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Evaluation Of Potential Biomarker And Genetic HLA Alleles Profile In Diabetes Mellitus Type 1 Patients

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Abstract

Background :type1 diabetes mellites is an autoimmune disease result from deterioration of glucose metabolism along with beta cell gradually distraction in Langerhans islets in pancreas by influence of genetic and environmental conditions. The major genetic determinants of this disease are polymorphisms of class II HLA genes encoding DQ and DR.

Aim: to compare presence of some auto antibodies, cytokines and class II HLA haplotypes in diabetes millets type1 patient and healthy individuals.

Methods. Case-control study included 70 blood samples from unrelated T1DM patients attended to diabetes center in Nasseriah city (Iraq), in addition to 30 apparently healthy persons as control, collected from October 2020 to June 2021. The diagnosis of T1D was set up according to American Diabetes Association criteria. Demographical, anthropometric and biochemical characteristics were evaluated: C-peptide level, (anti-GAD65Ab and, anti-IA), IL-6 and TNF-α titer, FBS, RBS and HbA1c concentrations were evaluated for all patients and control healthy cohorts. Finally, four of relevant HLA haplotypes expression (HLA-DR3, HLA-DR4, HLA-DQA1*05:01 and HLA-DQB1*02:01) were checked by means of the PCR-SSP method.

Results. T1DM patients have higher both autoantibodies, but have a significant low level of C-peptide ($P < 0.0001$); significant higher amounts of FBS, RBS and HbA1c ($P < 0.0001$), higher concentrations of IL-6 and TNF-α ($P = 0.048$ and $P < 0.003$ respectively), than control cohort. Class II HLA genotyping reveal that HLA=DR3 the most common haplotype followed by HLA-DQA1*05:01 and HLA-DQB1*02:01. The 11 -20 age group were the most affected people that have two of studied haplotypes followed by those have less or more than two. The majority of patients have two (DQA1*05:01, DQB1*02:01) haplotypes followed by whose carried one, three, or all four studied haplotypes.

Conclusion. newly diagnosed juvenile diabetics were positive anti-GAD65Ab, IA; had a significant relationship with low C-peptide level. These patients showed carrying distinct HLA-II haplotypes.

Key words: T1DM, IA, anti-GAD65, C peptide, Nasseriah, Iraq.

Introduction

The incidence of type 1 diabetes mellites (T1D), has been increasing worldwide by approximately 3% per year, with highest increasing rate among young children [1]. Diabetes mellitus (DM) is a chronic metabolic

and endocrine disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [2]. DM can be classified into type 1, type 2, gestational, and other suggested specific subtypes (e.g., latent autoimmune diabetes in adult LADA and MODY) [3]. Type 1 diabetes, formerly known as juvenile diabetes or insulin-dependent diabetes, is a chronic condition in which the pancreas produces little or no insulin. Insulin is a hormone that is required for sugar (glucose) to enter cells to produce energy. Although type 1 diabetes usually appears in childhood or adolescence, it can develop in adults to represents around 10% of the diabetes cases worldwide. Despite much research, no definitive cure for type 1 diabetes has been found. Treatment focuses on controlling blood sugar levels with insulin, diet, and lifestyle, to prevent complications that affect major organs in the body over time such as the heart, blood vessels, nerves, eyes, kidneys, foot damage, diseases related to the skin and mouth and pregnancy complications. Type 1 diabetes, a classical organ-specific autoimmune disease, characterized by autoimmunity against β-cell autoantigens. This chronic destructive process involves both cellular and humoral components detectable in the peripheral blood, months or even years, before the onset of clinical diabetes [4,5]. Autoimmune T1D comprises sensing of death-associated molecular patterns (DAMPs) released from apoptotic β-cells by innate immune receptors such as Toll-like receptor (TLR), which suggests that innate immunity is critical for the initiation and development of T1D[6]. The exact cause of type 1 diabetes is unknown, but possible causes include: genetics and environmental conditions such as exposure to virus (such as rubella and Enteroviruses), several dietary factors (such as cow's milk consumption, vitamin D deficiency, Early integration of cereals into the diet), weight gain and psychological stress [7,8]. Heredity, however, increases the susceptibility of predisposed children to diabetes. In type 1 diabetes, the strongest genetic influence is conferred by genes in the HLA complex, which are responsible for approximately half of the genetic susceptibility.[9]. The (MHC) region contains multiple susceptibility alleles DR and DQ of the MHC class II genes, probably influenced by class I genes, too. Thus, genetic markers for T1DM are present from birth, immune markers are detectable after the onset of the autoimmune process, and metabolic markers can be detected with sensitive tests once enough beta cell damage has occurred. These predictor markers can be detecting, before the onset of symptomatic hyperglycemia.

In spite of large number of studies that demonstrated specific alleles of HLA that strongly associated with diabetes. [10]. However, allelic variation at these loci cannot account fully for the pattern of HLA haplotype sharing among residents of our geographical area. So, this study is attempted to use serological and genetic characteristics with cytokine profiling to predict irregular progression of diabetes, hopefully to slowdown accelerated destructive phase occurring prior to the clinical diagnosis.

Patients and methods

The present study included 70 newly diagnosed Iraqi T1DM patients (37 females, 33 males), attending the from Diabetes and Endocrine center in Thi-Qar (Nasiriyah) governorate, while control samples, were taken from Mohammed Al Mousawi teaching hospital for children attenders suffering from diseases other than diabetes. Both cohorts were Nasiriyah city habitants, in period from November 2020 till June 2021. From all subjects anthropometric data like age, gender was obtained; examined for biochemical test, and typed for HLA class I and II antigens. Informed consent from all the included subjects in the study and the approval from Thi-Qar health office ethical committee was taken. Patients with other types of diabetes (T2DM, diabetic pregnant females, genetic forms of diabetes—MODY patients, basal hyperglycemia, decreased glucose tolerance or symptoms resembling diabetes presenting), were excluded from the study.

Serum samples processing

Approximately 5 ml of human blood was collected from each subject (patients and control), transferred into sterilized test tubes (Gel tube) and allowed for 30 minutes to clot at room temperature. the sample was centrifuged for 15 minutes at 3000 rpm and the serum was immediately separated, added to Eppendorf tubes (1.5 ml) and stored at (-48^{0}C) till used for Anti glutamic acid decarboxylase (Anti GAD65), Insulin autoantibody (IA), C peptide and another biochemical analysis.

- **Determination of blood glucose**
  fasting and random Blood sugar was determinate by enzymatic colorimetric method using Randox diagnostic kits (UK).
- **Anti-glutamic acid decarboxylase (Anti GAD65) (Snibe , China)** the analysis achieved according to company instructions
  Cut off (negative < 5.0 IU/ml, positive ≥ 5.0 IU/ml)
- **Insulin autoantibody (IA) ELISA kit (Bioassay, China)** the analysis achieved according to company instructions
  Cut off (negative < 2.4 U / ml, positive ≥2.4 U / ml)
- **glycosylated hemoglobin** (HbA1c) by High-Performance Liquid Chromatography). **(Roche, German)**
- TNFα and IL-6 determination kit **(Bioassay, China)**

- **HLA antigen typing:** the basic material for typing with (**GoTaq® G2 Green Master Mix kit**). The test procedure was done by using the sequence specific primers (SSP). This method is based on the fact that primer extension and hence successful PCR relies on an exact match at the 3` ends of both primers. PCR products were analyzed on 2% agarose gel.
- **C-peptide (Roche, German)** the analysis achieved according to company instructions
  Cut off
  Fasting (ng/ml)
  normal (0.78 – 1.9), increase (>1.9), decrease (<0.78), decline (>0.5)
  Random (ng/ml)
  normal (3 – 9), increase (>9), decrease (<3), decline (>1.1)
- **gSYAN DNA Extraction Kit** (Geneaid , Taiwan)

Statistical Analysis:

All data were presented as a mean and standard deviation SD. Statistically analyzed by GraphPad prism version 8 software. Unpaired non parametric t test was used to compare between the mean of different variables. The P value> 0.05 was considered statistically significant.

Results

70 patients with diabetes mellitus who's the mean of patient age 14.02±6.06, ranged between (2-26) years included in this study. Thirty-four (48%) male and thirty-six (52%) females. the patient's cohort was compared with 30 healthy individuals cohort consists (15 males and 15 females), aged between 1–26 years.

Autoantibodies Prevalence: In comparison with our previous result [10], 65 T2DM was patients were associated by 3% of both auto antibodies (anti-GAD+ IA), 15% with anti-Gad alone, and 12.3% with IA. Now, in study of T1DM, among 70 T1DM patients, 16 cases (21%) had positive for both autoantibodies, 69 (98%),

16 (21%) were positive for anti-GAD65 anti-IA. respectively. both traditional autoantibodies were significantly very higher (P<0.0005) in patients than in healthy cohort (figure1D and 1E). None of the control subjects (n = 30) was in positive range of anti-GAD and anti-IAA. The level of anti-GAD and anti-IAA, were varied widely in patient's cohort, from 2.82 to 280 mIU/ml, and from 6.3 to 23.2 mIU/ml.

c- peptide measurement: we got significantly lower amount of C peptide (0. 23 ±0.39ng/ml) produced in autoantibodies bearing patients (see figure1C). Mean (FBS and RBS) of all patients were 244.73±105.89 and 393.35±130.39 mg/dl respectively. This means the presence and increasing the concentration of autoantibody may lead to a decrease in the concentration of c-peptide. Mean of C peptide low levels (below normal < 0.5 ng/ml) in our study, came in contrast to elevated fasting and random sugar concentrations (figure1C). **HbA1C:** the mean of glycated hemoglobin HbA1C in the plasma of patients was 9.66±1.61% (figure1B) Mean HbA1c was positively correlate with RBS (Pearson Correlation 0.237) and statistically significant (p=0.048) (table 1). This study shows poor glycemic control in patients having insulin neutralization by IA. IL-6 (figure1F), and TNF (figure1G) that all came in significantly higher amount in patients in comparison with healthy individual cohort.

Interrelation between different biochemical characteristic: the associations between different biochemical characteristic were determined using Pearson's correlation. weak negative correlation was observed between FBS and RBS from one side and all of C-peptide, IA, Anti Gad, IL-6, TNF on the other side with correlation coefficient between -0.021 and -0.18.the strong association was between FBS and RBS from one side and HbA1C from another side with correlation coefficient (0.237). the strongest positive association was between antiGAD and IA titer from one side and IL-6 concentration with strong correlation coefficient (0.408 and 0.419) respectively as shown in table 1.

Frequency of haplotypes (HLA-DR3, HLA-DR4 , HLA-DQA1*05:01 and HLA-DQB1*02:01) among T1DM patient vs control: the detection of haplotypes that genes that hypothesized associated with T1DM , shown that the frequency of the these alleles is higher in patients than in control groups. The most to less predominant HLA haplotype arrangement in patient cohort were DR3(73%), DQA1*05:01(53%), DQB1*02:01(37%) than DR4(18.5%) in the same priority in healthy control cohort but in about ten-fold more (see figure 2A). The majority [n=32(45.7%)] of T1DM patients have two different haplotypes (DQA1*05:01, DQB1*02:01), while 27.14% have one, 17.1% have three, 7.14% haven't particular one and 2.85% have all four studied haplotypes (see red color column of fig2B). In contrast to patient cohort, the majority of healthy group (green colored column in the same figure) have no at all, five persons have each one or two, nobody healthy having three or four of studied haplotypes. The majority of studied T1DM patients were (11-20 years old) followed by (0-10) (21-30) even fewer group at age range (31-40). All age group of patients, has a common feature which is that it possesses two haplotypes of the HLA II complex (see figure 2C)

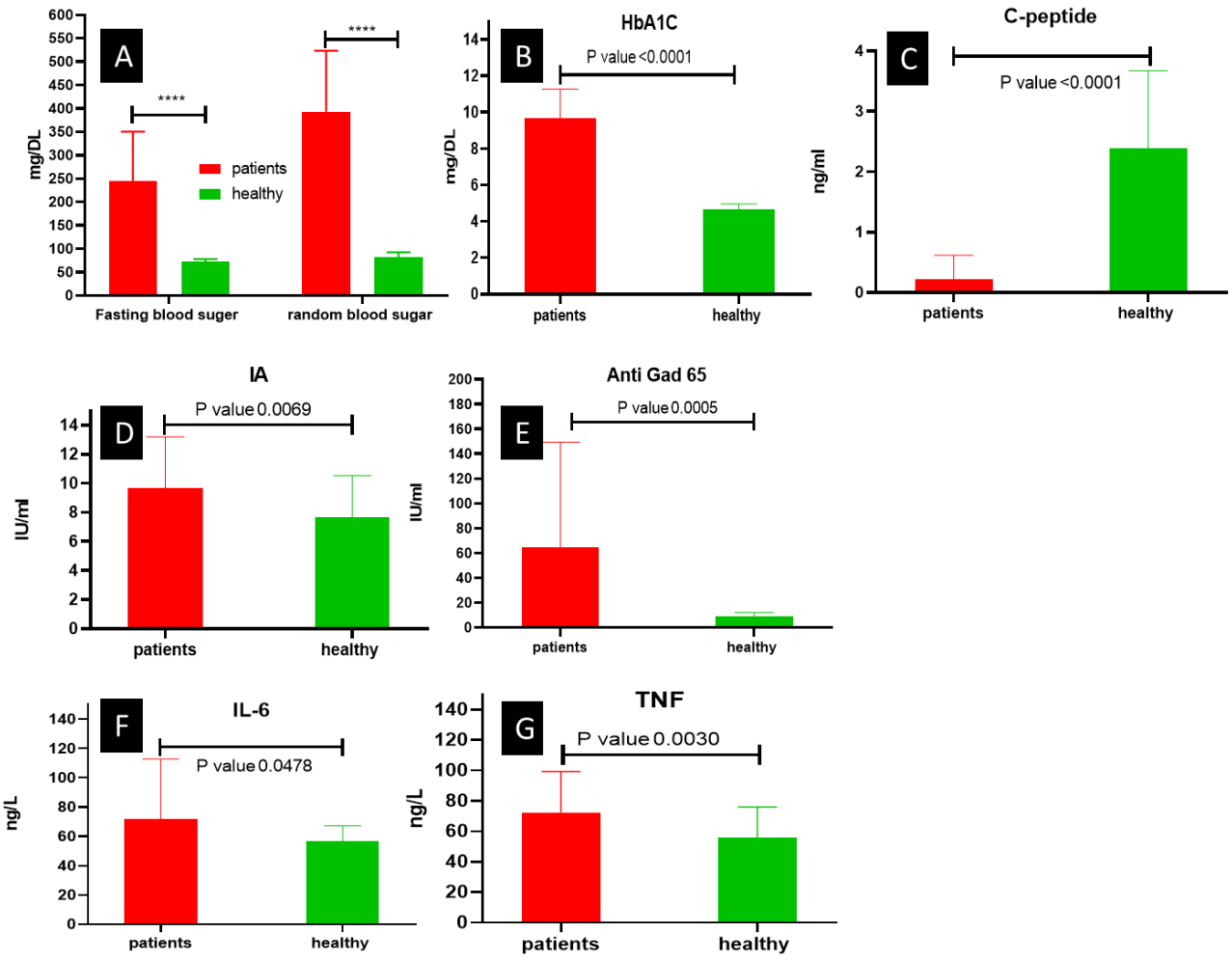

Figure 1: the significant clinical parameters of studied T1DM patients (red color column) in comparison with healthy control (green color column) groups. A-fasting and random blood sugar., B- Hb AIC concentration C- c -peptide D- anti insulin antibody IA, E- anti-GAD/65, F- IL-6 cons. G- TNF cons.

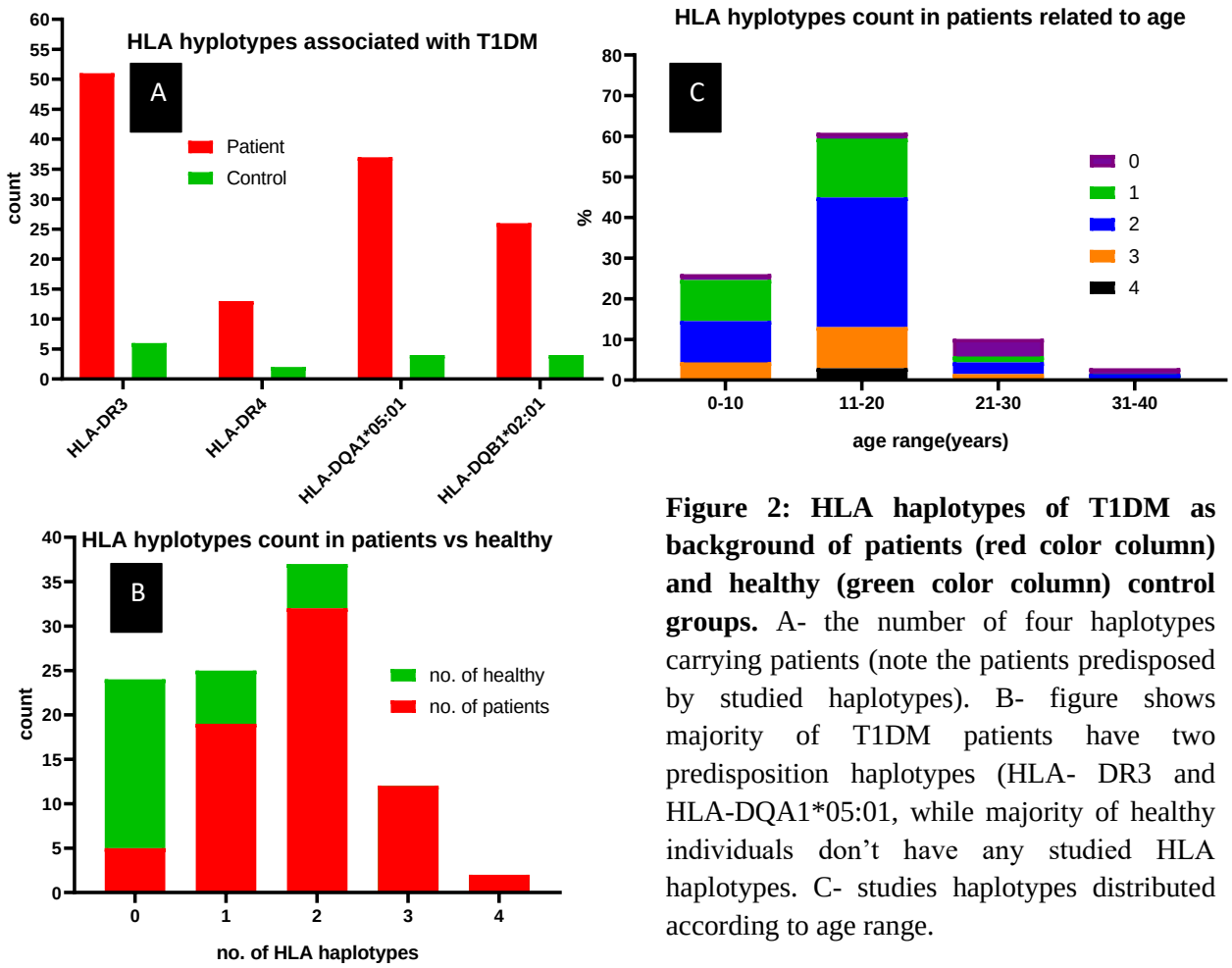

Figure 2: HLA haplotypes of T1DM as background of patients (red color column) and healthy (green color column) control groups. A- the number of four haplotypes carrying patients (note the patients predisposed by studied haplotypes). B- figure shows majority of T1DM patients have two predisposition haplotypes (HLA- DR3 and HLA-DQA1*05:01, while majority of healthy individuals don't have any studied HLA haplotypes. C- studies haplotypes distributed according to age range.

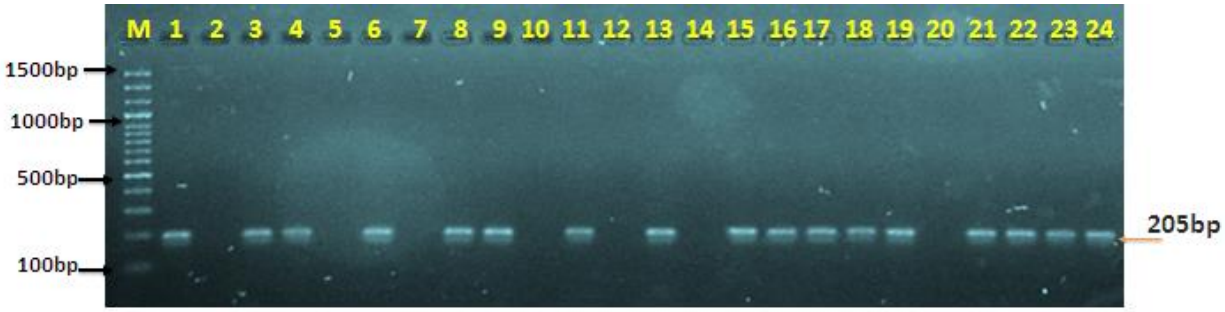

Figure 3: Agarose gel electrophoresis image that showed the SSP-PCR product analysis of HLA-DQB1*02:01mutation from patient and healthy control blood samples. Where, Lane (M) Marker ladder (1500-100bp), lane (1-24): showed some positive HLA-DQB1*02:01 mutation at 205bp PCR product size.

Table (1) the correlation values of different studied parameters (n=70)

		FBS	RBS	HbA1C	C_peptide	IA	Anti Gad	IL_6	TNF
FBS	Pearson Correlation	1	.772**	0.017	-.021-	-.119-	-.161-	-.127-	-.117-
	Sig. (2-tailed)		0	0.889	0.861	0.328	0.183	0.295	0.336
RBS	Pearson Correlation	.772**	1	.237*	-.097-	-.163-	-.189-	-.140-	-.174-
	Sig. (2-tailed)	0		0.048	0.426	0.178	0.118	0.248	0.151
HbA1C	Pearson Correlation	0.017	.237*	1	-.128-	0.086	-.064-	-.053-	0.076
	Sig. (2-tailed)	0.889	0.048		0.292	0.479	0.599	0.661	0.531
C_peptide	Pearson Correlation	-.021-	-.097-	-.128-	1	-.094-	-.011-	0.012	-.036-
	Sig. (2-tailed)	0.861	0.426	0.292		0.44	0.929	0.919	0.767
IA	Pearson Correlation	-.119-	-.163-	0.086	-.094-	1	0.192	.419**	0.143
	Sig. (2-tailed)	0.328	0.178	0.479	0.44		0.111	0	0.238
AntiGad	Pearson Correlation	-.161-	-.189-	-.064-	-.011-	0.192	1	.408**	0.126
	Sig. (2-tailed)	0.183	0.118	0.599	0.929	0.111		0	0.299
IL_6	Pearson Correlation	-.127-	-.140-	-.053-	0.012	.419**	.408**	1	0.143
	Sig. (2-tailed)	0.295	0.248	0.661	0.919	0	0		0.238
TNF	Pearson Correlation	-.117-	-.174-	0.076	-.036-	0.143	0.126	0.143	1
	Sig. (2-tailed)	0.336	0.151	0.531	0.767	0.238	0.299	0.238	
**. Correlation is significant at the 0.01 level (2-tailed).									
*. Correlation is significant at the 0.05 level (2-tailed).									

The discussion

Diabetes Mellitus is the commonest endocrine disorder in the population. Type 1 DM is caused by auto immune destruction of the beta cells of the pancreas, rendering it unable to synthesize and secrete insulin [2]. Antibodies to glutamic acid decarboxylase (GAD) inactivate of the enzyme that catalyzes the rate -limiting step in the conversation glutamic into a neurotransmitter in pancreatic islet β cells. In comparison with our previous results [11], clearly the distribution of both two types of autoantibody (anti-GAD and IA) were more common in T1DM than in T2DM (21% against 3% of patients respectively). anti-GAD and IAA each one alone was common in 15%, 12.3% with T2DM patient's cohort, while here these two antibodies were found in 98%, 21% respectively in very close manner from Graham study which reported 90% of anti GAD in T1D [12]. These results clearly indicate that the effect of autoimmune antibodies is more pronounced in the case of type 1 diabetes compared to type 2, by this way came in consistent with (Verge CF,2005) [13] and, so it is merit to get "autoimmune diabetes" name.

Another important serological marker analyzed here, was c- peptide, a widely used index for measuring pancreatic β cell function as well as differentiates T1D and T2D. Human insulin and c-peptide are synthesized as a single polypeptide chain known as Proinsulin in the pancreatic beta cells. 50% of produced insulin is extracted, when passes through the liver, therefore insulin measurement gives a wrong value of insulin secretion, and C-peptide, thus provides a better index of endogenous insulin production [2] also that because of the presence of insulin binding antibodies that neutralize insulin but not C peptide molecules. Our result shows reducing in c-peptide below normal range ((3 – 9 ng/ml) in all patient cohort, and were associated with increased fasting and random plasma glucose due to insulin resistance. These findings were in agreement with other studies such as [14]. A negative correlation exists in our study with r value of -0.021 (Table 1). Most studies showed decreasing in normal cellular function, alteration in enzyme activity and cytokine secretion in diabetic monocytes, macrophages, and neutrophils when compared to control cells [15], in our study we found increased level of IL-6 and TNFα in comparison with healthy control group. this result came in consistent with [16], in spite of other in vitro studies, that showed hyperglycaemia leads to macrophage dysfunction on long-term exposure resulting significant reduction in pro-inflammatory cytokines TNF-α and IL-6 production [17]. Stressed adipocytes secrete pro-inflammatory cytokines such as IL-6 or TNF-α, which activate T cells, B cells and macrophages to trigger an inflammation of pancreatic tissue. Our study showed that IL-6 are upregulated, (around 2-fold), in diabetic patients than healthy subjects and this result matches [18]. But still secretion of such cytokines is controversy may due to macrophage alteration by AKT/ERK pathway and epigenetic histone modification [19]. High-level expression of IL-6 are responsible for continuous active state which makes them less response to bacterial LPS stimuli for long time and made negative feedback mechanism of NET formation in diabetic neutrophils [20]. As in other autoimmune disorders, the relevance of the HLA class II in T1D undeniable, we analyzed prevalence of four HLA haplotypes among our two cohorts. As concluded in our study, several researchers reported certain haplotypes among T1D patients, for example Joshi, M., et al identified 30% DQ2 (DQA1*0501-DQB1*0201) haplotype prevalence among T1DM [21]. Also, DQB1*02:01 which previously documented associated with T1D in individuals from Bahrain, Lebanon, Tunisia as well as the Caucasian populations, came as third most common haplotype in thiqar province patients after DR3 and DQA1*05:01haplotypes prevalence. This finding came totally agree with another local study which found DQB1*0201 allele was high frequencies among T1DM patients in comparison with healthy controls [22]

Although taken genotypes here conferred extremely high risk, there is a spectrum of risk associated with HLA-DR/DQ genotypes— ranged from increased, to neutral, to protective, such as the HLA-DQA1*0102,

DQB1*0602 haplotype that have dominant protection from T1D, in spite of presence IA autoantibodies [23]. The HLA antigens DQ2 tightly linked with DR3 and DR4, to be the major common predisposal genetic factors that make Type 1 diabetes (T1D) and autoimmune thyroid disease (AITD), tend to occur together [24]. Therefore, we can predict the incidence of AITD in our T1D patients who have same aforementioned HLA haplotypes. We faced several limitations in this study such as, the differences in available number of patients vs control rendering the samples may be not enough to provoke further clues of the observed tendency, so further research with a larger cohort will be necessary. It is well known that HLA susceptibility haplotypes in Arab and Asian populations, differ from that found in European and American Caucasian populations. Therefore, comparing our results with the published results of others does not mean that there is an inaccuracy. In conclusion, the genetic, epigenetic and environmental factors may create a combined influence which lead to the onset of such autoimmune disorder within families.

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