Journal of Al Azhar University-Gaza *(Natural Sciences)*, **2014, 16** : **15-36**

Determination of anti-insulin and anti-glutamic acid decarboxylase in type 1 diabetic patients and their siblings in Gaza Strip

Abeer Al-Qatati [1] , Mohammed El-Khateeb[2]

[1] Faculty of Applied Medical Sciences
Al-Azhar University-Gaza, Palestine
[2] Faculty of Medicine, Jordan University, Amman, Jordan

a.qutati@alazhar.edu.ps

Received 5/9/2013 *Accepted* 09/02/2014

Abstract:

Several autoantibodies are associated with autoimmune type 1 diabetes. Measurements of islet autoantibodies can assist in the diagnosis of this disease, and the detection of islet autoantibodies in nondiabetic individuals indicates a significantly increased risk for the subsequent development of type 1 diabetes. When preventive therapies for type 1 diabetes become available, islet autoantibody screening of the general pediatric population should be considered to identify individuals at risk.

The goal of this study was to address two key questions concerning autoimmune type 1 diabetes: First: What are the prevalences of anti-insulin and anti-GAD autoantibodies in patients of type 1 diabetes mellitus and their siblings in Gaza Strip? and Second: Which of the siblings are at increased risk for developing type 1 diabetes? For this purpose, 47 type 1 diabetic patients and 154 of their siblings were examined for anti-GAD and anti-insulin autoantibodies. Our results indicated that there was a significant difference between type 1 diabetic patients and controls in relation to the positivity of anti-GAD [76.6% for the patients versus 2.6% for the controls; p = 0.001] and anti-insulin autoantibodies [89.4% for the patients versus 5.2% for the controls; p = 0.001].

In addition there was a significant difference between the siblings tested in this study and the controls according to the positivity of anti-GAD [45.5% for the siblings versus 2.6% for the controls; P = 0.001] and anti-insulin autoantibodies [38.3% for the siblings versus 5.2% for the controls; P = 0.001].

A total of 27 out of 154 (17.5%) of the siblings were found to be positive for both autoantibodies, anti-GAD and anti-insulin. So such siblings may be at increased risk of developing type 1 diabetes, they must be followed up by continuous medical examination and laboratory investigation which include testing for other islet autoantibodies and HLA typing to determine whether or not they have the susceptibility alleles for type 1 diabetes.

Introduction:

Type 1 diabetes mellitus or insulin-dependent diabetes mellitus is a lifelong metabolic disorder caused by cellular mediated autoimmune destruction of pancreatic β-cells (Bluestone *et al*. 2010), this process leads to progressive and irreversible failure of insulin secretion.

Type 1 diabetes mellitus is usually diagnosed among children, adolescents and young adult people. Worldwide, more than three hundreds and fourty six million people have diabetes (Jönsson *et al*. 2012), the incidence rate of type 1 diabetes is increasing by three percent per year in different countries around the world, particularly among younger children (Alderson *et al*. 2006, Rankin *et al*. 2012). The disease occurs more frequently among caucasians than in other ethnic groups. The age-adjusted incidence of type 1 diabetes varied from 0.1/100000 per year in China to 40.9/100000 per year in Finland (Liese *et al*. 2006).

Once β-cells are destroyed, there will be a loss in blood-glucose control, resulting in acute conditions such as ketoacidosis (Westerberg *et al*. 2013) and different complications as heart disease, blindness and kidney failure.

Biomarkers of the immune destruction of the β-cells include autoantibodies to islet cells, glutamic acid decarboxylase (GADA), insulin (IAA), the tyrosine phosphatases IA-2 and IA-2β, and zinc transporter 8 (ZnT8A). It was found that high percentage reaching eighty five to ninety percent of the newly diagnosed type 1 diabetic patients are positive for one or more of these autoantibodies, the differences in proportion depends on patient's age, number and quality of the assays used and ethnicity (Barker *et al*. 2004)

In the context of pathogenesis of type 1 diabetes mellitus, T-helper 1 (T_{H1}) cells infiltrating the pancreatic islets secrete significant

quantities of γ-interferon and tumor necrosis factor-β (TNF-β), these cytokines activate endothelial cells to recruit circulating leucocytes to the site of antigen challenge. Antigen-bearing cells are eliminated by activated macrophages by apoptosis which is activated by a number of alternative mechanisms (Kurrer *et al*. 1997) including: (i) interaction of Fas on β-cells with Fas Ligand on islet-infiltrating mononuclear cells, (ii) action of nitric oxide and oxygen-derived free radicals, and (iii) membrane disruption by perforin and granzyme B produced by cytotoxic T-cells. Persistent secretion of γ-interferon in the inflamed islets results in over-expression of HLA class I molecules on the β-cells, which potentiate their destruction (Pavlovic *et al*. 1997). Local high concentrations of IL-1, γ-interferon, TNFα and TNFβ have a direct pathogenic effect on the β-cells (Amrani *et al*. 2000).

An interplay between genetic susceptibility (polygenic) and a triggering environmental agent was thought to provide the fundamental elements for disease formation.

In both humans and non-obese diabetic (NOD) mice, type 1 diabetes mellitus arises as a complex polygenic trait, there is much evidence suggesting that the genetic association to type 1 diabetes is through the major histocompatibility complex class II alleles (Cucca *et al*. 2001). It was found that lymphocyte-defined HLA-D antigens, HLA class II DR3 (HLA-DRB1*04, DQB1*0302) were much more closely associated with type 1 (Jones *et al*. 2006), accounting for approximately fourty percent of the genetic risk for type 1 development, and the DR3/DR4 combination, two susceptible alleles, could produce a higher-risk genetic combination (Dorman *et al*. 2000, Noble *et al*. 2011). Besides HLA, the insulin gene (IDDM2) on chromosome 11 (Awata *et al*. 2007), the CTLA4 gene at the IDDM12 susceptibility locus (Nistico *et al*. 1996), PTPN22 lyp (Bottini *et al*. 2004) are strongly associated with type 1 diabetes onset.

Environmental risk factors are thought to be initiators or accelerators of β-cell autoimmunity or precipitators of obvious symptoms in the patients suffering from the destruction of isulin-producing cells. The environmental factors may have direct effect on the pancreas, or promote abnormal immune responses to proteins normally expressed in the cells.

It was shown that some viruses like enteroviruses, coxsackie virus B, mumps, rubella, cytomegalovirus, parvovirus, rotaviruses,

and encephalomyocarditis virus might contribute to type 1 pathogenesis (Van der Werf *et al*. 2007, Van Belle *et al*, 2011). Recently, *Mycobacterium avium subsp. Paratuberculosis* (MAP) has been proposed as a new environmental factor (Di Sabatino *et al*. 2011) that might play a role in the pathogenesis of type 1 diabetes (Rosu *et al*. 2009). The prevalence of MAP infection is high in type 1 diabetic patients in Sardinia (Rani *et al*. 2010) which is considered to be one of the areas with the highest type 1 prevalence all over the world.

The viral infection can provoke the development of type 1 diabetes through direct cytolytic effect or by triggering autoimmune response gradually leading to β-cell destruction because of the structural homology between viral structures and β-cell antigens (Ling Wu *et al*.2013).

Materials and methods:

Whole blood samples were collected from seventy four (twenty five males and twenty two females) patients diagnosed as having type 1 diabetes mellitus at Al-Rimal Clinic in Gaza city, Khan Yonis Clinic, Rafah Clinic, and Al-Naser hospital for children in Gaza city. Patients age ranged between six to thirty five years. In addition**,** Whole blood samples were collected from one hundred and fifty four (eighty seven males and sixty seven females) siblings of the patients, apparently don't have type 1 diabetes mellitus, sibling's age ranged between one and half to twenty years. Thirty eight age matched individuals were recruited as controls. Data concerning name, age, sex, the age at the disease onset were recorded for each patient. The name, age, sex of siblings and controls were also recorded. Venous peripheral whole blood samples were drawn from each patient, sibling and control using vacutainer system (Becton Dickinson Vacutainer System), (ten mL plain tube). Serum samples were separated within thirty minutes by centrifugation at 1500 g at 25°C for five minutes, then serum samples were aliquoted and stored at -20°C until they were analyzed.

Autoantibodies Detection:

1- Anti insulin antibody – 100 (AIA – 100):

Commercially available semi quantitative radioimmunoassay test for the measurement of free-anti-insulin antibodies in serum was used. (Bio source Europe S.A., Rue de l'Industrie, 8 B-1400 Nivelles Belgiu). In this test, one hundred µl of the standards and samples were dispensed in each of the respective tubes, then, one hundred µl of the tracer was dispensed into each tube, including the tubes for total counts and gently shaked to liberate any trapped bubbles, the tubes were then covered and incubated for two hours at 37°C. One ml of polyethylene glycol solution was then added into each tube, mixed gently by brief vortexing and incubated for fifteen minutes at room temperature. The tubes were then centrifuged for fifteen minutes at 1500 g and immediately the supernatants were removed carefully from each tube, avoiding to disturb the precipitate. The radioactivity was counted by gamma counter for sixty seconds (Gamma counter, LKB, Finland).

2- Anti-GAD65 RIA:

Commercially available semi quantitative radioimmunoassay test for the measurement of free-anti-GAD antibodies in serum was used (Bio source Europe S.A., Rue de l'Industrie, 8 B-1400 Nivelles Belgium). In this test, polystyrene tubes were labeled for each standard, sample and controls. Twenty five µl of each sample, standard and control were dispensed into the corresponding tubes. Then, fifty µl of the tracer was dispensed into each tube. The tubes were shaked, and incubated for two hours at room temperature. After the incubation, two ml of protein A was dispensed into each tube and incubated for thirty minutes at room temperature. After centrifugation at 1500 g for ten minutes, the supernatant was decanted and the tubes should stand for half a minute upside down on an absorbent paper. The radioactivity was measured by gamma counter.

Statistical analysis:

Data was presented as mean ± standard deviation. Statistical significance was performed by using coefficient correlation factor (P-

value) and Chi-square test (χ^2). P-values less than 0.05 were considered as significant.

Results:

1- Prevalence of anti-GAD and anti-insulin autoantibodies:

A total number of fourty seven patients with an age ranged between six to thirty five years, mean 16.7 ± 7.2 SD were examined for the presence of anti-GAD and anti-insulin autoantibodies. Thirty six (76.6%) of the patients were positive for anti-GAD autoantibodies (Figure 1) and fourty two (89.4%) were positive for anti-insulin autoantibodies (Figure 2).

A total number of one hundred and fifty four siblings with an age ranged between one and half to twent years, mean 11.4 ± 4.5 SD were screened for such autoantibodies. Seventy (45.5%) of the siblings were positive for anti-GAD autoantibodies (Figure 1) and fifty nine (38.3%) were positive for anti-insulin autoantibodies (Figure 2).

Age-matched control group of thirty eight individuals were examined for the presence of the same autoantibodies, one (2.6%) was positive for anti-GAD autoantibodies (Figure 1) and two (5.2 %) were positive for anti-insulin autoantibodies (Figure 2).

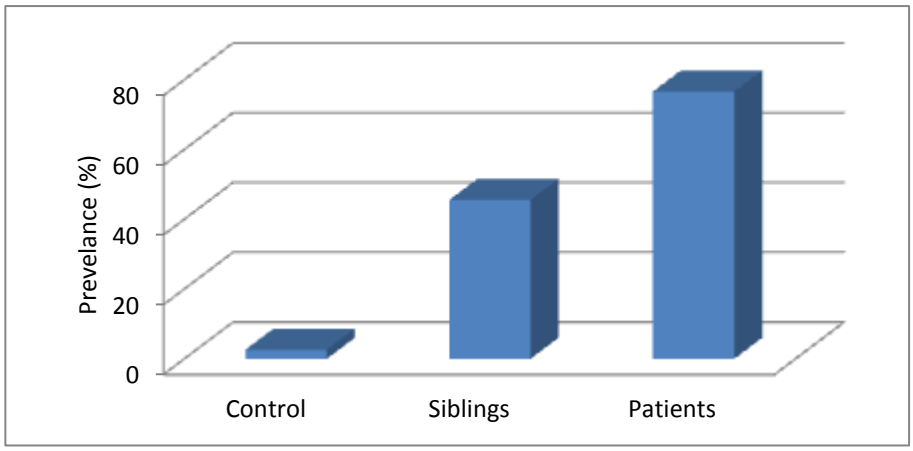

Figure 1: Prevalence of anti-GAD autoantibodies in patients, siblings and control group.

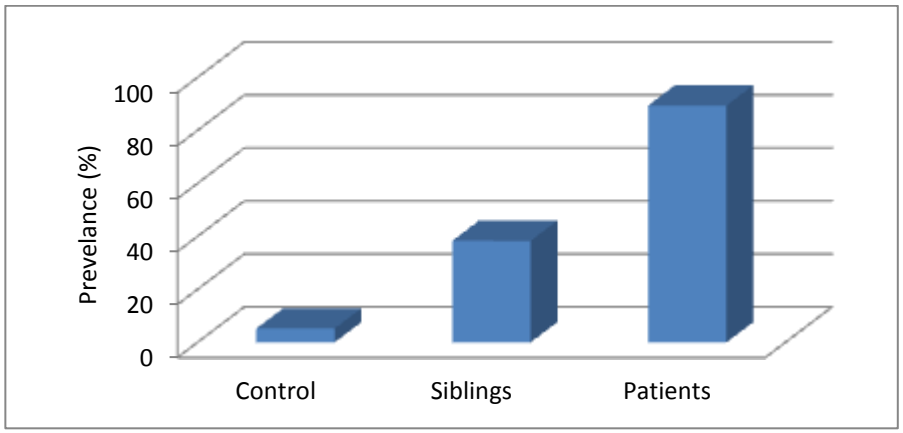

Figure 2: Prevalence of anti-insulin autoantibodies in patients, siblings and control group.

Comparison between study groups:

By the use of Chi-square test and p-value, it was found that there was a significant difference in the prevalence of positivity for anti-GAD and anti-insulin autoantibodies between patients and their siblings (P = 0.001), patients and controls (P = 0.001) and also between siblings and controls (P = 0.001).

2- Distribution of anti-GAD and anti-insulin autoantibodies according to gender:

A total of twenty two type 1 diabetic females were examined for anti-GAD and anti-insulin autoantibodies, it was noticed that seventeen (77.3%) were positive for anti-GAD (Figure 3) and twenty two (100%) were positive for anti-insulin autoantibodies (Figure 4). A total of twent five type 1 diabetic males were tested for the same autoantibodies, it was found that nineteen (76%) were positive for anti-GAD (Figure 3) and twenty (80%) were positive for anti-insulin autoantibodies (Figure 4).

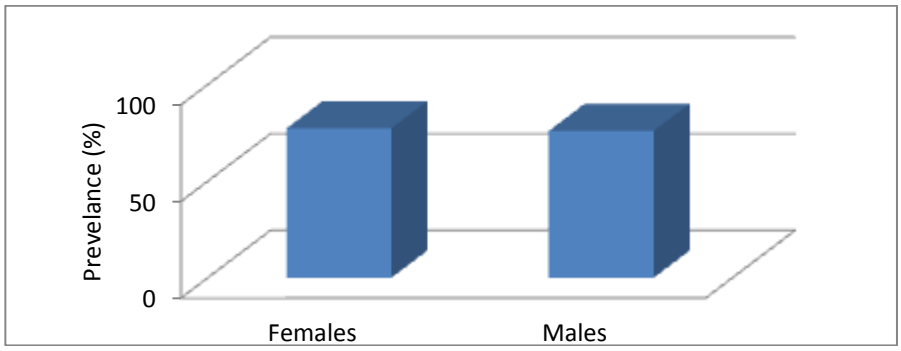

Figure 3: Prevalence of anti-GAD autoantibodies in both sexes of patients.

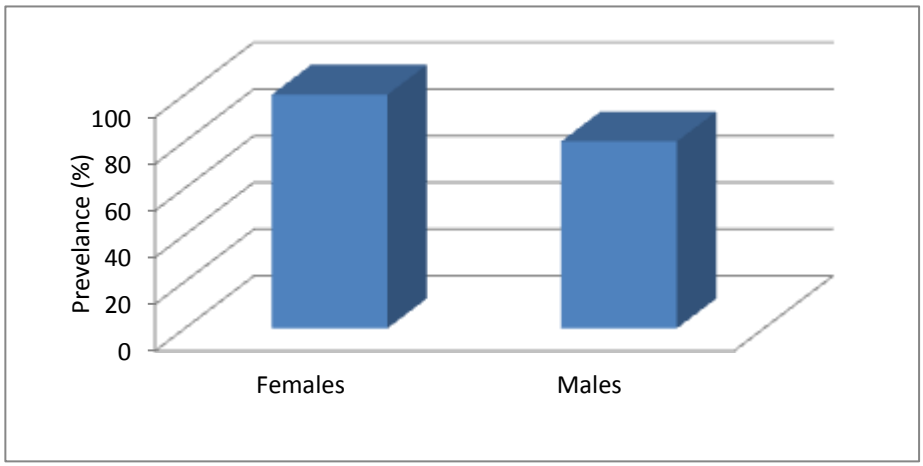

Figure 4: Prevalence of anti-insulin autoantibodies in both sexes of patients.

A total of sixty seven female siblings were screened for the same autoantibodies. Twenty eight (41.8%) were positive for anti-GAD (Figure 5) and twenty four (35.8%) were positive for anti-insulin autoantibodies (Figure 6).

A total of eighty seven male siblings were tested for anti-GAD and anti-insulin autoantibodies. Fourty two (48.3%) were positive for anti-GAD (Figure 5) and thirty five (40.2%) were positive for anti-insulin autoantibodies (Figure 6).

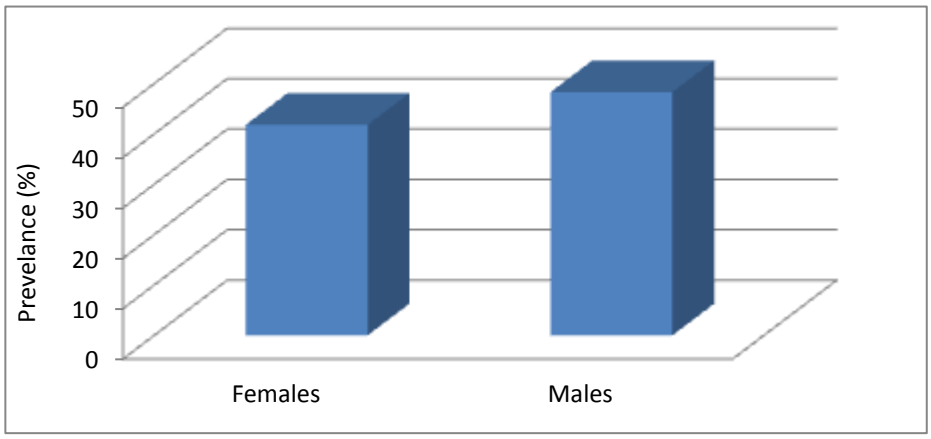

Figure 5: Prevalence of anti-GAD autoantibodies in siblings according to gender.

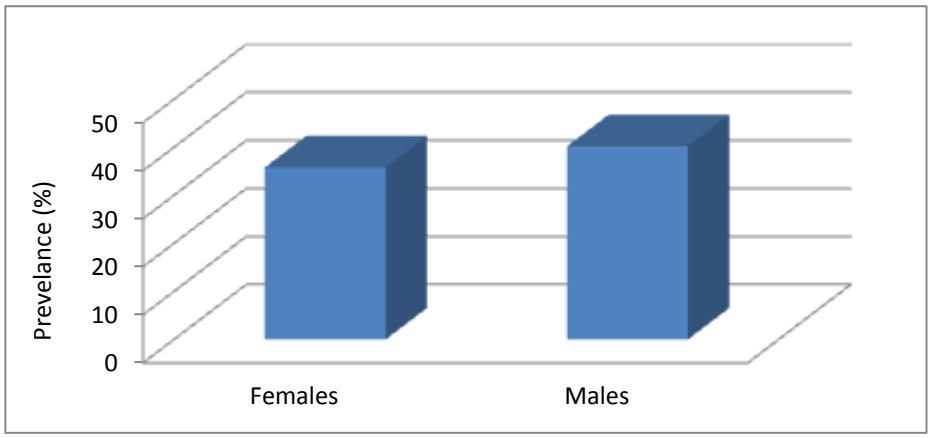

Figure 6: Prevalence of anti-insulin autoantibodies in siblings according to gender.

Comparison between both sexes in relation to the prevalence of the tested autoantibodies:

According to the prevalence of positivity of anti-GAD autoantibodies, it was found that there was no significant difference between males and females diabetic patients (P = 0.92), but there was a significant difference between them in relation to the prevalence of positivity of anti-insulin autoantibodies (P = 0.026). It was found that there was no significant difference between both sexes in siblings in

relation to the prevalence of positivity of anti-GAD (P = 0.158) and anti-insulin autoantibodies (P = 0.635).

3- Distribution of anti-GAD and anti-insulin autoantibodies according to age groups:

Our results for the prevalence of positivity for anti-GAD and anti-insulin autoantibodies in patients in different age intervals are summarized in Table (1) and Figure (7).

Table 1: Distribution of autoantibodies according to the age groups in patients.

Age (year)	Anti-insulin (%)		Anti-GAD (%)	
≤ 10	21.4	(9/42)	22.2	(8/36)
11-15	26.2	(11/42)	27.8	(10/36)
16-20	16.7	(7/42)	16.7	(6/36)
21-25	21.4	(9/42)	25.0	(9/36)
26-30	9.5	(4/42)	8.3	(3/36)
> 30	4.8	(2/42)	0.0	(0/36)

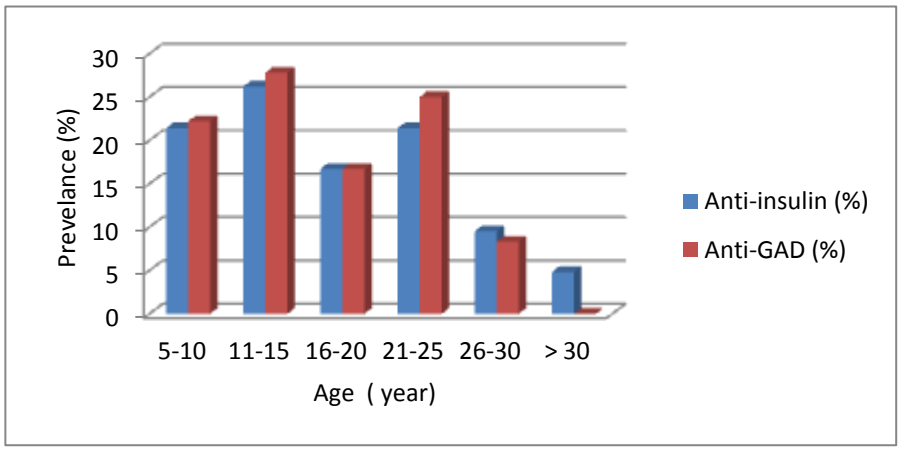

Figure 7: Prevalence of anti-GAD and anti-insulin autoantibodies in age intervals in patients.

From these results it was observed that the highest percentage of the positivity of anti-GAD and anti-insulin autoantibodies in the patients occur in the age interval of eleven to fifteen years.

Our results for the prevalence of positivity for anti-GAD and anti-insulin autoantibodies in the siblings in different age intervals are summarized in Table (2) and Figure (8).

Table 2: Distribution of autoantibodies according to the age groups in siblings.

Age group (years)	Anti-insulin (%)		Anti-GAD (%)	
< 5	3.4	(2/58)	7.1	(5/70)
5-10	28.8	(17/58)	25.7	(18/70)
11-15	45.8	(27/58)	41.4	(29/70)
16-20	22.0	(13/58)	25.7	(18/70)

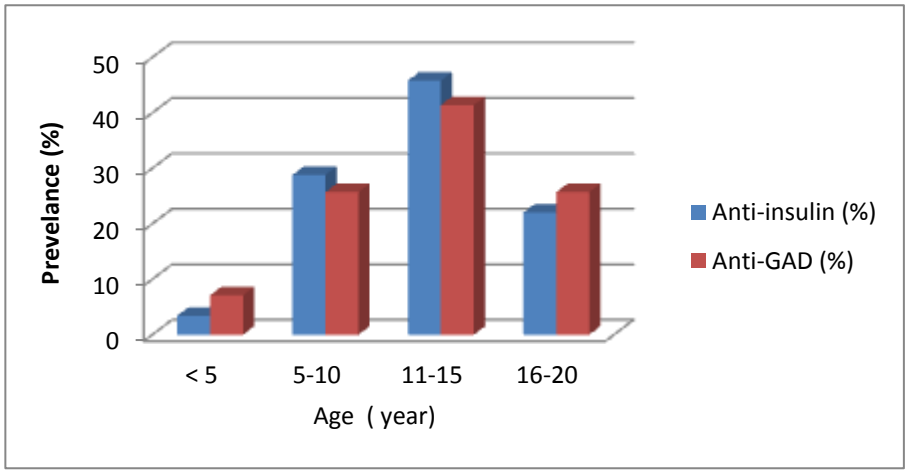

Figure 8: Prevalence of anti-GAD and anti-insulin autoantibodies in age intervals in siblings.

From these results it was observed that the highest percentage of the positivity of anti-GAD and anti-insulin autoantibodies in the siblings occur in the age interval of eleven to fifteen years.

Comparison between age intervals in relation to the positivity of anti-GAD and anti-insulin autoantibodies:

ANOVA test showed that there was a significant difference between the different age intervals in patients in relation to the positivity of anti-GAD (P = 0.041) whereas there was no significant difference between them in relation to the positivity of anti-insulin autoantibodies (P = 0.348). For the siblings there was no significant difference between the age intervals in relation to the positivity of anti-insulin (P = 0.323) and anti-GAD autoantibodies (P = 0.251).

4- Presence of two antibodies, one antibody and no antibody in patients regardless to the sex:

As indicated in Table 3 and Figure 9, the highest percentage of patients have two different autoantibodies

Table 3: Percentage of patients having one, two and no autoantibody

Number of autoantibodies	Percent of patients
2	70.2 (22/47)
1	25.5 (12/47)
0	4.3 (2/47)

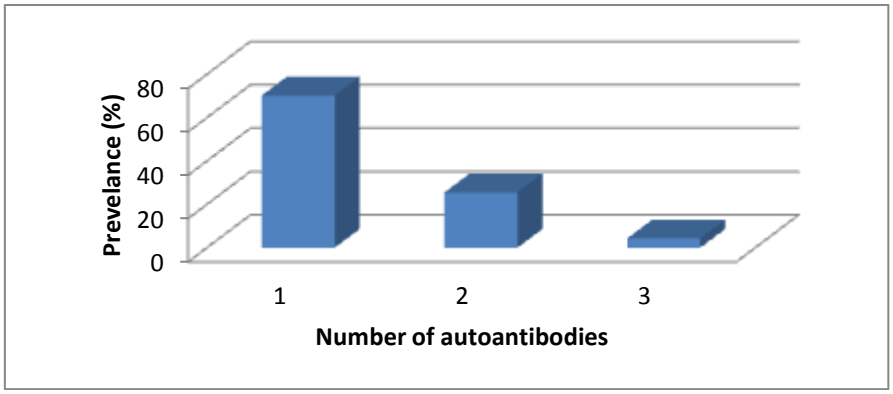

Figure 9: Presence of one, two and no autoantibody in patients.

5- Presence of two antibodies, one antibody and without any antibody in siblings:

As noted in Table 4, the highest percentage of siblings have one autoantibody, either anti-GAD or anti-insulin antibody.

Table 4: Percentage of siblings having one, two, and no autoantibody

Number of autoantibodies	Percent of patients
2	17.5 (27/154)
1	48.7 (75/154)
0	33.8 (52/154)

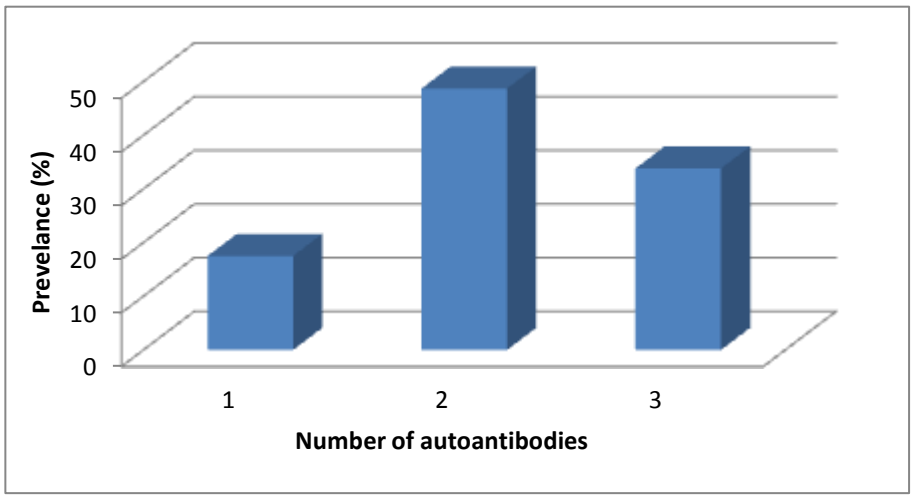

Figure 10: Presence of one, two and no autoantibody in siblings.

Discussion:

Type 1 diabetes mellitus was formerly known as insulin-dependent diabetes mellitus or juvenile-onset diabetes. Approximately five to ten percent of all individuals with diabetes mellitus are in this category. Most type 1 diabetes results from a cellular-mediated autoimmune destruction of the insulin-secreting cells of pancreatic β-cells (Atkinson *et al*. 2001). In most patients, the destruction is mediated by T-cells. This is termed type 1A or immune-mediated

diabetes. The islet cells have a chronic mononuclear cell infiltrate called insulitis. The autoimmune process leading to type 1 begins months or years before the clinical presentation, and an eighty to ninety percent reduction in the volume of the β-cells is required to induce symptomatic type 1 diabetes.

This study was designed to assess the prevalence of anti-GAD and anti-insulin antibodies in type 1 diabetic patients in Gaza Strip and to examine whether the presence of immune markers could be useful for the prediction of type 1 diabetes in their siblings. Our results showed that approximately seventy six percent of the patients have an anti-GAD, which was similar to a previous study in Italy (seventy seven percent) (Hawa *et al*. 1997) and higher than the results obtained in Sweden (sixty six percent) (Borg *et al*. 1997), Germany (seventy five percent), (Wiest *et al*. 1997), Australia (sixty nine percent) (Feeney *et al*. 1997), UK (seventy four percent) (Bingley *et al*. 1997), USA (seventy two percent) (Libman *et al*. 1998), Finland (seventy three percent) (Sabbah *et al*. 1999), Hong Kong (twenty six percent) (Kelly *et al*. 2001), Japan (seventy percent) (Yokota *et al*. 1998), Southern brazil (sixty three percent) (Moreira *et al*. 2011), but our results were lower than that obtained in France (eighty six percent) (Maugendre *et al*. 1997) and Russia (eighty three percent) (Trofimenko *et al*. 1994).

Our results indicated that the patients have an overall prevalence of anti-insulin autoantibodies of eighty nine percent which was higher than that reported before in Sweden (fifty six percent) (Hagopian *et al*. 1995), Australia (sixty five percent) (Feeney *et al*. 1997), UK (sixty nine percent) (Bingley *et al*. 1997) and Germany (fourty three percent) (Christie *et al*. 1997).

The prevalence of positivity of anti-GAD and anti-insulin autoantibodies among type 1 diabetic patients in Gaza Strip differs from those reported for different countries in the world, this may be due to differences in the genetic and environmental factors that may be responsible for triggering the autoimmunity in such patients, or may be due to the high frequency of consanguinity marriage in the population of Gaza or may be due to delay in the diagnosis because of the shortage in the advanced diagnostic tools and techniques or poor economical situations . The higher incidence of GADA in the patients

with age more than ten years (eighty percent) in comparison to patients with age less than ten years (seventy three percent) may indicate a difference in the environmental factors in our patients. The social, cultural and financial factors, due to unstable situation may complicate the follow up and early diagnosis of the disease.

It was shown by this study that seventy out of one hundred and fifty four (fourty five percent) of unaffected siblings were positive for anti-GAD autoantibodies which was higher than previously reported by Savola *et al* (1999) in Finland where fifty seven out of seven hundreds and fifty eight (seven percent) of the siblings were positive for GADA.

Our results indicated that fifty nine out of one hundred and fifty four (thirty eight percent) siblings were positive for IAA which were higher than what was reported by others, for example, in Mexican-Americans, five out of seventy eight (six percent) of siblings were positive for IAA (Zeidler *et al*. 2001) and in Finland, twenty eight out of seven hundreds and fifty eight (three percent) of the siblings were positive for IAA (Savola *et al*. 1999). The rates of GADA and IAA of the siblings in our study may be the highest reported in the literature so far.

Hagopian *et al*. (1995) showed that the prevalence of GADA and IAA were four percent and three percent respectively in a large population, which is comparable with our results where we found that two percent of the controls were positive for GADA and five percent were positive for IAA.

In this study, we were unable to demonstrate any difference between males and females in regard to the prevalence of GADA in type 1 diabetic patients. GADA (seventy seven percent for females versus seventy six percent for males; $p = 0.92$). This was in disagreement with previously published report by Sabbah *et al*. (1998) in Finland in which there was a sex related difference in the prevalence of GADA (seventy four percent for females versus sixty four percent for males; $p = 0.05$).

Our study showed a difference between females and males of type 1 diabetic patients in the prevalence of IAA [one hundred percent for female versus eighty percent for males; $p = 0.026$], this is in

agreement with a previously published report by Zimmet *et al* (1994) in Australia in which sixty percent of the females versus eighty two percent for the males were positive for IAA ; $p = 0.02$.

In the current study, the highest percentage of patients having GADA are in the age group of eleven to fifteen years, whereas the lowest percentage was observed in the age group of twenty six to thirty. These results were in agreement with those reported by Vandewalle et al. (1995) where they found that ninety two to ninety eight percent of the patients in the age group of ten to nineteen years were positive for GADA.

We were able to demonstrate that IAA occurred in twenty one percent of the patients in the age group of five to ten years and in four percent of the patients in the age group of more than thirty years. In a previous study done in Belgium, Vandewalle CL *et al*. (1995) showed that IAA occurred in over ninety percent of patients under the age of twenty and in sixty five percent of the patients between the ages twenty and fourty. Our results confirmed the Vandewalle's results where an inverse correlation between age and IAA was observed.

In this study, thirty three out of fourty seven (seventy percent) patients were positive for both autoantibodies and none of the controls were positive for both autoantibodies. In a previous study done by Sellers *et al* (2000), it was found that two hundreds and thirty seven out of two hundreds and fifty six (ninety two percent) of type 1 Canadian patients were positive for both autoantibodies versus twenty out of two thousands, eight hundreds and fifty five (point seven percent) controls. By our findings, we were able to confirm the fact that type 1 diabetes is an autoimmune disease associated with multiple autoantibodies against islet cell antigens.

In the present study, the presence of both autoantibodies in twenty seven out of one hundred and fifty four (seventeen percent) of the unaffected siblings may be an indication of increased risk for developing type 1 diabetes and they require careful and continuous follow up. In 1999, Savola and coworkers reported that point nine percent of the siblings of type 1 diabetic patients were initially presented without autoantibodies, six percent of them had only one, twenty three percent had two, and sixty five percent of these siblings

had 3 autoantibodies. All of these groups were presented with clinical signs of type 1 diabetes.

It is important to note that anti-islet cell autoantibodies are frequently observed in non-diabetic monozygotic twin siblings of patients with type 1, ranging from fourty two to seventy six percent (Hawa *et al*. 1997, Petersen *et al*. 1997), which is in concordance with their high progression to diabetes. Life table analysis and long term follow-up studies showed the highest rate for the progression of diabetes in monozygotic twin siblings (Petersen *et al*. 1997).

In conclusion, the prediction of the risk of developing type 1 diabetes in the siblings of type 1 diabetics is gaining an increased importance. A proposal of two-step screening strategy for estimating the risk of progression to type 1 DM over the next ten years for initially unaffected siblings of type 1 diabetics is recommended (Bingley et al. 1999). The first step would commence at the time of diagnosis of type 1 diabetes in the first child in the family, all siblings below the age of twenty years should be screened for the autoantibodies. All the newborn siblings thereafter should also be screened, e.g. at the age of one to two years. All siblings positive for one or more autoantibodies at the first step would further be tested for islet cell cytoplamic antibodies (ICAs) and their HLA-DQB_1 should be defined.

The screening is very important because (i) early treatment of type 1 diabetes with tight glycemic control preserves β-cell function, (ii) early diagnosis and treatment of type 1 diabetes should prevent the development of diabetic ketoacidosis and (iii) prediction of type 1 diabetes provides an opportunity for entrance into trials to prevent type 1 diabetes.

References:

Alderson P, Sutcliffe K, Curtis K. Children as partners with adults in their medical care. *Arch Dis Child*. 2006; 91: 300 -303

Amrani A, Verdaguer J, Thiessen S, Bou S, Santamaria P. IL-1 alpha, IL-1 beta and IFN-gamma mark beta cells for Fas-dependent

destruction by diabetogenic CD(+) T lymphocytes. J Clin Invest. 2000; 105(4): 459-68.

Atkinson Mark A, Eisenbarth George S. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. The Lancet 2001; 358: 221-229.

Awata T, Kawasaki E, Ikegami H, et al . Insulin gene/IDDM2 locus in Japanese type 1 diabetes: contribution of class I alleles and influence of class I subdivision in susceptibility to type 1 diabetes. *J Clin Endocrinol Metab*. 2007; 92(5): 1791-1795.

Barker JM, Barriga KJ, Yu L, et al. Diabetes Autoimmunity Study in the Young. Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). *J Clin Endocrinol Metab*. 2004; 89: 3896-3902.

Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA: Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. *Diabetes* 1997; 46: 1701-1710.

Bingley PJ, Williams AJK, Gale EAM. Optimized autoantibody-based risk assessment in family members; implications for future intervention trials. *Diabetes care* 1999; 22: 1796-801.

Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature*. 2010; 464: 1293-1300.

Borg H, Femlund P, Sundkvist C. Tyrosine phosphatase-like protein 1A2-antibodies plus glutamic acid decarboxylase 65 antibodies (GADA) indicates autoimmunity as frequently as islet cell antibodies assay in children with recently diagnosed diabetes mellitus. *Clin Chem* 1997; 43:2358-2363.

Bottini N, Musumeci L, Alonso A, et al. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet*. 2004; 36: 337-338.

Cucca F, Lampis R, Congia M, et al . A correlation between the relative predisposition of MHC class II alleles to type 1 diabetes

and the structure of their proteins. *Hum Mol Genet*. 2001; 10 (19): 2025-2037.

Di Sabatino A, Paccagnini D, Vidali F, et al. Detection of Mycobacterium avium subsp. paratuberculosis (MAP) -specific IS900 DNA and antibodies against MAP peptides and lysate in the blood of Crohn's disease patients. *Inflamm Bowel Dis*. 2011; 17: 1254-1255.

Dorman JS, Bunker CH. HLA-DQ locus of the human leukocyte antigen complex and type 1 diabetes mellitus: a HuGE review. *Epidemiol Rev*. 2000; 22: 218-227.

Feeney SJ, Myers MA, Mackay IR, Zimmet PZ, Howard N, Verge CF, Rowley MJ. Evaluation of ICA512As in combination with other islet cell autoantibodies at the onset of IDDM. *Diabetes Care* 1997; 20: 1403-1407.

Hagopian WA, Sanjeevi CB, Kockum I, Landin-Olsson M, Karlsen AE, Sundkvist C, Dahlquist G, Palmer J, Lemmark A. Glutamate decarboxylase, insulin, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. *J Clin Invest* 1995; 95:1505-1511.

Hawa M, Rowe R, Lan MS, Notkins AL, Pozzilli P, Christie MR, Leslie RU. Value of antibodies to islet protein tyrosine phosphatase-like molecule in predicting type 1 diabetes. *Diabetes* 1997; 46:1270-1275.

Jones EY, Fugger L, Strominger JL, et al . MHC class II proteins and disease: a structural perspective. *Nat Rev Immunol*. 2006; 6(4): 271-282.

Jönsson L, Hallström I, Lundqvist A. "The logic of care"-parents' perceptions of the educational process when a child is newly diagnosed with type 1 diabetes. *BMC Pediatr*. 2012; 12: 165.

Kelly M.A, Chant J.C.N, Heward J, Mijovic C.H, Zimmet P.Z, Yeung V.T.F, Cockram C, Barnett A.H. HLA typing and immunological characterization of young-onset diabetes mellitus in a Hong Kong Chinese population. Diabetes UK. *Diabetic Medicine* 2001; 18: 22-28.

Kurrer MO, Pakala SV, Hanson HL, Katz JD. Beta cell apoptosis in T cell-mediated autoimmune diabetes. Proc Natl Acad Sci USA. 1997; 94(1): 213-8.

Libman IM, Pietropaolo M, Trucco M, Dorman JS, LaPorte RE, Becker U. Islet cell autoimmunity in white and black children and adolescents with IDDM. *Diabetes Care* 1998; 21:1824-1827.

Liese AD, D'Agostino RB Jr, Hamman RF, et al. The burden of diabetes mellitus among US youth: Prevalence estimates from the SEARCH for diabetes in youth study. *Pediatrics* . 2006; 118: 1510-1518.

Ling Wu Yan, Ding Yan-Ping, Gao Jian, Tanaka Yoshimasa, Zhang Wen. Risk factors and primary prevention trials for type 1 diabetes. *International Journal of Biological Sciences*. 2013; 9(6): 666-679.

Maugendre D, Chaillous L, Rohmer V, Lecomte P. Marechaud R, Sai P, Marre M, Charbonnel B, Allannic H, Delamaire M. Multiple antibody status in type 1 diabetic patients and subjects at various risk with islet-cell antibodies. *Diabetes Metab* 1997; 23: 320-6.

Moreira Marina, Lara Gustavo, Linden Rafael, Feska Luciane, Tavares Rejane, Almeida Sabrina, Berlese Daiane. Frequency of the anti-glutamic acid decarboxylase immunological marker in patients with diabetes during longer than three years in Southern Brazil. *Sao Paulo Med J*. 2011; 129(3): 130-3.

Nisticò L, Buzzetti R, Pritchard LE, et al . The CTLA -4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry. *Hum Mol Genet*. 1996; 5: 1075- 1080.

Noble JA, Valdes AM. Genetics of the HLA region in the prediction of type 1 diabetes. *Curr Diab Rep*. 2011; 11(6): 533 - 542.

Pavlovic D, Vande Winkle M, Vander Auwera B, Chen MC, Schuit F, Bouwens L, Pipeleers D. Effect of interferon-gamma and glucose on major histocompatibility complex class I and class II expression by pancreatic beta-and non-beta-cells. J Clin Endocrinol Metab.1997; 82(7): 2329-36.

Petersen JS, Kyvik KO, Bingley PJ, et al . Population based study of prevalence of islet cell autoantibodies in monozygotic and dizygotic Danish twin pairs with insulin dependent diabetes mellitus. *BMJ*. 1997; 314: 1575- 1579.

Rani PS, Sechi LA, Ahmed N. Mycobacterium avium subsp. paratuberculosis as a trigger of type - 1 diabetes: destination Sardinia, or beyond? *Gut Pathog*. 2010; 2: 1.

Rankin D, Cooke DD, Elliott J, et al. Supporting self -management after at-tending a structured education programme: a qualitative longitudinal investigation of type 1 diabetes patients' experiences and views. *BMC Public Health*. 2012; 12: 652.

Rosu V, Ahmed N, Paccagnini D, et al . Specific immunoassays confirm association of Mycobacterium avium Subsp. paratuberculosis with type-1 but not type -2 diabetes mellitus. *PLoS One*. 2009 ; 4: e4386.

Sabbah E, Kulmala P, Veijola R, Vähasälo P, Karjalainen J, Tuomilehto-Wolf E, Akerblom HK, Knip M. Glutamic acid decarboxylase antibodies in relation to other autoantibodies and genetic risk markers in children with newly diagnosed insulin-dependent diabetes. Childhood Diabetes in Finland Study Group. *J Clin Endocrinol Metab*. 1996; 81(7): 2455-9.

Sabbah E, Savola K, Kulmala P, Reijonen H, Veijola R, Karjalainen J, Ilonen J, Akerblom H.K, Knip M. Disease associated autoantibodies and HLA-DQB_1 genotypes in children with newly diagnosed insulin-dependent diabetes mellitus (IDDM). *Clin Exp Immunol* 1999; 116: 78-83.

Savola. Kaisa, Mrena, Samy. Staging of preclinical type 1 Diabetes in sibling of affected children. *Pediatrics* 1999; 14: 925-931.

Sellers E, Eisenbarth G, Kue Young, Heather J. Diabetes-associated autoantibodies in Aboriginal Children. *Lancet* 2000; 355: 1-4.

Trofimenko EV, Zlobina EV, Labedev NV, Martynova MI, Piliutik VF, Shchegolkova TS, Zlobina EN, Dedov II. Autoantibodies to glutamate decarboxylase in children with newly detected insulin-dependent diabetes mellitus. Russian study. *Prob Endokrinol* 1994; 40: 18-21.

Van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev*. 2011 ; 91:79-118.

van der Werf N, Kroese FG, Rozing J, et al . Viral infections as potential triggers of type 1 diabetes. *Diabetes Metab Res Rev*. 2007; 23(3): 169 -183.

Vandewalle CL, Falorni A, Svanholm S, Lernmark A, Pipeleers DG, Gorus FK. High diagnostic sensitivity to glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. The Belgian Diabetes Registry. *J Clin Endocrinol Metab* 1995; 80: 846-51.

Westerberg DP. Diabetic ketoacidosis: evaluation and treatment. *Am Fam Physician*. 2013; 87(5): 337-346.

Wiest-Ladenburger U, Hartmann R, Hartmann U, Berling K, Bohm BO, Richter W: Combined analysis and single-step detection of GAD65 and IA2 in IDDM can replace the histochemical islet cell antibody test. *Diabetes* 1997; 46:56-571.

Zeidler A, Raffel LJ, Costin G, Shaw SJ, Buchanan TA, Noble J, Rotter JI, Palmer J, Krischer JP, Wait C, Maclaren NK. Autoantibodies and human leucocyte antigen class II in first-degree family members of Mexican-American type 1 diabetic patients. *J Clin Endocrinol Metab*. 2001; 86(10): 4957-62.

Zimmet PZ, Elliot RB, Mackay IR, Tuomi T, Rowley MJ, Pilcher CC, Knowles WJ. Autoantibodies to GAD and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender. *Diabet Med* 1994; 11: 866-71.