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Assessment of Specific and Non-specific Autoantibodies among Newly Diagnosed Type 1 Diabetes Mellitus Sudanese Patients

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Pathogenesis of type 1 diabetes mellitus is associated with the presence of specific autoantibodies and viral infection. Herein we aim to assess specific and nonspecific autoantibodies in newly diagnosed type 1 diabetes mellitus patients. In this case-control study 200 subjects were enrolled, classified into 100 newly diagnosed type 1 diabetes mellitus patients ages ranged from 1 to 16 years old, and 100 apparently health control age matched group. Serum anti-glutamic acid decarboxylase/tyrosine Phosphatase 2 (anti-GAD/IA2), anti-tissue transglutaminase (anti-tTG) and antinuclear antibodies were measured. Of 100, 62(62%) were males, and 38(38%) were females. Anti-GAD/IA2, anti-tTG, and antinuclear antibodies were found to be significantly higher in the case than in the control group. Anti-GAD/IA2 and anti-tTG were associated with higher risk of type 1 diabetes mellitus (OR= 5.44, P= 0.000) and (OR=5.82, P= 0.009) respectively. Anti-GAD/IA2, anti-tTG and antinuclear antibodies are higher in type 1 diabetes mellitus patients. Moreover, anti-GAD/IA2 and anti-tTG are associated with a high risk of type 1 diabetes mellitus.

Keywords: ANAs; Anti-tTG; Anti-GAD/IA2; Celiac Disease; Type-1 DM.

Type 1 diabetes mellitus (T1DM) accounts for over 90% of childhood diabetes globally, including in Africa and the Middle East[1].A previous study has reported a constant global rise in incidence, which is considered a significant health problem worldwide[2]. Moreover, the Epidemiological studies from Sudan reported that the incidence is 10.1/100,000 population/ year and the prevalence is 0.74/1,000 population/ year among children aged ranged from 6 months to 19 years living in Khartoum State, and the overall annual increase is estimated to be around 3%[3]. Furthermore, T1DM is frequently related to other organ-specific autoimmune diseases like autoimmune thyroid disease (AIT), celiac disease (CD), Addison's disease (AD), and vitiligo[4]. Children with T1DM have an increased risk of developing these diseases, which cause morbidity

and deteriorate diabetes control[5]. In T1DM, the major autoantigens are 65GAD and IA2 which are sensitive and specific markers of T1DM[6]. And at clinical onset, the prevalence of anti-GAD is estimated to be 80%[7].Moreover, the prospective birth cohorts have demonstrated that infections, particularly viral infections, diet, and toxins, are considered significant environmental factors of T1DM.Furthermore, few previous studies reported that some patients with T1DM contain non-organ-specific autoantibodies in their serum, which might involve the pathogenesis of T1DM[8].In addition, few studies demonstrated the association between the systemic lupus erythematosus (SLE) which characterizes by the high titers of antinuclear antibodies and T1DM[9].Additionally, the presence of ANAs was found in T1DM in studies done in Greece and Poland [8,10]. (Kota *et al.*, 2012)[11]reported the co-exiting of autoimmune conditions in patients with T1DM, and (1.2%) had associated SLE. On the other hand, T1DM and SLE are strongly associated with human leukocyte antigen (HLA) class II alleles defining the overlap between organ-specific and non-organ-specific autoimmune diseases. Moreover, rare studies have discussed bacterial infections[12]. In Sudan, one previous study has indicated the high prevalence and widespread of Coxsackievirus within T1DM patients at Khartoum State, and it might have a significant role in the causation of T1DM[13]. Therefore investigating the risk factors associated with T1DM in Sudan could make it possible to be prevented and treated.

Accordingly; this study aims to assess specific and nonspecific autoantibodies in newly diagnosed type 1 diabetes mellitus Sudanese patients

MATERIALS AND METHODS

This case-control study was carried out in Khartoum state from June 2016 to December 2019. The ethics committee of Sudan University of Science and Technology and the Ministry of Health approved this study. The ethical approval number (DSR-IEC-02-1-2016). After obtaining the informed consent from the parents, 100 patients (age ranged from 1 to 16 years old) and 100 healthy matched individuals age ranged from (1-15 years old) were included as a control group. All participants were already diagnosed according to the International Diabetes Federation criteria (Fasting plasma glucose e"126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8h or two –hour plasma glucose e"200 mg/dL (11.1mmol/L) during oral glucose load (OGTT). The world health organization (WHO) recommend using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water. Or HbA1c e" 6.5% (48 mmol/mol) or in a patient with classic symptoms of hyperglycemia, a random plasma glucose e"200 mg/dL (11.1 mmol/L)[14]. The patients were attending Omdurman teaching hospital (Mustafa al Amin) for Children and Pediatric and Ahmed Qasim hospital) for follow-up and routine investigation. We have collected demographic data by using a structured questionnaire. Children diagnosed with T1DM for more than six month, or have other acute or chronic diseases, and type 2 diabetic patients were excluded from this study. Venipuncture (5 ml) blood specimens were collected. Serum was obtained by centrifugation at 3000 rpm for 10 min and stored at "20°C. Anti- GAD/IA2, anti-tTG, and antinuclear antibodies (ANAs) were measured using indirect ELISA techniques. BMI was calculated using (Weight Kg/ Height m^2) formula.

Estimation of Anti-GAD/IA2 ELISA

Briefly, according to the manufactured of Euroimmun company, autoantibodies against GAD and IA2 were measured simultaneously using the ELISA test for a semi-quantitative in vitro assay in serum samples, 75ìl of patient's diluted serum was incubated into microplate wells which coated with the specific antigen. During incubation patient's antibodies were bound to the antigen while the unbound fraction was removed by washing, 100ìl of biotin-labeled antigens (pool of GAD and IA2) was added. Bound antibodies were able to act divalently and formed a bridge between the antigen on reagent wells and biotin-labeled antigens, 100 ìl of enzyme conjugate (peroxidase-labeled avidin) was added to detect the bound biotin, and 100 ìl chromogen/substrate solution was added to promote a color reaction. The color intensity is proportional to the concentration of antibodies against GAD and IA2 in the patient's samples. For semi quantitative interpretation, Samples results were evaluated by calculating a ratio of the extinction value of the patients sample over the extinction value of calibrator 6.The ratio e" 1 was

considered positive and the ratio <1 was considered negative.

Estimation of Anti-tTG

Briefly, according to the manufactured of Aeskulisa company, anti-tTG were measured using a solid-phase enzyme immunoassay against neo-epitopes of tissue transglutaminase in human serum, 100 ìl of patient's diluted serum was incubated in the microplates coated with the specific antigen. Patient's antibodies were bound to the antigen while the unbound fraction was removed by washing three times. Anti-human immunoglobulin conjugated to horseradish peroxidase (conjugate) (100ìl) was added, and then reacted with the antigen-antibody complex in the microplates. Tetramethylbenzidine (TMB) (100 ìl) was added, and a color reaction was formed. The color intensity is proportional to the concentration of respective antibodies in the patient's samples. For qualitative interpretation, Samples results were considered within a range of 20% around the cut-off value. The results >1.2 was considered positive. The results < 0.8 was considered negative.

Estimation of ANAs

Briefly, according to the manufactured of Aeskulisa company, ANAs were measured using a solid-phase enzyme immunoassay against HEp2 cells in human serum, 100 ìl of patient's diluted serum was incubated in the microplates coated with the specific antigen. Patient´s antibodies were bound to the antigen while washing removed the unbound fraction. Anti-human immunoglobulins conjugated to horseradish peroxidase (Conjugate), 100 ìl was added, so then reacted with the antigen-antibody complex in the microplates. Tetramethylbenzidine (TMB) (100 ìl) was added, and a color reaction was formed. The color intensity is proportional to the concentration of respective antibodies in the patient's samples. For qualitative interpretation, Samples results were considered within a range of 20% around the cut-off value. The results >1.2 was considered positive. The results < 0.8 was considered negative.

Statistical analysis

Data were analyzed by the SPSS statistical package of social science (version 20).

Table 1. Characteristics of study participants

Characteristics	Cases Frequency (%)	Controls Frequency (%)	P-value
Gender			
Male	62 (62.0%)	46 (46.0%)	0.0232
Female	38 (38.0%)	54 (54.0%)	
Age group			
≤ 10 Years	37 (37.0 %)	49 (49.0%)	0.0865
>10 Years	63 (63.0 %)	51(51.0%)	
BMI grade			
Underweight	60 (60.0 %)	25 (25.0%)	0.001
Normal weight	34 (34.0 %)	63 (63.0%)	
Over weight	4 (4.0 %)	7 (7.0%)	
Obese	2 (2.0%)	5 (5.0%)	
Duration			
≤ 3 month	87 (87.0%)		
>3 month	13 (13.0%)		
Anti-GAD/IA2			
Anti-GAD/IA2 positive	82 (82.0%)	0 (0.0%)	<0.001
Anti-GAD/IA2 negative	18 (18.0%)	100 (100.0%)	
Anti-tTg			
Anti-tTG positive	13 (13.0%)	0 (0.0%)	0.009
Anti-tTG negative	87 (87.0%)	100 (100.0%)	
ANAs			
ANAs positive	0 (0.0%)	0 (0.0%)	1.0
ANAs negative	100 (100.0%)	100 (100.0%)	
Total	100 (100.0%)	100 (100.0%)	

Demographic variables were expressed as numbers and percentages. An Independent T-test was used to compare mean concentrations level between cases and the control group. Moreover, the Chi square test was used to identify risk factors associated with T1DM. Results are expressed as percentages (%), odds ratio (OR), and confidence interval (CI). A *P*-value d" 0.05 was considered statistically significant.

RESULTS

The demographic characteristics outlined that, out of 100 T1DM patients, 62 (62%) were males and 38 (38%) were females. Male: female ratio was 1.6:1. Whereas 37 (37%) were less or had ten years old and 63 (63%) had more than ten years old. Of the 100 participants, 60 (60 %) of them were underweight, followed by 34 (34%) normal weight, four (4%) overweight, and only two (2%) were obese. In addition, 87 (87%) of the total had T1DM onset for three months or less while, 13 (13%) of them were for more than three months. Results showed that the percentage of anti-GAD/IA2 is 82 (82%), anti-tTG is 13 (13%),and no positive ANAs were found in Sudanese newly diagnosed T1DM (Table 1).Furthermore, the results of the independent T-test found that significant increase of means anti-GAD/IA2, anti-tTG, and ANAs of cases compared to control group with p-value >0.001, >0.001 and >0.001 respectively (Table 2). In addition, The results of chi-square revealed that, positive anti-GAD/IA2 and anti-tTG subjects are 5.4 and 5.8 times higher risk to have T1DM in comparison with control group (OR= 5.44, CI = 3.58 – 8.26, P= 0.000)and (OR=5.82, CI= 1.27- 26.63, P= 0.009) respectively. Meanwhile, no association was observed between ANAs and T1DM (Table 3).

DISCUSSIONS

Concurrent with the previous study, a significantly higher mean of anti-GAD/IA2 was found in T1DM patients compared to the control group[15]. In fact, anti-GAD has been found in approximately 50-80% of newly diagnosed T1DM and is considered a significant auto-antibody in T1DM. Also, it serves as a [16]. Meanwhile, the current study shows that mean anti-tTG were significantly higher inT1DM children compared to healthy individuals, which in agreement with a

Table 2. Comparison of mean concentrations level of autoantibodies among T1DM patients and control group

Autoantibodies	Case (Mean ± SD)	Control (Mean ± SD)	P-value
Anti-GAD/IA2	4.47 ± 2.81	0.80 ± 0.07	>0.001
Anti-t TG	0.68 ± 0.45	0.33 ± 0.29	> 0.001
ANAs	0.40 ± 0.17	0.31 ± 0.14	>0.001

Table 3. Association between Anti-GAD/IA2, Anti-tTG and ANAs among T1DM patients and control group

	Case	Control	CI (lower-upper)	OR	P-value
Anti-GAD/IA2					
Positive	82 (82.0%)	0 (0.0%)	3.587-8.265	5.444	0.0001
Negative	18 (18.0%)	100 (100.0%)			
Anti-tTG					
Positive	13 (13.0%)	0 (0.0%)	1.275-26.638	5.828	0.009
Negative	87 (87.0%)	100 (100.0%)			
ANAs					
Positive	0 (0.0%)	0 (0.0%)			
Negative	100 (100.0%)	100 (100.0%)			

previous study[17] but disagreed with another which observed insignificant difference between cases and healthy individuals [18].Moreover, our results demonstrated that significantly higher mean ANAs of T1DM patients compared to the control group. Furthermore, the present study determined that anti-GAD/IA2 was found to be a risk for T1DM.Recent studies have demonstrated that the co-occurrence of two or more pancreatic autoantibodies might increase the risk of developing T1DM with high sensitivity and specificity[19]. This is consistent with *Simon E Regnell and Ake Lernmark*'s study which indicatedthat, underlying type 1 diabetes is a genetic etiology dominated by the influence of specific HLA haplotypes involving primarily the class II DR-DQ region. In genetically predisposed children with the DR4-DQ8 haplotype, exogenous factors, yet to be identified, are thought to trigger an autoimmune reaction against insulin, signaled by insulin autoantibodies as the first autoantibody to appear[20]. Moreover, the data of the present study demonstrated that anti-tTG was found to be a risk for T1DM in our population. Indeed, Castellaneta *et al.* stated that children with T1DM have a seven times higher risk of having celiac disease[21]. In fact, the risk of autoimmune diseases is higher among T1DM[17]. Also, there is a well-known association of celiac disease with T1DM. Thus, the presence of celiac disease is associated with an increased risk of diabetes-related morbidity, so early diagnosis and treatment are recommended.

On the other hand, our study determined that there was no association between ANAs and T1DM. Few previous scientific reports contradict our finding that the presence of ANAs is 24% in T1DM, Litwinczuk stated, moreover, the presence of ANAs is associated with developing diabetic neuropathy[10].

In addition, an Indian study determined the co-existing autoimmune condition in patients with T1DM meanwhile concluded that 1.2% of T1DMwas associated with SLE[11].The contradiction finding is justified by that the prevalence of ANAs is higher in adults T1DM, which is included in the previous studies. Most previous studies suggested that aging is associated with autoimmunity, and the higher risk of ANAs positivity is associated with increasing age peaking at 20-30 and 40-50 years old[22, 23].

CONCLUSION

Anti-GAD/IA2, anti-tTG, and ANAs are higher in T1DM patients. Moreover, anti-GAD/IA2 and anti-tTG are associated with a higher risk of T1DM. Therefore, further prospective study is needed to investigate the risk factors associated with the severity onset of disease in positive autoantibodies patients.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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