Role of Beta-Cell Autoantibodies as a Predictor Marker in Diabetic Patients and Their Relationship to Glycemic Control

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To evaluate glutamic acid decarboxylase autoantibodies (GAD65), islet cell autoantibodies (ICA) and insulin autoantibodies (IAA) as disease markers and their relationship to certain residual beta-cell function as well as glycemic control among patients with diabetes mellitus. Also, to evaluate of the level of CD4+CD25+(Treg) out of CD4 cells among patients with immune mediated diabetes mellitus (DM). The study included 80 individuals divided into: 40 diabetic patients (group A) and 20 risk siblings (group B) of diabetic father or mother or both. 20 healthy individuals enrolled as control group (group C) all were with no family history of DM. GAD, ICA, IAA autoantibodies and C-peptide were determined by ELISA. HbA1 by ion exchange chromatography and measurement of the expression of CD4+CD25+ (T reg) by flowcytometry. The most frequently encountered antibody in adult and children groups was GAD65, followed by ICA. But in risk group the most frequently antibody was ICA, followed by GAD. In the risk group, there was no statistical difference in the level of CD4+CD25+ in comparison with control group. There was significant decrease in the percentage of CD4+CD25+ in adult and children patients groups with positive autoantibodies than those with negative autoantibodies. In conclusions, at the time of diagnosis the majority of patients with type I diabetes have autoantibodies that are reactive to islet antigens. GAD, ICA, IAA are of value for predicting IDDM in sibling of diabetic parents type I. CD4+CD25+ Treg cells may actively suppress activation of the immune system and prevent pathological self-reactivity.

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defect in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels (ADA 2007). The diagnosis should be made according to criteria established, e.g. WHO guidelines (Seisster & Scherbaum, 2006) Type 1 diabetes mellitus (T1DM) is a chronic disease associated with the selective destruction of pancreatic β-cells. The exact etiology of the disease is unclear, however, the insulin deficiency results primary from an auto-immune destruction of pancreatic β-cells (Chang *et al.,* 2004).

It seems now that the appearance of autoantibodies to beta cell antigen, such as those against glutamic acid decarboxylase (GAD65) and the protein tyrosine phosphatases like insulinoma associated protein (IA-2) in the peripheral circulation may be used as predictive signs of clinical disease in non--diabetic individuals and for identification of diabetes type, e.g. in patients with late-onset autoimmune diabetes (LADA) (Lohmann *et al.,* 2000).

GAD65 and IA-2 may not be directly involved in the pathogenic processes in beta-cell destruction, however, they are good markers in assessing the risk of disease manifestation. In addition, the simultaneous presence of more than one autoantibody type, such as GAD 65, IA-2 and insulin autoantibodies IAA together with islet cell autoantibodies (ICAs), seem to be highly predictive of progression to clinical disease (Matti *et al.,* 2003). To function properly, the immune system must discriminate between self and non-self. However, when this

discrimination fails, the immune system destroys self, meaning cells and tissues of the body; as a result such process causes autoimmune diseases. Regulatory T cells actively suppress activation of the immune system and prevent pathological self-reactivity, i.e. autoimmune disease. $CD4^+CD25^+$ regulatory T cells (T reg) play a central role in the prevention of autoimmunity and in the control of immune responses by down regulating the function of effectors $CD4^+$ or $CD8^+$ cells (Wenhao *et al.,* 2007).

The development of T cell-mediated autoimmune diseases such as thyroditis, gastritis and insulin- dependent diabetes mellitus (IDDM) can be prevented in various animal models by inoculating $CD25^+CD4^+$ T cells prepared from histocompatible normal animals, so regulatory T cells play important roles in immune system homeostasis and may also be involved in tumor immunotolerance by suppressing Th1 immune response which is involved in anti- tumor immunity (Cao *et al.,* 2004).

The objective of this study was to evaluate glutamic acid decarboxylase autoantibodies (GAD65), islet cell autoantibodies (ICA) and insulin autoantibodies (IAA) as disease markers. In addition, to study markers' relationship to certain residual beta-cell function determined by C-peptide of insulin in serum and glycemic control determined by HBA1c in diabetic patients, also to determine the level of T regulatory cells ($CD4^+CD25^+$ out of CD4) among patients with immune mediated diabetes mellitus.

Patients and Methods

This study was carried out at Clinical Pathology, Pediatrics and Internal Medicine Departments. Faculty of Medicine, Zagazig University Hospitals during 2009 and 2010.

The study included 80 subjects. University Research Ethics Committee approved this study. All enrolled subjects, adult and children's parents - gave an informed written consent prior to the study. This study included 40 patients diagnosed as diabetes mellitus type II in adults and type I in child Patients were randomly chosen every fifth patients from out-patient clinics of Zagazig University Hospital during 2009 and 2010.

Group A1 (adult patient group): included 20 patients diagnosed as type II diabetes mellitus not responding to oral hypoglycemic treatment. They were 8 males (40%) and 12 females (60%); age mean ± SD: (37±12.3).

Group A2 (children patient group): included 20 patients diagnosed as type I diabetes mellitus according to WHO classifications (ADA 2010). They were 11 males (55%) and 9 females (45%); age ranged from 3-16 years (mean±SD: 10.6± 4).

Group B (risk group): included 20 siblings of common diabetic father or mother or both (type I diabetes mellitus). They were 11 males (55%) and 9 females (45%); age ranged from 18-25 years (mean±SD: 21±2.5).

Group C (control group); included healthy individuals with no family history of diabetes mellitus.

Group C1 (adult control group) included 10 healthy adults, 5 males (50%) and 5 females (50%) and their age ranged from 25-60 years (mean±SD: 44±13.5)

Group C2 (children control group): included 10 healthy children; 5 females (50%) and 5 males (50%) and their age ranged from 5-16 years old (Mean±SD: 10.8±2.8).

All groups were subjected to history taking, clinical examination and laboratory investigations including fasting and postprandial plasma glucose levels (FPG, PPPG), liver and kidney functions and lipid profile tests. All previous tests (except HDL-C, and LDL-C) were determined using a Dade Behring Dimention RXL Chemistry analyzer (WS-DADERXL, Simmenes USA). HDL-C was determined using a commercial kit (ELI-TECH- Diagnostics, France)(Burstein 1970). LDL-cholesterol (LDL-C) was calculated using Friedewald's equation (Friedewald *et al.,* 1972).

Autoantibodies against GAD were determined using commercially available kits forGADEIA1910 kit, ICA EIA1594 kit, IAA1593 kit and EIA1293 ELISA kits, (DRG® international Inc, USA).

Glycosylated Hb was determined using ion-exchange chromatography catalogue number10658 (Gannon *et al.,* 2004) was done according to the kit provided by (human gesellschaft - Germany).

Measurement of the expression of the $CD4^+$/ $CD25^+$ T-regulatory cells out of $CD4^+$ T-cells was performed by flowcytometry kit provided by DAKO Denmark A/S. Briefly, from each enrolle 5 ml blood were aseptically withdrawn by sterile veinpuncture and collected in 2 tubes; one containing EDTA (1.5mg EDTA/ml blood) for determination of HbA1c and flowcytometric analysis of $CD4^+$/ $CD25^+$ T regulatory cells and other tube -in which blood was left to clot. Serum was separated by centrifugation, stored frozen in aliquots for use in routine investigations, as well as, for the detection of GAD65, ICA and IAA auto antibodies and C- peptide of insulin.

Statistical Analysis

Data were presented as mean ± standard deviation (X±SD) or percentage (%).The means of two groups were compared using student "t" test which was used to test the significance of the difference between two independent sample means. Linear correlation and regression were used to test the correlation between the measured parameters and the studied groups.

A *P*-value<0.05 was considered significant. The Chi-square test was used for comparing categorical variables. Data were carried out with the statistical package for Social Sciences (SPSS), version 10 software (Noursis *et al.,* 1997).

Results

The most frequently encountered antibody in adult, and children group was GAD65, as observed in 20% of adults, in 60% in children, and in 10% in risk cases, followed by ICA in 15% of adults and in 40% of children, while it was the most frequently encountered antibody in risk group 15%. When taken together, both GAD65 and ICA were detected in 10 % of adult, 30% in children, and 10% in risk cases. IAA was only detected in 10% of adults, 30% in children, and 10% in risk cases. When both GAD65 and IAA were taken together, they were detected in 5 % of adults, 25% of children, 5% in risk cases. Also ICA and IAA were detected in 5 % of adults, 15% of children, and 5% in risk cases. When all three markers were taken together, GAD, ICA and IAA were detect in 5% of all three study groups (Table 1).

Table 1. Positivity of autoantibodies (GAD, ICA, IAA) in study groups.

Autoantibodies	Frequency		
	Adult= 20	Children= 20	Risk= 20
	n (%)	n (%)	n (%)
GAD	4 (20)	12 (60)	2 (10)
ICA	3 (15)	8 (40)	3 (15)
IAA	2 (15)	6 (30)	2 (10)
GAD & ICA	2 (10)	6 (30)	2 (10)
GAD & IAA	1 (5)	5 (25)	1 (5)
ICA & IAA	1 (5)	3 (15)	1 (5)
GAD & ICA & IAA	1 (5)	1 (5)	1 (5)

GAD: Glutamic acid decarboxylase autoantibodies., ICA: Islet cell autoantibodies., IAA: Insulin autoantibodies.

The comparative study between adult and children patients groups showed the presence of a significant difference between the two groups for GAD65 autoantibody prevalence (*P*<0.009), while there was no significant difference for ICA autoantibody (*P*<0.07),and for IAA autoantibody prevalence(*P*<0.2) (Table 2).

Table 2. Positivity of autoantibodies in adult versus children patients.

Autoantibodies	Group A1	Group A2	*P value
GAD	4 (20%)	12 (60%)	0.009
ICA	3 (15%)	8 (40 %)	NS
IAA	2 (10%)	6 (30 %)	NS

GAD: Glutamic acid decarboxylase autoantibodies., ICA: Islet cell autoantibodies., IAA: Insulin autoantibodies.

P>0.05 is not significant NS= not significant.

In table (3), no gender differences (P =0.92) for Ab prevalence in adult group was observed. There was significant relation between age of adult patients and Ab positivity (P = 0.017).There was significant relation between history of weight loss, peripheral neuritis and autoantibodies positivity (P = 0.037). There was no significant relation between family history of DM, polyuria, polydipsia, and autoantibodies positivity respectively where (P = 0.87, 0.62, 0.92). There was significant relation between presence of ketonuria and autoantibodies positivity (P =0.02), while there was highly significant relation between autoantibodies and insulin requirement. (P<0.001).There was no significant relation between autoantibodies positivity and HBA1c (P=0.63), cholesterol level (P =0.69), triglyceride level (P=0.79), HDL level (P=0.11) and LDL level (P=0.18).

Table 3. Comparison between the presence/absence of autoantibodies and clinical and laboratory data in adult patients.

	Positive autoantibodies *n*= 6 (%)	Negative autoantibodies *n*=14 (%)	*P value
Sex (M/F)	2/4	6/8	NS
Age (years)	27.5 ± 6.8	41 ± 11.97	0.017
Family History of DM	1 (16.7%)	1 (7.1 %)	NS
History of Weight loss	4(66.7 %)	2 (14.3 %)	0.037
Polyuria	3(50.3 %)	7 (50 %)	NS
Polydypsia	3(50 %)	5 (35.7 %)	NS
Ketonuria	3(50 %)	0.0 (0 %)	0.02
Insulin Requirement	5(83.3 %)	1(7.1 %)	<0.001
Peripheral Neuritis	4(66.7 %)	2 (14.3 %)	0.037
HBA1c %	9.5±0.8	9.3±1.0	NS
Cholesterol (150-240mg/dl)	180±34.1	173.5±24.6	NS
Triglycerides (60-160 mg/dl)	111.7±26.4	107.8±32.4	NS
HDL(>37 mg/dl)	42.3±2.6	44.1±2.1	NS
LDL(<130 mg/dl)	115.0±21.7	102.8±16.4	NS

**P*>0.05 is non significant NS= not significant.

In table (4), no gender differences (*P*=0.67) for Ab prevalence in children group was observed. Also there was neither significant relation between age of children patients (*P*=0.79), nor family history of DM (*P*=0.51) and Ab positivity. There was significant relation between history of weight loss and autoantibodies positivity (*P*=0.035), and no significant relation between polyuria and polydipsia and autoantibodies positivity (*P* =0.43 and 1.0 respectively). There was significant relation between presence of ketonuria and autoantibodies positivity (*P*=0.035), while there was significant relation between autoantibodies and insulin requirement (*P*=0.02).There was no significant relation between autoantibodies positivity and HBA1c (*P*=0.51), cholesterol level (*P*=0.82), triglyceride level (*P*=0.6), HDL level (*P* =0.59) and LDL level (*P*=0.66).

Table 4. Comparison between the presence/absence of autoantibodies and clinical and laboratory data of children patient group.

Clinical Data	Positive autoantibodies *n*= 18	Negative autoantibodies *n*= 2	**P* value
Sex M/F	10/8	1/1	NS
Age years	10.3 ± 4.2	11.2± 2.7	NS
Family History of DM	4 (26.7 %)	0 (0.0 %)	NS
History of Weight loss	8 (53.3 %)	0 (0.0 %)	0.035
Polyuria	8 (53.3 %)	1 (20.0 %)	NS
Polydypsia	5 (33.3 %)	1 (20.0 %)	NS
Ketonuria	8 (53.3 %)	0 (0.0 %)	0.035
Insulin Requirement	0.33± 0.07 0.2-0.4 u/kg/day	0.244±0.06 0.2-0.35 u/kg/day	0.02
HBA1c %	9.6±1.0	9.3±0.58	NS
Cholesterol (120-200 mg/dl)	168±24	166±11.4	NS
Triglyceride (60-160 mg/dl)	111.6±23.5	101.4±18.4	NS
HDL>37 mg/dl	43.9±2.5	44.8±2.8	NS
LDL<130 mg/dl	94.8±9.6	100±11.7	NS

**P*>0.05 is non significant NS= not significant.

Statistical comparison of fasting level of serum C-peptide between patient groups and control groups found a highly significant increase in the level of fasting C-peptide in control groups than patients groups. There was significant increase in the level of serum C-peptide in patients with negative autoantibodies than those with positive autoantibodies (*P*<0.003) (Data not shown).

A negative correlation between the presence of autoantibodies and C-peptide of insulin among patients groups was detected, while there was a positive correlation between the presence of autoantibodies and HBA1c level in patients groups (Table 5).

Table 5. Correlation between presence of individual autoantibodies and level of both serum fasting C-peptide of insulin, and level of HBA1c among patients group.

Autoantibodies	C-peptide		HBA1c	
	r	*P value	r	*P value
GAD	-0.32	<0.05	0.55	<0.001
ICA	-0.35	<0.05	0.4	<0.001
IAA	-0.36	<0.05	0.38	<0.001

GAD: Glutamic acid decarboxylase autoantibodies., ICA: Islet cell autoantibodies., IAA: Insulin autoantibodies.

*P<0.05 is significant

Flowcytometric analysis showed a highly significant increase in the level of $CD4^+CD25^+$ in control group than adult patients group (P<0.001), also highly significant increase in the level of $CD4^+CD25^+$ in control group than children patients group (P<0.001), but there was no significant statistical difference (P= 0.9) in the level of $CD4^+CD25^+$ in risk group than control group (Figure 1), (Table 6). There was significant decrease in percentage of $CD4^+CD25^+$ in adult patients group with positive autoantibodies than those with negative autoantibodies (P<0.05), also there was highly significant decrease in percentage of $CD4^+CD25^+$ in children patients group with positive autoantibodies than those with negative autoantibodies (P<0.001), but there was no significant difference (P= 0.57) in percentage of $CD4^+CD25^+$ in risk group with positive autoantibodies than those with negative autoantibodies (Table 7).

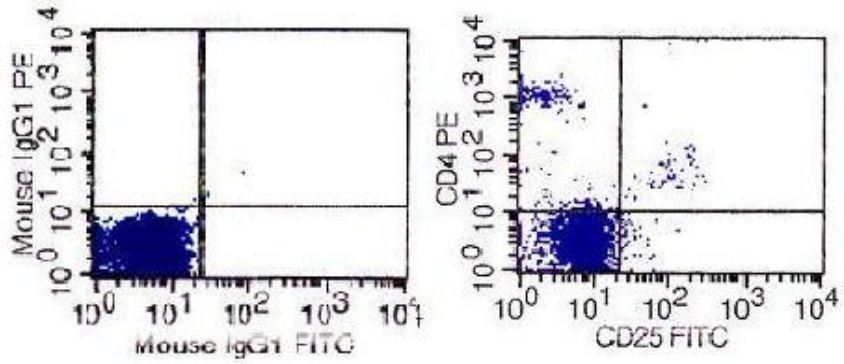

Figure 1. Results of flowcytometric analysis show the isotypic control and the expression of CD4+CD25+ out of CD4 cells in one of the child cases revealed that the percentage of CD4+CD25+ out of CD4 in this case was 0.95.

Table 6. Comparative study between the studied groups and their control according to the mean and SD of the percentage of $CD4^+/CD25^+$ out of CD4 cells:

Variables	Mean± SD	Range	*P
Adult group	0.96±0.55	0.2 – 2.7	< 0.001
Control group	2.85±0.92	1.09 – 3.90	
Children group	0.96±0.46	0.5-2.2	< 0.001
Control group	2.96±0.62	2.2 – 3.74	
Risk group	2.99±0.7	2.1 – 4.0	NS
Control group	2.96±0.6	2.2 – 3.74	

*P>0.05 is non significant NS= not significant.

Table 7. Association between the percentage of $CD4^+CD25^+$ cells and the presence or absence of autoantibodies in the studied groups.

Autoantibodies	CD4/CD25 Mean ± SD (Range)	*P value
Adult -ve (n=14)	1.399 ± 0.73 (0.5 -2.2)	0.019
Adult +ve (n= 6)	0.625 ± 0.06 (0.56 –0.7)	
Children -ve (n=2)	1.37 ± 0.27 (0.9 -1.53)	0.001
Children +ve (n=18)	0.75 ± 0.26 (0.53 –1.5)	
Risk -ve (n=16)	1.36± 0.53 (0.56 -2.1)	NS
Risk +ve (n= 4)	1.17± 0.74 (0.5 –2.1)	

*P>0.05 is non significant NS= not significant.

Discussion

Type 1 diabetes results from the autoimmune destruction of insulin-producing ß cells in the pancreas (Litherland, 2008). It is characterized by selective loss of insulin-producing ß-cells in the pancreatic islets in genetically susceptible subjects.

The most important genes contributing to disease susceptibility are located in the HLA class II locus on the short arm of chromosome 6 (Mikael *et al.,* 2005). Type 1 diabetes is an autoimmune disorder caused by autoreactive CD4+ and CD8+ T-cells that recognize pancreatic antigens such as insulin or GAD and subsequently destroy insulin-producing β-cells (Christophe & Matthias, 2008). Although the appearance of autoantibodies does not follow a distinct pattern, the presence of multiple autoantibodies has the highest positive predictive value for type 1 diabetes (Pihoker *et al.,* 2005). Latent autoimmune diabetes in adults (LADA) is a genetically

linked, autoimmune form of type 1 diabetes mellitus that is commonly seen after age 30 in patients who often have a normal body mass index without overt signs of metabolic syndrome. They have positive circulating antibodies reflecting the autoimmune nature of beta cell destruction (Jane 2008; Chul *et al.,* 2006).

The present study revealed that GAD65 auto antibody was present in adult patients group followed by ICA, then IAA as observed elsewhere (Schiel & Muller*,* 2000; Harvey *et al.,* 2007) unlike results obtained by Takeda *et al.,* (2002). This may be due to differences in the number of studied subjects as well as the difference in method of determination, as they detected GAD antibodies in cross 67-sectional study. Furthermore, another study concluded that the presence of GAD antibody in type 2 diabetic patients can predict their course of β-cell function and identify in advance who are likely to require insulin treatment (Chul *et al.,* 2006). Richard *et al.,* (2007) agree with this study in the percent of ICA and IAA.

In children group we found that percent of GAD65 antibody is higher than ICA, IAA as observed elsewhere, however, others found that the prevalence of ICA is higher than GAD antibody and IAA (Lotfy *et al.,* 2001; Urakami *et al.,* 2009, Zanone *et al.,* 2003)) This may be due to difference in the method of determination, and the number of the studied subjects which were children with slowly and rapidly progressive forms of type 1 diabetes. Also, results obtained in this study are in disagreement with that of Chang et al.,(2004) who found that percent of IAA, GAD in children group was 23.6% and 47% respectively. The results of Schiel and Muller (2000) was more or less comparable with the present study where GAD antibody was positive in 55% of diabetic patients type I. Falorni & Brozzetti, (2005) concluded that GAD antibody assay should be offered to every diabetic patient and in cases of positivity screening for other autoimmune diseases should be carried out.

In risk group, we found that ICA antibody was present in higher frequency (15%) followed by IAA, GAD antibody present in 10% of individuals where GAD antibody and ICA present in 10%, GAD antibody, IAA in 5% ICA, IAA in 5%, three antibodies were found in 5%. Such results differ from other reports as they studied large groups of subjects, aged below 20 years and used different methods of markers determination such as immunofluorescence unlike this study (Petri *et al.,* 1998; Kimpimaki *et al.,* 2002; Jennifer *et al.,* 2004).

There was significant relation between age of adult patients and Ab positivity, in agreement with this study Chul *et al.,* 2006.

Human proinsulin C-peptide is a cleavage product of insulin in the beta cells of the islets of langerhans. It is released in amount equal to insulin into portal circulation. Its main function is to enable the folding of the proinsulin molecules by facilitating the formation of disulphide bonds of the α and β chains (Thai *et al.*, 2007; Steiner, 1978). In agreement with other studies, there was highly significantly decrease in the level of C-peptide in patient than control group (Zanone *et al.,* 2003; Lotfy *et al.,* 2001). In this study, there was a significant increase in the level of serum C-peptide in patients with negative autoantibodies than those with positive autoantibodies as mentioned before (Takeda *et al.,* 2002; Richard *et al.,* 2007; Peter *et al.,* 2004). In addition, a positive correlation between the presence of individual autoantibody and HBA1c was observed in agreement with others (Zanone *et al.,* 2003).

In the present study we found no significant differences in the percentage of CD4+ CD25+ between risk group and control group, however, the percent of CD4+ CD25+ in the peripheral blood of adult patients group (LADA) was significantly lower in patient

than in control group in agreement with other studies (Longhi *et al.,* 2006). Furthermore, similar results were obtained for children patients group agreeing with others studies which showed that the level of CD4+ CD25+ cells in IDDM was significantly lower than normal control (Yang *et al.,* 2007; Ana *et al.,* 2006). Such data suggest that CD4+ CD25+ T cells might play an essential role in the pathogenesis of IDDM patients. Regulatory T cells are believed to be critical in the maintenance of immune tolerance and they exert their effect on other immune cells by cell-to-cell contact as well by a set of powerful cytokines like TGF-b and IL-10 (Tihamer *et al.,* 2012).

There was highly significant difference between percent of CD4+ CD25+ and the presence and absence of autoantibodies in the children patients group and in the adult group, while no significant correlation was found in the risk group. So, autoantibodies against islet antigens are found in most patients with type I diabetes and are now established markers for the clinical diagnosis and the preclinical phase of this disease (Peter & Anette, 2005).

In conclusion, at the time of diagnosis the majority of patients with type I diabetes have autoantibodies that are reactive to islet antigens, auto antibodies (GAD, ICA, IAA) are of value for predicting IDDM in sibling of diabetic parents type I, CD4+ CD25+ T-regulatory cells actively suppress activation of the immune system and prevent pathological self-reactivity. So, we recommend early detection of diabetes by testing for the presence of autoantibodies and prevention of diabetes by treating the deficiency in T-regulatory cells, if possible.

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References

1. American Diabetes Assocciation (2010). Standards of Medical care in diabetes (position statement).Diabetes care, vol. 33, suppl. 1.
2. American Diabetes Association (2007). Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care; suppl. 1; 25: 5-20.
3. Ana I, Janine LC, Fiona P.(2006). Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. Immunol. Rev. 212(1): 256 – 71.
4. Burstein M, Scholnick HR, Morfin R. (1970). Rapid method for isolation of lipoproteins from human serum by precipitation with polyanion. J Lipid Res; 11(6):583-95.
5. Cao D, Vollenhoven R, Klareskog R,Christina TE, Vivianne ME.(2004). CD25 bright CD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res & Ther; 6: 335-46.
6. Chang YH, Shiau MY, Tsai ST, Lan MS. (2004). Autoantibodies against IA-2, GAD, and topoisomerase II in type I diabetic patients. Biochem. & Bioph. Res. Comm. 320(3): 802-9.
7. Christophe MF, Matthias GVH. (2008). Viral Trigger for Type 1 Diabetes. Diabetes; 57(11): 2863-71.
8. Chul SK, Jae HN, Ji SN, Park JS, Kang ES, Ahn CW, (2006). Clinical and biochemical characteristics of non-obese type 2 diabetic patients with glutamic acid decarboxylase antibody in Korea. Metab. Clin. & Exp. 55(8):1107– 12.
9. Falorni A., Brozzetti A. (2005). Diabetes-related antibodies in adult diabetic patients. Clin. Endocrinol. & Metab. 19(1):119-33.
10. Friedewald WT, Levy RI, Fredrickson DS. (1972). Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin Chem; 18: 499-502.
11. Gannon MC, Nuttall FQ. (2004). Effect of a High-Protein, Low-Carbohydrate Diet on Blood Glucose Control in People with Type 2 Diabetes. Diabetes; 53(9): 2375-82.
12. Harvey K C, Elaine CT, Rattan J, James S, Barbara BW, Amit G. (2007). Equivalent insulin resistance in latent autoimmune diabetes in adults (LADA)

and type 2 diabetic patients. Diab. Res. & Clin. Pract; 77(2): 237-44.

13. Jane K. (2008). Latent Autoimmune Diabetes in Adults. J. For Nurse Practitioners; 4(9): 681-87.
14. Jennifer MB, Katherine JB, Liping Y, Miao D, Erlich HA, Norris JM, (2004). Prediction of Autoantibody Positivity and Progression to Type 1 Diabetes: Diabetes Autoimmunity Study in the Young (DAISY). J. Clin. Endocrinol. & Metabol. 89(8): 3896-902.
15. Kimpimaki T, Kulmala P, Savola K, Kupila A, Korhonen S, Simell T. (2002). Natural history of β-Cell autoimmunity in young children with increased genetic susceptibility to type 1 diabetes recruited from the general population. J. clin. Endocrinol. & Metabol. 87(10):4572-9.
16. Litherland SA. (2008). Immunopathogenic Interaction of Environmental Triggers and Genetic Susceptibility in Diabetes. Diabetes; 57(12): 3184-86.
17. Lohmann T, Hawa M, Leslie RD, Lane R, Picard J, Londei M. (2000). Immune reactivity to glutamic acid decarboxylase 65 in stiff man syndrome and type I diabetes mellitus. Lancet; 356(9223): 31-5.
18. Longhi MS, Hussain MJ, Ragai RM, Sunil KA, Giorgina MV, Diego V. (2006). Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. J. Immunol. 176: 4484-91.
19. Lotfy AM, Mohamed A, El-Shenawy FA. (2001). Correlation between allele specific class II HLA antigens and autoantibodies in IDDM. Thesis, MD, Clinical Pathology, Faculty of Medicine El-Mansoura University.
20. Matti A, Annette WK, Jorma I, Mikael K, Kaisa S, Pasi K. (2003). Time-resolved fluorometric assay for detection of autoantibodies to glutamic acid decarboxylase (GAD65). Clin. Chem. 49:908-915.
21. Mikael K, Riitta V, Suvi MV,Hyöty H, Vaarala O, Akerblom HK. (2005). Environmental Triggers and Determinants of Type1 Diabetes. Diabetes; 54:125-36.
22. Noursis MJ. (1997). Statistical Package for social Sciences (SPSS), base 10.0 for windows. User' s Guide, Chicago, IL-SPSS.
23. Peter A, Anette GZ. (2005). Diabetes-related antibodies in euglycemic Subject. Clin. Endocrinol. & Metab. 19 (1): 101–117.
24. Peter N, Marie C, Katarina K, Pavlina C, Emanuel Z, Dana N. (2004). Diabetes mellitus in adults: association of HLA DRB1 and DQB1 diabetes risk alleles with GAD ab presence and C-peptide secretion. Immunol. Letters; 95: 229–32.
25. Petri K, Kaisa S, Jacob SP, Paula V, Jukka K, Tuija L. (1998). Prediction of Insulin-dependent Diabetes Mellitus in Siblings of children with diabetes. J Clin Invest; 101(2): 327-36.
26. Pihoker C, Gilliam LK, Hampe CS, Lernmark Å (2005). Autoantibodies in Diabetes. Diabetes; 54:552-61.
27. Richard AJ, Lisa KG, Carina T, Landin-Olsson M, Karlsson FA, Palmer JP. (2007). Multiple factors affect the loss of measurable C-peptide over 6 years in newly diagnosed 15- to 35-year-old diabetic subjects. J. Diab. & Its Complications; 21(4): 205– 13.
28. Schiel R., Muller UA. (2000). GAD autoantibodies in a selection-free population of insulin-treated diabetic patients: indicator of a high prevalence of LADA. Diab. Res. & Clin. Pract. 49(1):33-40.
29. Seissler J., Scherbaum WA (2006). Autoimmune diagnostics in diabetes mellitus. Clin Chem Lab Med; 44(2): 133-7.
30. Steiner DF. (1978). On the role of pro-insulin C-peptide. Diabetes; 27:145-8.
31. Takeda H, Kawasaki E, Shimizu I, Konoue E, Fujiyama M, Murao S. (2002). Clinical, autoimmune, and genetic characteristics of adult-onset diabetic patients with GAD autoantibodies in Japan. Diabetes Care 25(6): 995-1001.
32. Thai AC, Mohan V, Khalid BA, Cockram CS, Pan CY, Zimmet P. (2007). Islet autoimmunity status in Asians with young-onset diabetes (12–40 years): Association with clinical characteristics, beta cell function and cardio-metabolic risk factors. Diab. Res. & Clin. Pract. 80(2): 224-30.
33. Tihamer O, Klara F, Heyam J, Janos K, Andras T, Ben F,. (2012). Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy. J. Autoimmunity; 34:408-15.
34. Urakami T, Yoshida A, Suzuki J, Saito H, Wada M, Takahashi S. (2009). Differences in prevalence of antibodies to GAD and IA-2and their titers at diagnosis in children with slowly and rapidly progressive forms of type 1 diabetes. Diab. Res. & Clin. Pract. 83(1):89-93.

35. Wenhao C, Jun D, Stanislaw MS, Zhang L. (2007). Both infiltrating regulatory T cells and insufficient antigen presentation are involved in long- term cardiac xenograft survival. J Immunol; 179: 1542-48.

36. Yang Z, Zhou Z, Huang G, He L, Xiang Y, Jian P, (2007). The CD4(+) regulatory T-cells is decreased in adults with latent autoimmune diabetes. Diabetes Res Clin Pract; 76 (1): 126-31.

37. Zanone MM, Catalfamo E, Pietropaolo S, Rabbone I, Sacchetti C, CeruttF.(2003). Glutamic Acid Decarboxylase and ICA512/IA-2 Autoantibodies as Disease Markers and Relationship to Residual Cell Function and Glycemic Control in Young Type 1 Diabetic Patients. Metab. 52(1): 25-29.