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Beta-cell Death and/or Stress Biomarkers in Diabetes Mellitus Type 1

Madha Mohammed Sheet[1] Hasan Abd Ali Khudhair[2]*

[1]Assistant Professor/Middle Technical University/College of Health and Medical Technology/Baghdad.
[2]Assistant Lecturer/Southern Technical University/Al-Nasiriya Technical Institute, hasanabdali89@stu.edu.iq.

Abstract

Diabetes Mellitus type 1 (T1D) occurs due to disturbance intolerance of the immune system and invasion of β-cells by auto-reactive immune T cells and inducing deterioration of β-cells activity and viability and a prolong therapy with external insulin. This study was aimed to detect the predictive power as well as the degree of correlation of defined glutamic acid decarboxylase autoantibody (GADA), protein tyrosine phosphatase type A2 (IA-2A) and total antioxidant capacity (TAC) biomarkers on β-cells stress and/or death in T1D individuals as well as their first degree relatives (FDRs). Three groups of T1D patients, FDRs and apparently diseases free subjects had been enrolled in this work and evaluated for their serum GADA, IA-2A, C-peptide and TAC. The results revealed positive GADA and IA-2A in 88.6% and 40%, respectively, of T1D patients. The frequency of normal level and mean titer of C-peptide were significantly low among T1D and their FDRs, also the frequency of normal level and mean titer of TAC was significantly low among T1D patients. However, no significant difference in C-peptide level was noticed between the $GADA^+$ and $GADA^-$ subjects with no significant effect of TAC level on the concentration of C-peptide. Finally, the concentration of C-peptide was significantly lower in IA-2-A positive than IA-2-A negative individuals. In conclusion, GADA, IA-2A and C-peptide combination can suggest the most powerful and cost-effective diagnostic approach in patients with T1D and their FDRs. In addition, IA-2A in T1D patient's serum can be predicted for β-cell death and/or stress, however, GADA and TAC was found of no effect on the C-peptide level.

Key Words: Type 1 diabetes, Beta cells stress and death.

Introduction

Type 1 diabetes is one of the disturbance intolerance of immune system and invasion of beta (β)-cells by auto-reactive immune T cells, inducing deterioration of β-cells activity and viability and a prolong therapy with external insulin (1). Beta-cells loss occurs in its majority in the preclinical stages of T1D that when the diagnosis of the disease is completed, it became too late as about 90 percent of the β-cells were destructed. To preserve β-cells from destruction, certain immune therapy was used to yield only the lowest conservation of β-cells activity without a true recovery from T1D (2).

There are other few selected intrinsic pathways candidates in which certain factors might play a role in the death/stress of the slow generative and developmental pancreatic β-cells during the prodromal stages and early clinical stages (3). Of these are; the auto-antibodies (Abs), the oxidative stress (OS), the connecting (C) peptide/insulin ratio and few others.

Anti-glutamic acid decarboxylase auto-Abs, as well as IA-2-A were considered as the most specific cytoplasmic tests in the detection of T1D (4). Measuring this circulatory auto-Abs is a more respectable biological marker for the preclinical time of this disease as they are detected before the clinical stages by many years (5).

On the other hand, increasing findings speculates that reactive oxygen species (ROS) and other oxidative plays a certain role in the occurrence of T1D and their later advancement. In addition, antioxidant mechanisms are depleted in diabetic individuals, which may further increase OS (6). Accordingly, manipulating the progressive pathologic processes in the pre-clinical era would sparkle for new hope in β-cells preserved viability and function (7 and 8).

Furthermore, C-peptide is well known to fulfill an important function in insulin synthesis. Connecting peptide is useful as an indicator of β-cell function, and has been used as a surrogate biomarker for monitoring the course of TID and T2D and illustrating the effects of interventions designed to preserve and improve residual β-cells function (9). Levels of C-peptide consider as an excellent marker for serum insulin concentration and the function of the β-cell within the pancreatic gland (10). Connecting peptide evaluation regarded as a method that directly assesses the autoimmune attack on pancreatic β-cells (4).

This study investigates the predictive power and the degree of correlation of defined GADA, IA-2A and TAC biomarkers on β-cells stress and/or death (as represented by the C-peptide serum level) in newly diagnosed T1D and their 1st degree relatives. Such predictive power and degree of correlation presumably might provide new approaches for both earlier diagnosis and immunomodulatory therapy of preclinical stages and early insidious clinical stages of T1D.

Subjects, Materials and Methods

- ***Study groups*:** Three groups were employed in this study. The first comprised 35 new onset T1D individuals (17 males and 18 females with an age range from 10 to 18 years) attending the Diabetes and Endocrinology Specialist Center at Thi-Qar Province (Iraq) during the period from May 2018 to November 2018. The second group included a total of 35 FDRs (22 males and 13 females) of T1D patients with an age range from 4–39 years. The third group comprised 20 apparently healthy individuals (10 males and 10 females with an age range of 5-30 years) and tagged as healthy control (HC) group.
- ***Sample collection and serum separation*:** From each subject; a whole blood volume of 3-4 milliliter was drawn by puncture of the vein. The collected samples were left to complete the clotting processes at room temperature and then centrifuged at 1500 round per minute (min) for 10 min for serum production. Each serum sample was divided into several aliquots and stored at ˉ20 C° until needed for serological investigation.
- ***Serological tests:*** Serology of the study tests were executed in Imam Hussein Teaching Hospital/Health Department of Thi-Qar within three months after samples collection. Serology includes:
    - Detection of IA-2A auto-Abs (*Human IA-2A, Cusabio, China*). This assay employs the indirect qualitative enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with antigen (Ag). Samples are pipetted into the wells with anti-human immunoglobulin conjugated horseradish peroxidase (HRP). Any Abs specific for the Ag present will bind to the pre-coated Ag. Following a wash to remove any unbound reagent, a substrate solution is added to the wells and color develops in proportion to the amount of human IA-2A bound in the initial step. The color development is stopped and the intensity of the color is measured.
    - Detection and titration of GAD auto-Abs (*GAD auto-Abs, Demeditec, Germany*). In anti-GAD enzyme linked immuno-sorbent assay (ELISA) kit, anti-GAD Ab in patient's sera, calibrators and controls are allowed to interact with GAD_{65} coated Ag onto ELISA plate wells. After one hour incubation, the samples are discarded leaving anti-GAD Ab bound to the immobilized GAD_{65} Ag on the plate. GAD_{65}-biotin Ag is added in a second incubation step where, through the ability of anti-GAD Ab in the samples to act divalently, a bridge is formed between GAD_{65} immobilized Ag on the plate and GAD_{65}-biotin. The amount of GAD_{65}-biotin Ag bound is then determined in a third incubation step by addition of streptavidin peroxidase, which binds specifically to biotin. Excess, unbound streptavidin peroxidase is then washed away and addition of tetra-methyl-benzidine results in formation of a blue color. This reaction is stopped by addition of stop solution causing the well contents to turn yellow. The absorbance of

the yellow reaction mixture at 450 nanometer (nm) is then read using an ELISA plate reader.

- Detection and titration of serum C-peptide (*C-peptide, Demeditec, Germany*). The C-peptide kit is a solid phase ELISA, based on the principle of competitive binding. The microtiter wells are coated with anti-mouse Abs, which binds a monoclonal Ab directed towards a unique antigenic site on the C-peptide molecule. Endogenous C-peptide of a patient sample competes with a C-peptide-HRP conjugate for binding to the coated Ab. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of C-peptide in the sample. After addition of the substrate solution, the intensity of color developed is inversely proportional to the concentration of C-peptide in the patient sample.
- Detection and titration of TAC by using ABTS (2, 2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) method (*Human TAC, Mybiosource, USA*). The principle of the ABTS method for determining the TAC is as follows. ABTS is oxidized to green $ABTS^+$ by appropriate oxidant, which can be inhibited if antioxidants exist. The TAC of the sample can be determined and calculated by measuring the absorbance of $ABTS^+$ at 405 nm. Trolox is an analog of vitamin E and has a similar antioxidant capacity to that of vitamin E. Trolox is used as a reference for other antioxidant. For example, the TAC of Trolox is 1, and then the antioxidant capacity of the other substance with the same concentration is showed by the ratio of its antioxidant capacity to Trolox antioxidant capacity.

- ***Statistics***: Statistical descriptive analyses were included. Chi-Square was employed to assess the correlations among the parameters. Statistical values were regarded significant in case of *P*. values were less than 0.05. Finally, SPSS (*ver*, 24) was employed for the above mentioned statistical methods.

Results

The results in Figures (1) showed that anti-GAD Abs positivity was with the highest frequency in the T1D group (88.6%), followed by FDRs group (11.4%) with significance difference (*p. 0.04*). The lowest frequency % of anti-GAD Abs positivity (5%) was among HC group with significance differences (*p. 0.03*) in comparison with T1D patients and with absence significance different (*p. 0.07*) in comparison with FDRs subjects. Anti-GAD Abs titer within T1D patients revealed a significantly (*p. 0.01*) elevated mean titer (711.5 U/ml) in comparison with FDRs groups (59.3 U/ml) and HC group (1.9 U/ml). The difference between FDRs group and HC group was not significant.

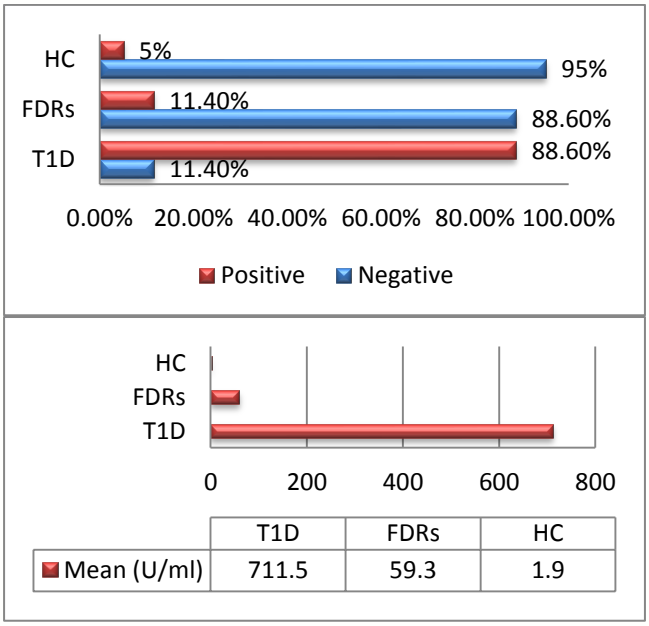

Figure (1): The results of frequency (percentage) and mean titer of anti-glutamic acid decarboxylase in all study groups. (**U**=unit, ml=milliliter, **T1D**=type 1 diabetes, **FDRs**=first degree relatives and **HC**=healthy control).

Islet antigen-2 Abs results are shown in Figure (2). The IA-2A positivity revealed a significant (*p.0.02*) elevation in frequency % among T1D patients (40%) than FDRs group (11.4%) and HC group (5%). The frequency differences for IA-2A positivity between the FDRs group (11.4%) and HC group (5%) were not significant (*p>0.05*).

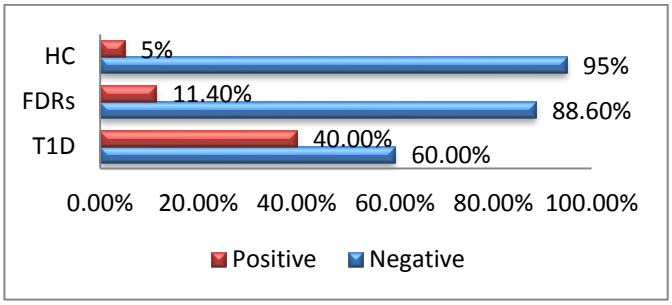

Figure (2): The results of frequency (percentage) of islet antigen-2 autoantibody in all study groups. (T1D=type 1 diabetes, **FDRs**=first degree relatives and **HC**=healthy control).

Figure (3) showed that the vast majority of T1D patients were below the normal level for serum C. peptide (65.7%) compared to FDRs (8.6%) and HC (5%) with a statistical different (*p.0.01*). No statistical different (*p.0.08*) for this value was recorded between FDRs and HC groups. Concerning the mean titers of C. peptide in the same figure, the lowest was in the T1D group (2.6 ng/ml) compared to the FDRs (5.3 ng/ml) and HC (6.5 ng/ml) with statistical differences (*p. 0.03*). Furthermore, the difference in C-peptide titers between FDRs group and HC group was significant (*p. 0.04*).

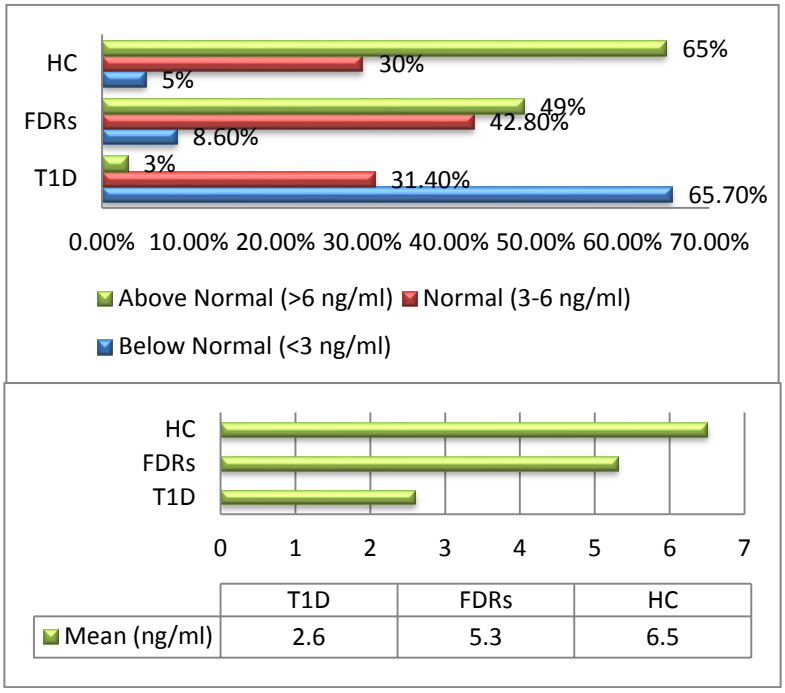

Figure (3): The results of frequency (percentage) and mean titer of connecting peptide in all study groups. (**ng**=nanogram, **ml**=milliliter, T1D=type 1 diabetes, **FDRs**=first degree relatives and **HC**=healthy control**).**

In Figure (4), the frequency percent of TAC below the normal level was statistically higher (*p. 0.03*) among T1D group (85.7%) in comparison to the FDRs group (65.7%) and HC group (60%). No statistical differences (*p. 0.1*) were found in FDRs group in comparison to HC individuals. Figure (4) also illustrated the mean titers of TAC which exhibited a decreased level in T1D group (0.42 mM) compared to the elevated level among the FDRs group (0.48 mM) and HC (0.46 mM). The difference in mean titer between the T1D and FDRs groups was significant (*p. 0.03*), whereas all other differences between study groups were not significant (*p>0.05*).

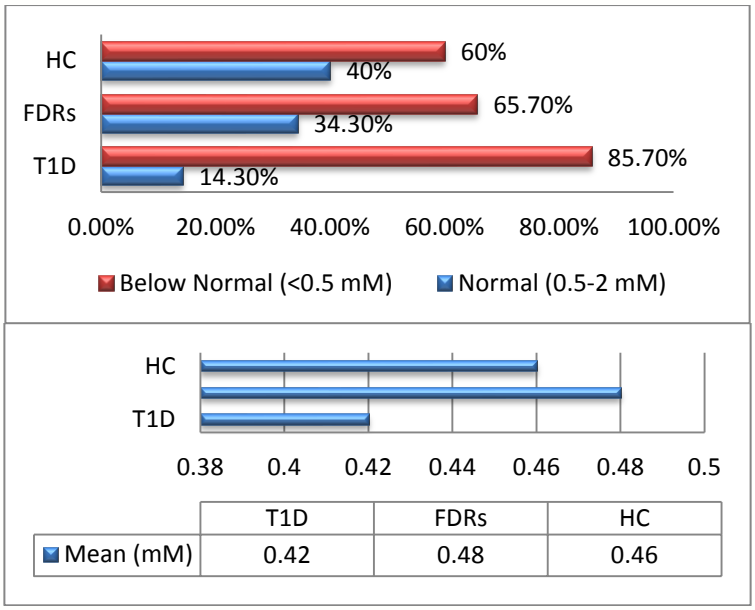

Figure (4): The results of frequency (percentage) and mean titer of total antioxidant capacity in all study groups. (**mM**=millimolar, **T1D**=type 1 diabetes, **FDRs**=first degree relatives and **HC**=healthy control).

The correlation between the anti-GAD Abs and the serum C-peptide among various groups of this work is illustrated in Table (1). No statistically significant correlation (*p. 0.07*) between those with positive anti-GAD Abs (low, moderate or high) or negative anti-GAD subjects with the frequency % of C-peptide level among all study groups. The same results were reported for C-peptide mean titer.

Table (1): Correlation between anti-glutamic acid decarboxylase autoantibody and connecting peptide in all study groups

Parameters				Connecting peptide (ng/ml)								p. value
				Below N (<3)		Normal (3-6)		Above N (>6)		Total		
				FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	
Anti-GAD Abs (U/ml)	T1D (n=35)	Positive	L (n=7)	4(57.1)	1.6	3 (42.9)	3.9	0(0)	0	7(100)	2.6	p>0.05
			M (n=4)	3 (75)	1.7	1 (25)	5.2	0(0)	0	4(100)	2.6	
			H (n=20)	13(65)	1.9	6(30)	3.8	1(5)	7.2	20(100)	2.7	
			T (n=31)	20(64.5)	1.8	10(32.3)	4	1(3.2)	7.2	31(100)	2.7	
		-ve (n=4)		3(75)	2.1	1(25)	3.2	0(0)	0	4(100)	2.4	
		Total (n=35)		23(65.7)	1.8	11(31.4)	3.9	1(2.9)	7.2	35(100)	2.6	
	FDR (n=35)	Positive	L (n=3)	0(0)	0	3(100)	4	0(0)	0	3(100)	4	p>0.05
			M (n=0)	0(0)	0	0(0)	0	0(0)	0	0(0)	0	
			H (n=1)	0(0)	0	0(0)	0	1(100)	6.9	1(100)	6.9	
			T (n=4)	0(0)	0	3(75)	4	1(25)	6.9	4(100)	4.7	
		-ve (n=31)		3(9.7)	2.3	12(38.7)	4	16(51.6)	7	31(100)	5.4	
		T (n=35)		3(8.6)	2.3	15(42.8)	4	17(48.6)	7	35(100)	5.3	
	HC (n=20)	Positive	L (n=1)	0(0)	0	1(100)	3.7	0(0)	0	1(100)	3.7	p>0.05
			M (n=0)	0(0)	0	0(0)	0	0(0)	0	0(0)	0	
			H (n=0)	0(0)	0	0(0)	0	0(0)	0	0(0)	0	
			T (n=1)	0(0)	0	1(100)	3.7	0(0)	0	1(100)	3.7	
		-ve (n=19)		1(5.3)	2.8	5(26.3)	5.2	13(68.4)	7.5	19(100)	6.6	
		Total (n=20)		1(5)	2.8	6 (30)	5	13(65)	7.5	20(100)	6.5	

N=normal, **L**=low, **M**=moderate, **H**=high, **FR**=frequency, **-ve**=negative,**T**=total, **%**=percentage, **GAD Abs**=glutamic acid decarboxylase antibody, **n**=number, **U**=unit, **ng**=nanogram, **ml**=milliliter, **T1D**=type 1 diabetes, **FDRs**=first degree relatives and **HC**=healthy control.

In Table (2), the correlation between the IA-2A and C-peptide in all study groups is shown. For positive IA-2A within T1D and FDRS groups, the results revealed that 12/14 (85.7%) and 3/4

(75%), respectively, were with below normal C-peptide level with a total mean titer of 2.1 ng/ml for T1D and 2.6 ng/ml for FDRs, whereas the negative IA-2A were 11/21 (52.4%) and 0/31 (0%), respectively, had below normal C-peptide level with a total mean titer 3 ng/ml for T1D and 5.6 ng/ml for FDRs. The differences between positive and negative IA-2A regarded to C-peptide level were significant ($p<0.05$).

In the HC group, the above normal level of C-peptide was the highest among negative IA-2A 13/19 (68.4%) compared to positive IA-2A 0/1 (0%) with a significant difference between them (*p. 0.02*). Connecting peptide mean titer was highest in negative IA-2A (6.6 ng/ml) compared to positive IA-2A (4.3 ng/ml) with a statistical different (*P. 0.03*).

Table (2): Association between islet antigen-2 autoantibodies and connecting peptide in all study groups

Parameters			Connecting peptide (ng/ml)								*p. value*
			Below N (<3)		Normal (3-6)		Above N (>6)		Total		
			FR (%	Mean	FR (%)	Mean	FR (%	Mean	FR (%	Mean	
IA-2A	T1D (n=35)	+ve (n=14)	12((85.7)	1.8	2(14.3)	3.6	0(0)	0	14(100)	2.1	*P<0.05*
		-ve (n=21)	11(52.4)	1.8	9(42.8)	3.9	1(4.8)	7.2	21(100)	3	
		T(n=35)	23(65.7)	1.8	11(31.4)	3.9	1(2.9)	7.2	35(100)	2.6	
	FDR (n=35)	+ve (n=4)	3(75)	2.3	1(25)	3.5	0(0)	0	4(100)	2.6	*P<0.05*
		-ve (n=31)	0(0)	0	14(45.2)	4	17(54.8)	7	31(100)	5.6	
		T(n=35)	3(8.6)	2.3	15(42.8)	4	17(48.6)	7	35(100)	5.3	
	HC (n=20)	+ve (n=1)	0(0)	0	1(100)	4.3	0(0)	0	1(100)	4.3	*P<0.05*
		-ve (n=19)	1(5.3)	2.8	5(26.3)	5.1	13(68.4)	7.5	19(100)	6.6	
		T(n=20)	1(5)	2.8	6(30)	5	13(65)	7.5	20(100)	6.5	

N=normal, **FR**=frequency, **+ve**=positive, **-ve**=negative, **T**=total, **n**=number, **%**=percentage, **IA-2A**=islet antigen-2 autoantibody, **ng**=nanogram, **ml**=milliliter, **T1D**=type 1 diabetes, **FDRs**=first degree relatives and **HC**=healthy control.

Table (3) illustrates the relationship between the serum C-peptide level and serum TAC level. The difference in the frequency % of TAC level between subjects with below normal, normal and above the normal level of serum C-peptide was not significant ($p>0.05$) for all study groups. The same results profile was documented with TAC mean titer as it is expressed in the table.

Table (3): Correlation between connecting peptide and total antioxidant capacity in all study groups

Parameters			Total antioxidant capacity (mM)						p. value
			Below N (<0.5)		Normal (0.5-2)		Total		
			FR %	Mean	FR %	Mean	FR %	Mean	
Connecting peptide (ng/ml)	T1D (n=35)	Below N (n=23)	18(78.3)	0.38	5(21.7)	0.56	23(100)	0.42	P>0.05
		Normal (n=11)	11(100)	0.4	0(0)	0	11(100)	0.4	
		Above N (n=1)	1(100)	0.44	0(0)	0	1(100)	0.44	
		Total (n=35)	30(85.7)	0.39	5(14.3)	0.56	35(100)	0.42	
	FDR (n=35)	Below N (n=3)	1(33.3)	0.49	2(66.7)	0.53	3(100)	0.52	P>0.05
		Normal (n=15)	11(73.3)	0.42	4(26.7)	0.59	15(100)	0.46	
		Above N (n=17)	11(64.7)	0.44	6(35.3)	0.56	17(100)	0.48	
		Total (n=35)	23(65.7)	0.43	12(34.3)	0.56	35(100)	0.48	
	HC (n=20)	Below N (n=1)	0(0)	0	1(100)	0.52	1(100)	0.52	P>0.05
		Normal (n=6)	4(66.7)	0.4	2(33.3)	0.53	6(100)	0.45	
		Above N (n=13)	8(61.5)	0.4	5(38.5)	0.56	13(100)	0.46	
		Total (n=20)	12(60)	0.4	8(40)	0.54	20(100)	0.46	

FR=frequency, **N**=normal, **n**=number, **%**=percentage, **ng**=nanogram, **mM**=millimolar, **ml**=milliliter, **T1D**=type 1 diabetes, **FDRs**=first degree relatives and **HC**=healthy control.

Discussion

Diabetes mellitus type 1 is characterized by massive damage in insulin-producing β-cells. Presumably, this could happen due to immune response deregulation as triggered by infection or other environmental factors. Genetically susceptible individuals would be more vulnerable to these factors and their immune system could deviate towards an abnormal autoimmune humoral and cell-mediated response against β-cells altered antigens (11 and 12). In agreement with these findings, the results of this study exhibited a significant elevation of anti-GAD Abs and IA-2A among T1D patients compared to FDRs and HC groups.

The present study found the highest frequency percentage of anti-GAD Abs positivity and IA-2A positivity were 88.6% and 40%, respectively among newly onset T1D patients. This result is inconsistent with 67.5% and 33.3%, respectively, of the same biomarkers as estimated by Mahdi *et al*., (11). This study result was higher than those recorded by another study (13) which conducted in Saudi Arabia in which the frequency percentage of positivity for GADA was 54% and IA-2A was 27%. In Taiwan, another study revealed an anti-GAD of 47% and IA-2A of 23% positivity among T1D patients (14). These variations in autoantibody frequency percentage in different studies could be due to multifactorial causes associated with genetic constitutions of the patients, environment, as well as test's sensitivity.

It has been assumed that existence of islet cell antigen (ICA) autoantibodies is important proves for the immune system deregulation in T1D which can help in the prediction of the disease occurrence among patient's relatives (15). In respect to our results, modest frequency percentage of GADA and IA-2A positivity were observed within FDRs groups (11.4% for both). Detection of autoantibodies against β-cells antigens would refer to an early development of autoimmunity directed towards the insulin-producing cells (16).

The presence or absence of one or more types of autoantibodies in the sera of T1D patient's relatives would represent an important cumulative risk for the disease development in the near or far future (17). This study finding concluded that the FDRs individual with positive islets auto-Abs may eventually be affected by the disease in the near future because of the high association between these antibodies and the β-cell death and/or stress mainly during the prodromal stages of the disease.

Connecting peptide serum level has been used recently to assess the amplitude of autoimmune-mediated β-cell destruction (4). A lower level in C-peptide concentration among the T1D patients in comparison with other study groups had been reported in this study, a result with high consistency with the above mentioned study.

Very few previous studies had prospectively elucidated a gradual deterioration in the C-peptide during the successive stages of the disease until an immeasurable level can be reached years after the disease onset. This pattern of C-peptide levels was noticed by many studies (18 and 19) in which they reported that C-peptide dropped down slowly at first during the pre-diabetic time and then accelerated during the clinical stages. The results of this study were in consistency with these findings, as it is illustrated in Figure 3.

Fluctuation in C-peptide values among children could be more sophisticated which can be overlapping with the age-associated phenomenon of C-peptide elevation which interpreted as no increase in a growing child is equivalent to depletion of this biomarker (18, 19 and 20).

The activities of antioxidant enzymes are declined in individuals with T1D and in latent autoimmune diabetes patients. The depletion was worse in those with bad control of diabetic (21). Current research revealed that TAC level in patients suffering from T1D was statistically lowest compared to healthy people as in some other studies (11 and 22). In diabetic patients, the antioxidant enzymes are inhibited by means of the high level of H_2O_2 generated from the auto-oxidation of glucose (11). Consequently, a high blood glucose level, the diabetes tag, declines the levels of antioxidants then promotes the generation of ROS, which can interact with many components like lipids, proteins, carbohydrates, and DNA and produce toxic actions on metabolic activities (23).

Hyperglycemia would aggravate the OS according to the fact that monosaccharaides and their glycolysis intermediates can produce reactive oxidation molecules (11). The current report revealed a clear decrease in TAC concentration in T1D patients, mostly due to an increased OS. Decreased TAC values lead to elevated ROS and massive accumulation of lipid peroxidation products (11).

The results of the current study (Figure 4) confirmed the finding of dysfunctional anti-oxidative defense among T1D patients and low anti-oxidative protection in diabetic's FDRs which is in agreement with another study (21). The same findings had been recorded by Kural *et al*., (24). These findings speculate the pathogenic role of OS in T1D.

Several studies have demonstrated the effect of auto-Abs, mainly of GADA and IA-2A, on the β-cells viability and residual function (represented by a decreased level of C-peptide in this study).

Many workers had reported paradoxical findings concerning the autoantibodies levels and β-cells residual mass and function, revealing the absence of association (25), negative association (26), or a positive association (27). The present study showed no statistical differences between C-peptide concentration and positive or negative GADA subjects among all study groups. Such paradoxical findings could be a result of the difference in sample inclusion conditions (e.g., patient's age at diagnosis) as well as biomarkers measurement methods. Table 2 results are fully coordinated with Christie *et al*. (28) report, who found that the positivity of IA-2A could be related to an obvious decreasing β-cells residual mass and activity. Many possible explanations for the decreased level of C-peptide among the positive IA-2A in comparison to negative IA-2A subjects; first, IA-2 has a major effect on insulin hormone secretion in which the absence of IA-2 and/or IA-2β lead to reduced insulin level and excretion which is as a result of dense core vesicle

number reduction (29). Second, the presence of IA-2A in particular, which is often associated with other Abs, confers a higher risk of rapid development toward clinical onset than multiple Abs. It is proposed that the production of IA-2A associated with a captious shift in T1D development, where the intracellular part of IA-2 may only become encountered with the immune system at the outer cell surface in the case of β-cell damage or dysfunction (30).

The results of the current study showed no significant difference in TAC level in relation to the C-peptide level in all study groups (Table 3). This result was unusual when compared to potential deteriorated effects of OS on β-cells (31). Beta-cells damage or reduced activity due to OS was observed in a previous study (32). A possible explanation for this could be due to oxidative molecules which act as a reversed effect in inflecting insulin action, as needed for insulin hormone to employ its physiologic role, but also involved in the pathological process of insulin hormone resistance (33). Thus, a beneficial role of or a need for, ROS or reactive nitrogen species for pancreatic β-cells activity may exist. Reports for such a benefit do exist. Therefore, a luxurious report by Leloup *et al*., (34) exhibited that ROS produced by mitochondria is essential in glucose-mediated insulin stimulation. Whereas in the former report, it has been stated that antioxidant would participate in the impairment of insulin excretion. Another study (35) has confirmed these findings, exhibited that ryanodine receptor-mediated Ca_2^+ excretion triggered by ROS is an important stride in glucose-induced insulin excretion. These observations indicate that antioxidant and OS have a relatively equal role of insulin hormone release by β-cells, however, in a harmony with these findings, this study results illustrated no significant difference in C-peptide level in correlation with TAC level.

However, many mysterious issues are still existing about the actual participate of ROS and other oxidative in the advancement of disrupted glucose tolerance and more research is needed to find out their role in pathologic processes and potentiality to neutralize their effects in treatment methods. For example, what is the amplitude that OS would affect β-cells identity and therefore activity, independently of cell vitality? Other researches required to answer this and other possibilities which identified new methods in the modulation of the OS impact in treatment purposes.

In conclusion, the combination of GADA, IA-2-A, and C-peptide can be proposed as the major powerful and cost-effective diagnostic way in patients with T1D and their FDRs. The presence of IA-2A in subjects serum can be predicted for β-cells stress and/or death during prodromal and early onset stages of T1D in which IA-2A positivity was inversely correlated with β-cell function (as represented by C-peptide level). Finally, GADA and TAC have no effect on the C-peptide level in all study groups.

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