the primary signal that triggers glucagon secretion may be the recognition by α cells of a sudden decrease in Zinc in the islet peri-portal circulation, which closes α cell ion channels, allowing calcium to enter and stimulate glucagon production (Ishihara and Wollheim, 2016). Selenium (Se) is essential for the proper functioning of several physiological functions in the human body. Due to its antioxidant characteristics, it also plays a critical role in the pathogenesis and pathophysiology of a variety of ailments, including oxidative stress, inflammation, apoptosis, reproductive problems, diabetes, thyroid issues, cancer, and immunological responses (Giri *et al.,*2018; Kieliszek *et al.,* 2022). Selenium is an insulin-mimetic element, according to several studies, since it regulates enzymes in the insulin signaling cascade, the production of lipogenic enzymes, and carbohydrate metabolism in the liver (Ogawa-Wong *et al.,* 2016). It has also been reported that adequate concentrations of selenium play a key role in the secretion and action of insulin, but an excess of selenium in the body is associated with the pathogenesis of insulin resistance and the development of diabetes mellitus (Fontenelle *et al.,* 2018). Many pathological disorders are caused by rising Se deficiency in various parts of the world (Barchielli *et al.,* 2022). Selenium administration has been shown to increase the number of endocrine islets, as well as the cellularity and viability of the pancreatic tissue (Xu *et al.,*2022). Thus, the study aimed at assessing the levels of fasting blood glucose, serum amylase, zinc transporter 8, glutamic acid decarboxylase (GAD), zinc, selenium and zinc : selenium ratio in type 2 diabetic and control subjects because of their impact in hyperglycemic condition.

Materials and Methods

Ethical approval was obtained from Human Research and Ethics Committee, Federal Teaching hospital, Ido-Ekiti, Nigeria with clearance number ERC/2022/06/22/799B. The study is a case - control investigation conducted on type 2 diabetic patients between the ages of 30 – 70 years and age matched control subjects. All the subjects gave their consents to participate in the study. A total of 92 blood samples were collected from diabetic and non diabetic subjects and the samples consisted of 50 diabetic and 42 control samples. Fasting venous blood samples of 3 milliliters were collected, part of the sample was dispensed into fluoride oxalate bottle for analysis of glucose level while the remaining blood sample was put into plain sample container for the analysis of other parameters. The samples were separated and centrifuged at 12000 rpm for 5 minutes to

separate plasma or serum from cells. The samples were kept frozen until they were ready for analysis except the samples for fasting blood glucose that were analyzed immediately.

Estimation of glucose

Glucose was determined by using enzymatic method.
Principle:
Glucose oxidase catalyzes the oxidation of glucose to give hydrogen peroxide and gluconic acid. In the presence of peroxidase, the hydrogen peroxide is broken down to oxygen and water. Oxygen reacts with 4-aminophenazone and phenol to give a pink color and absorbance of the color produced is measured at 505nm wavelength using a spectrophotometer. The intensity of the light path is directly proportional to the amount of glucose concentration present in the sample (Trindler, 1969).

Procedure:
1000µl of Glucose Reagent and 10µl of sample/standard was pipetted into test tube and incubated at 37^{o}c for 15 minutes. The intensity of color was measured at 505 nm using a spectrophotometer.

Estimation of amylase using enzymatic method

Principle
α-Amylase (AMY) hydrolyzes the 2-chloro-4-nitrophenyl-α-*D*-maltotrioside (CNPG3) to release 2-chloro-4-nitrophenol (CPNP) and form 2-chloro-4-nitrophenyl-α-*D*-maltoside (CNPG2), maltotriose, and glucose. The rate of formation of the 2-chloro-4-nitrophenol can be detected spectrophotometrically at 404 nm to give a direct measurement of α-amylase activity in the sample (Lorentz, 2000).

Procedure:
Sample of 20ul and 500ul of amylase reagent (R1 buffer) was pippeted and mixed thoroughly. 100ul of substrate (R2) was added, and the solution was mixed and read at 405 nm.

Estimation of Serum Zinc Transporter 8 using Enzyme Linked Immunosorbent Assay method

Principle: The principle is based on the sandwich enzyme linked immunosorbent assay (ELIZA) technology. Specific polyclonal antibodies pre-coated onto the microwell plates and the enzyme labelled antibody and a serum containing native antigen is mixed, resulting into a sandwich complex. After equilibrium is attained, the antibody bound fraction is separated from unbound antigen by decantation. The density of color produced is proportional to the concentration of analyte present in the sample captured in the plate (Alhajj *et al.,* 2023).

Procedure:
All reagents and samples were brought down to room temperature. The wells were identified for standard, blank and samples. 50ul of standard/ samples was added

to their determined wells, covered with a plate sealer and incubated for 80 minutes at 37 ℃. The plates were decanted and washed with 200ul of 1x wash solution and allowed to sit for 1-2 minutes and washed three times. 100ul of biotinylated antibody working solution was added, the plate was covered and incubated for 50 minutes at 37℃.The plate was decanted and washed three times.100ul of Streptavidin-HRP working solution was added, the plate was covered and incubated for 50 minutes at 37℃. The plate was decanted and washed five times and 90ul of TMB Substrate solution was added; the plate was covered and incubated for 30 mins at 37℃ and 50ul of stop reagent was added. The plate was immediately read at 450 nm.

Estimation of serum Glutamic acid decarboxylase (GAD**)**

This was estimated using Enzyme-Linked Immunosorbent assay (ELISA) method
Principle: Standards and samples are added to the micro ELISA plate wells precoated with specific antibody to human GAD2. A biotinyalted detection antibody specific for human GAD2 and Avidin-Horseradish Peroxidase (HRP) conjugate are added to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human GAD2, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is stopped by the addition of stop solution and the colour turns yellow. The optical density (OD) of the yellow product formed is measured spectrophotometrically at a wavelength of 450 nm (Li *et al.,* 2015).

Procedure:
Sample volume of 100ul of and standard volume (100µl) was added to the wells and incubated at 37°C for 90 minutes. The liquid was discarded immediately and 100ul of biotinylated detection Ab working solution was added to each well and incubated at 37°C for 60 mins. The solution was aspirated and washed 3 times. 100ul of HRP conjugate working solution was added and incubated for 30 minutes at 37°C, the solution was aspirated and the plate was washed 5 times. 90ul of substrate reagent was added to each well and it was incubated for 15 minutes at 37°C. 50ul of stop solution was added to each well and the plate was read immediately at 450 nm.

Estimation of Serum zinc and selenium using Atomic Absorption Spectrophotometry (AAS) method

Principle: A metal in its ground state absorbs light of the same wavelength as that emitted by the metallic cathode lamp. The amount of the light absorbed by the metal is proportional to the concentration of toxic essential metal in the solution and was determined at appropriate wavelengths for each toxic or essential metal (Visser, 2021).

Procedure
Working standards (5) of known concentrations were prepared from the stock standard by pipetting 10ml of the stock solution into a volumetric flask and diluted to 100ppm. Working standards were poured into their respective sample bottles and used to calibrate the atomic absorption spectrophotometer.

Afterwards, samples were analysed by placing each samples under the aspirator for aspiration using AAS Buck Scientific 211VGP.

Statistical analysis

The data obtained were subjected to statistical analysis using Statistical Package for Social Science (SPSS) version 23.0 software, SPSS Inc. Chicago, Illinois, USA. Values were expressed as Mean ± Standard Error Mean (SEM). Student 't' test was the tool of choice in comparing means. Correlation was also done to determine relationship between parameters. Values at $p<0.05$ or ($p<0.01$) were taken to be statistically significant. The results were reported on tables and chart.

Results

Table 1 **s**hows the level of the selected pancreatic enzymes, zinc transporter 8, zinc and selenium when compared in diabetic and control subjects. There was significant increase at $p<0.05$ in the levels of FBS, GAD and Zn when diabetic subjects were compared with control while there was significant decrease in the levels of amylase and ZnT8. There was no significant difference in the levels of the parameters when the female subjects were compared with male subjects (table 2). Significant negative correlations ($p<0.01$) were observed (table 3) between age and selenium; selenium and the Zn: selenium ratio. Figure 1 shows the distribution of the parameters based on age; there was significant increase in Zn: selenium ratio when those in age group 61-70 years were compared with others.

Table 1: Levels of selected pancreatic enzymes, zinc transporter 8, zinc and selenium in diabetic patients and controls

Parameters	Diabetes N= 50	Control N=42	P value
Age	51.64 ± 1.41	52.24 ± 1.46	0.771
FBS (mmol/L)	10.30 ± 0.65	4.59 ± 0.06	0.000*
AMY (u/l)	53.24±1.92	79.39±5.33	0.001*
GAD (ng/ml)	75.36±0.79	68.69±3.31	0.037*
ZNT8 (u/ml)	0.17 ± 0.02	0.22 ± 0.02	0.043*
ZN (ppm)	0.26 ± 0.04	0.14 ± 0.12	0.004*
SEL (ppm)	0.24 ± 0.03	0.21 ± 0.03	0.461
Zn/selenium ratio	4.60 ± 1.74	1.76 ± 0.44	0.147

Values are given as Mean ± SEM
* Significant at $p<0.05$

Table 2: Levels of selected pancreatic enzymes, zinc transporter 8, zinc and selenium in diabetic patients based on gender classification

Parameters	Female N=29	Male N=21	P value
Age	51.41 ± 1.89	51.95 ± 2.19	0.853
FBS (mol/dm3)	10.28 ± 0.84	10.33 ± 1.05	0.969
AMY(u/l)	52.28±2.30	54.57±3.33	0.561
GAD((ng/ml)	75.67±1.10	74.93±1.14	0.647

Parameters	Female N=29	Male N=21	P value
ZNT8 (u/ml)	0.17 ± 0.02	0.17 ± 0.03	0.982
ZN (ppm)	0.29 ± 0.06	0.3 ± 0.01	0.396
SEL (ppm)	0.25 ± 0.05	0.22 ± 0.04	0.617
Zn/selenium ratio	6.49 ± 2.95	1.98 ± 0.43	0.204

Values are given as Mean ± SEM
* Significant at p<0.05

Table 3
Correlation between selected parameters in type 2 diabetic patients

Parameters	ZN (ppm)	SEL (ppm)	Zn/selenium ratio
Age	0.057 (0.69)	0.386**(0.006)	-0.216 (0.132)
FBS (mmol/L)	0.243 (0.08)	0.024 (0.870)	-102 (0.482)
Amylase(u/l)	-0.013 (0.928)	-0.051 (0.727)	-0.061 (0.676)
GAD(ng/ml)	-0.001 (0.995)	-0.033 (0.818)	0.081 (0.571)
ZNT8 (u/ml)	-0.111(0.443)	-0.159 (0.270)	0.046 (0.753)
ZN (ppm)	1	-0.022 (0.882)	0.250 (0.08)
SEL (ppm)	-0.022 (0.882)	1	-0.306** (0.031)
Zn/selenium ratio	0.250 (0.08)	-0.306**(0.031)	1

*Correlation is significant at the 0.05 level.
** Correlation is significant at the 0.01 level.

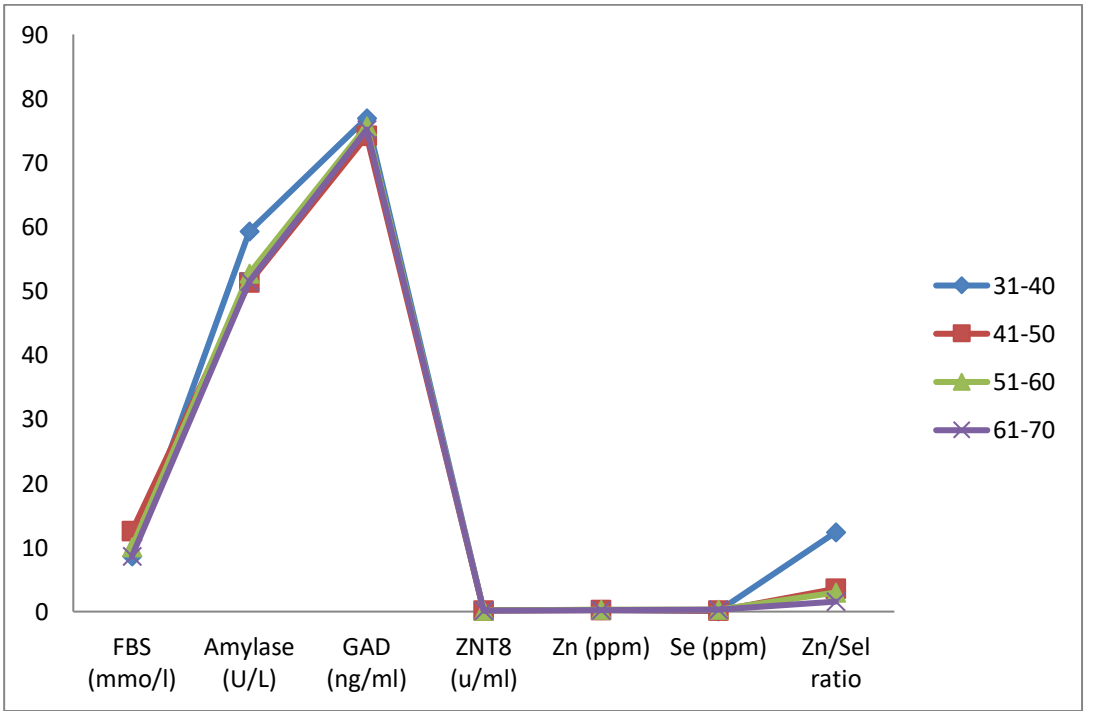

Figure 1: Levels of selected pancreatic enzymes, zinc transporter 8, zinc and selenium in diabetic patients based on age distribution

Discussion

In this study, there was significantly low level of serum amylase while there was a significant increase in the level of fasting plasma glucose in diabetic subjects compared with control and this is in agreement with the work of Zhuang *et al.,* (2016) and Yedjou *et al.,* (2023). They stated that low serum amylase levels may be associated with impaired islet β cell function in type 2 diabetes. This is because amylase is an enzyme that is produced by pancreatic acinar cells, and low serum amylase levels may be associated with endocrine diseases, such as metabolic syndrome and diabetes. Low serum amylase is also associated with insulin deficiency in patients with type 1 diabetes and, less commonly, with type 2 diabetes (Aughsteen *et al*, 2005; Kumar *et al.,*2022). This may reflect an increased risk of metabolic abnormalities and abnormal glucose metabolism (increased glucose level is expected in DM patients compared with control), which are associated with insulin resistance and impaired insulin secretion leading to diabetes. It was also discovered in this study that there was no significant decrease in the level of amylase in female diabetic subjects when compared to male subjects which is in line with the findings of (Yadav & Lowenfels, 2013) which observed no significant decrease in the level of serum amylase in female subjects when compared with male subjects.

This study observed significantly low level of serum zinc transporter 8 (ZnT8) in diabetic subjects compared with the control subjects. Serum zinc transporter 8 (ZnT8) is a protein highly specific to pancreatic insulin-producing β cells and it is vital for the biosynthesis and secretion of insulin. It is more specifically expressed in insulin-containing secretory granules (Yi *et al.,* 2016). Furthermore, the low level of ZnT8 (observed in this study) which is necessary for the delivery of the zinc into the insulin-containing granules could be deficient which in turn might have led to the deficiency or reduced level of insulin in the type 2 DM patients. It has been shown that zinc is required for insulin crystallization, storage, and secretion into the secretory granules of the pancreatic β cells as well as the delivery of zinc into the insulin-containing granules is largely accomplished by the ZnT8 protein (Huang, 2014). This finding is in line with the work of (Shan *et al.,*2014) where increased level of zinc was associated with zinc transporter gene. It has been reported that auto immune attack against the β cells of the pancreas could lead to reduced levels of islet- associated antigens e.g glutamic acid decarboxylase, insulinoma-associated antigen 2, and zinc transporter 8 (Chen *et al.,*2016). This is in agreement with the study done by Yi *et al.,* (2016) which showed that autoantibodies directed against the zinc transporter 8 (ZnT8) peptide were found to be significantly increased in diabetes mellitus. This peptide has been shown to be encoded by the SLC308 gene located on chromosome 8q24.11 and is found in the membrane of insulin secretory granules located in pancreatic β-cells. The ZnT8 peptide mediates the uptake of Zn^{2+} into the secretory granules which in turn stabilizes insulin by allowing hexamer formation. Our study is also in line with the study done on diabetic rats by Bhola *et al.,* (2021) which has shown that zinc transporter 8 (ZnT8) is a novel islet autoantigen and is specifically expressed in insulin-containing secretory granules of β-cells. According to their report, the ZnT8 protein expression as well as zinc content in β-cells, was decreased in diabetic mice. The finding of reduced ZnT8 in type 2 DM is also in support of the work of Bhatty *et al.,* (2019) which stated that

zinc transporter 8 auto antibodies are associated with Type 2DM. Stressing further on this fact is the report which had associated zinc transported 8 gene with Type 2 DM in Chinese patients (Xiang *et al.,*2008). Also, it has been shown in both invitro and animal studies that alterations in ZnT8 expression strongly modulate insulin secretion (Chimienti *et al.,*2006; Nicolson *et al.,*2009; Fu *et al.,*2009) and that zinc transporter could be targeted to develop therapeutic option for type 2 DM.

This study also observed an increase in the level of glutamic acid decarboxylase in diabetic subjects compared with control. Glutamic acid decarboxylase (GAD) is an enzyme that is produced primarily by pancreatic islet cells. Our finding is in agreement with the study of Crotti & Selmi (2014) which indicated that the presence of the enzyme glutamic acid decarboxylase in the body is associated with diabetes. The reduced level of zinc transporter 8 in this study could not be said to be due to autoimmune attack because the glutamic acid decarboxylase level was significantly increased. Other factors could be responsible for the increase since the development of Type 2 DM is more complex and could be affected by environmental factors or dysfunctional metabolic processes that could lead to increase level of glucose. GAD has also been implicated as a key auto antigen in the induction of IDDM (Morran *et al.,*2015).

The finding of significantly high level of zinc in type 2 diabetic group compared with control in this study could mean that there is adequate level of zinc that is required for insulin biosynthesis and release but there is problem with the transportation. Zinc plays a key role in the synthesis, secretion and insulin action in both physiological and pathological situations (Tamura 2021; Li *et al.,*2022). Zinc is known to play a role in insulin sensitivity through the activation of the phosphoinositol-3-kinase/protein kinase B cascade. Due to its insulin–mimetic action, zinc also stimulates glucose uptake in insulin-dependent tissues. However, this finding is not in agreement with the study done by (Farooq *et al.,*2020) where diabetic patients have low Zn level compared to healthy population.

Selenium (Se) is a trace mineral that is necessary for the body's many processes to function properly, it is a basic component of selenoprotein. Selenoproteins play a functional role in redox homeostasis, thyroid hormone metabolism and protection from oxidative stress and inflammation Kieliszek *et al.,* (2021). In this study, results showed that there is an increase in the selenium levels in diabetic subjects when compared with the control subjects and this is in agreement with the study done by Fontenelle *et al.,* (2018) which stated that selenium could play a role in the secretion and action of insulin, but an excess of selenium in the body is associated with the pathogenesis of insulin resistance and the development of diabetes mellitus. This is because selenium is an anti-inflammatory and antioxidant micronutrient that is essential for the activity of selenoproteins. Two selenoproteins (glutathione peroxidase and selenoprotein P) are known to be involved in the insulin signaling pathway. There was no significant difference in the parameters when the levels were compared between the males and females in diabetes subjects. There is a positive correlation between age and FBS while there was negative correlation between selenium and Zinc/ selenium ratio as reported previously by Yi *et al.* (2016) and Fukunaka & Fujitani (2018).

Conclusion

This study found that diabetic subjects have a significant decrease in the levels of amylase and serum zinc transporter 8 when compared to control subjects. It also found that there were significant increases in the levels of GAD and zinc in diabetic subjects relative to control subjects. These findings could lead to the pathogenesis of insulin resistance and the development of type 2 diabetes mellitus. Therefore, assessing the levels of these parameters especially zinc transporter 8, GAD, zinc, and selenium could provide insight to the diagnosis of type 2 DM and also be targeted in developing therapeutic option for type 2 DM.

Authors' contribution
AO conceived the idea, designed the study and collated the data. EA and FC analysed the specimen while BO prepared the manuscript. All the authors read and approved the final manuscript.

Conflicts of interest: None to declare

Data Availability: The data is available with the author and could be made available on request to qualified researchers who provide a methodologically sound proposal and whose proposed use of the data has been approved.

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