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PREVALENCE OF ANTI-INSULIN ANTIBODIES IN TYPE 2 DIABETIC PATIENTS IN BENGHAZI

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Dedication

I would like to dedicate this work to the dearest and prettiest daughters in the world ***Farah*** *and* ***Malak,*** *praying and hoping that Allah Subhanahu wa Ta'ala may preserve and protect them so that they become better than their father in everything*

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Abstract

Introduction: type 2 diabetes mellitus is characterized by decreased insulin secretion or increased insulin resistance or both. Insulin antibodies could be one of the causes for insulin resistance that may affect glycemic control of the patients. This study was performed to evaluate the prevalence of insulin antibodies in type 2 diabetic patients in Benghazi. Those treated with exogenous insulin and those treated with oral hypoglycemic drugs, in order to determine effect of insulin and oral hypoglycemic drugs on induction of IAs .

Materials and methods: Study was carried out on tow groups. Group (A) carried out on 200 diabetic patients (140 insulin users, and 60 oral hypoglycemic drug users) followed up BDC were subjected to history taking, blood samples were obtained from them, sera were tested for IAs by ELISA(DRG kit), and plasma glucose was measured twice fasting and after lunch. Group (B) carried out on 95 diabetic patients (insulin users) followed up BDC were subjected to history taking, blood samples were obtained from them, sera were tested for IAs by ELISA(Medipan kit). RFT, LFT, Lipid profile and HbA1c were measured in blood, and microalbumine was measured in urine. Results were analyzed using student T-test.

Results: Overall prevalence of IAs for group (A) was 11% for whole group (10% among insulin users and 3.3% among OHD users). The IAs titer was (10.11-71.05 u/ml). Results did not show any influence of patient's age, weight, diet control, metformin intake, or insulin dose on prevalence of IAs. However IAs were more prevalent among males, inactive patients, insulin users, and with chronic use of insulin. Results showed significant influence of IAs on glycemic status. Overall prevalence of IAs for group (B) was 26.3%, the IAs titer was (3.1-60.5 u/ml) . Results showed influence of aging, increased weight, uncontrolling diet, inactivity, untaking metformin, increasing dose and duration of insulin treatment on prevalence of IAs. Results did not show any influence of IAs on blood levels of RFT, LFT and insulin, or urine microalbumin, however results showed influence of IAs on blood levels of lipid profile and HbA1c.

Conclusion: IAs are significantly prevalent in type 2 diabetic patients attending BDC. IAs may be detectable in insulin-naïve diabetic patients. IAs were more prevalent in males, inactive subjects, and with chronic use of insulin treatment. Prevalence of IAs and its correlation with patient's age, weight, diet control, metformin intake and insulin dose was affected by the difference in sensitivity of ELISA test used and by size of sample subjected to the study. IAs obviously correlated with high blood levels of HbA1c and lipid profile.

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Abbreviations

ALP	Alkaline Phosphatase
ALT	Alanin aminotransferase
APC	Antigen Presenting Cell
AST	Aspartate transaminase
AUC	Area Under the Curve
BDC	Benghazi Diabetic Center
BIL	Bilirubin
CHOL	Cholesterol
Dpm	Disintegrations per minute
ELISA	Enzyme Linked Immunosorbant Assay
FPG	Fasting Plasma Glucose
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotien
HRP	Horse Radish Peroxidase
IAs	Anti-Insulin Antibodies
IAAs	Insulin Autoantibodies
IAS	Insulin Autoimmune Syndrome
Ig	Immunoglobulin
IPPs	Intra Peritoneal Pumps
K	Potassium
Ka	Association Constant
Kd	Dissociation Constant

LDL	Low Density Lipoprotien
LFT	Liver Function Test
MAU	Microalbuminuria
MHC	Major Histocompatibility Complex
Na	Sodium
NOD	Non Obese Diabetic
OHD	Oral Hypoglycemic Drug
PLPG	Post Lunch Plasma Glucose
RFT	Renal Function Test
RLB	Radio Labeled Binding
TG	Triglycerides

CHAPTER 1

INTRODUCTION

Introduction

1.1 Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Type 2 diabetes, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency at least initially, and often throughout their lifetime [(1)]. Causes of insulin resistance include metabolic syndrome, infection, steroid use and presence of insulin antibodies(secondary to exogenous insulin). 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 [(1)].

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the beta cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is due to the deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia [(1)].

Pathophysiology of diabetes:

Insulin auto antibodies (IAAs) may be detectable in insulin-naïve individuals who have a high likelihood of developing type 1 diabetes or in patients who have had viral infections have been treated with various drugs, or those who have autoimmune disorders or paraneoplastic syndromes [(2)].

In T2 Diabetes, Insulin antibodies (IAs) are antibodies that developed against exogenous therapeutic insulin. Regardless of purity and origin, therapeutic insulins continue to be immunogenic in humans [(2)].

Today's human insulins are free of noninsulin peptides and variations in insulin structure that contributed to the antigenicity seen with pancreatic insulins in the past; however, insulins must

be manufactured, stored, and delivered by nonphysiological means, and these possibly contributing to the humoral and cellular immune responses seen in patients treated with insulin [2]. Several other factors can influence the production of IAs, some are related to the host (age, sex), others are related to the antigen (dose, duration).

Epidemiology of diabetes:

Diabetes is a worldwide problem that is has global distribution. prevalence of diabetes in north America is 10.5%, 6.7% in Europe, 4.3% in Africa and 10.9% in middle east [3], as shown in (figure 1.1).

In Benghazi the prevalence of Type2 diabetes was reported to be 14.1% [4].The clinical characteristics and prevalence of non-insulin-dependent diabetes mellitus (NIDDM) diagnosed in Benghazi were reported. Data were based on the analysis of records BDC for the period 1981 to 1990. The overall prevalence rate of NIDDM (Type2) was 0.19%; it was significantly higher in females (0.21%) than males (0.17%) The prevalence rate in patients aged 20years was 3.8% which was significantly higher in females (4.7%) than in males (2.9%) ($P < 0.01$). Prevalence rates increased with each higher age group and peaked in the 50-54 years age group [5].

Prevalence of hypertension was 22.5% and prevalence of overweight was 59.4%. (5)The most common complications of diabetes were neuropathy (45.7%), retinopathy (30.5%) and nephropathy (25.2%). ($P < 0.01$) [5].

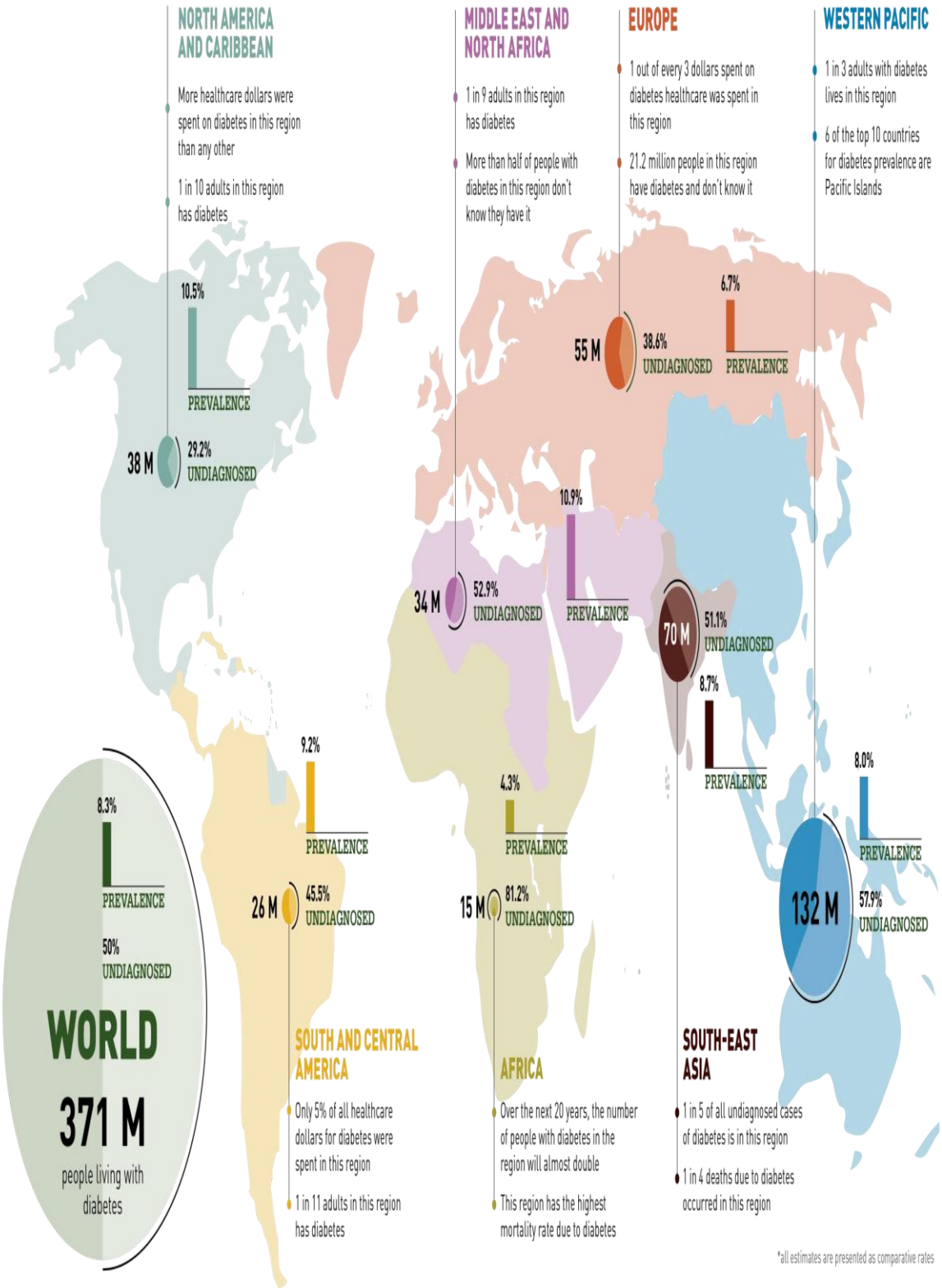

Figure 1.1: Prevalence of diabetes around the world

1.2 Recombinant human insulins.

The first human insulins for clinical use were derived from porcine insulin by semisynthetic conversion[6]. The next advance in insulin therapy was the development of recombinant DNA (rDNA) human insulin made by fermentation of Escherichia coli or Saccharomyces cerevisiae yeast that contained DNA for insulin A and B chains, proinsulin, or modified proinsulin. Later steps after cell lysis include removal of bacterial and yeast components, recombination of insulin chains, or removal of connecting peptides [7,8].

Clinical trials revealed that semisynthetic human insulin was less immunogenic than porcine monocomponent insulin in patients newly diagnosed with type 1 diabetes. Schernthaner *et al* [9] found IAs of the IgG class in 14% of patients receiving human insulins and in 29% of the patients receiving nonhuman insulins. Heding *et al* [10] reported that 44% of pediatric patients with type 1 diabetes receiving human monocomponent insulin were antibody-positive, compared with 59% of similar patients receiving porcine monocomponent insulins. These differences may be attributable to antibody measurement methodologies, In a 1-yr trial, Fineberg *et al* [11] compared development of antibodies to rDNA human insulin in 100 insulin-naive children and adults with 121 similar individuals treated with purified porcine insulin. At the end of the trial period, 44% of patients treated with rDNA human insulin and 60% of patients treated with porcine insulin had developed significant levels of IAs. In a study in Japan anti insulin antibodies were demonstrated in most (89%) of sera of diabetic patients who had been treated with human insulin for 0.5-8.2 months [12].

In a retrospective review, injection site reactions with highly purified mixed bovine (70% bovine, 30% porcine), purified porcine, and rDNA human insulin were reported to be 12, 3.9, and 2.4% of patients, respectively [13]. Sporadic case reports have been published regarding individuals who developed insulin resistance and/or systemic hypersensitivity reactions while being treated with rDNA human insulins, including short-acting and long-acting human insulin analogs [14-18].

1.3 Modifying factors effects of insulin sequence differences and impurities on Immunoginicity.

Differences between the amino acid sequence of a protein and the sequence of a self-protein will increase its inherent immunogenicity [19-31]. The sequences of bovine and porcine insulin differ from human insulin by three amino acids and one amino acid, respectively. Therefore,

the administration of bovine insulin or bovine-porcine insulin mixtures resulted in greater IAs responses than porcine or human insulin [32-35]. Studies of insulin aspart, which differs from human insulin by one amino acid, have shown a robust antibody response in a subpopulation of patients [36]. However, insulin lispro is thought to have comparable rates of antibody formation as regular human insulin [37].

Owing to the presence of contaminants (including glucagon, pancreatic polypeptide, somatostatin, proinsulin, and insulin complexes) in early bovine and mixed bovine-porcine preparations, some of the antibodies generated may have been against noninsulin proteins [14]. Moreover, other contaminants may also have adjuvant effects and thereby enhance the generation of IAs[38]. This finding is consistent with studies that have shown minimal difference in immunogenic potential between purified porcine and human insulin[11, 33, 39].

Most commonly, IAs that develop in response to exogenous bovine, porcine, or human insulins are cross-reactive with all three species (Fig. 1.2) [40- 43]. In rare cases, however, antibodies bind differentially to insulins from different species, correlating with clinical responses observed when switching insulin sources [44, 45].

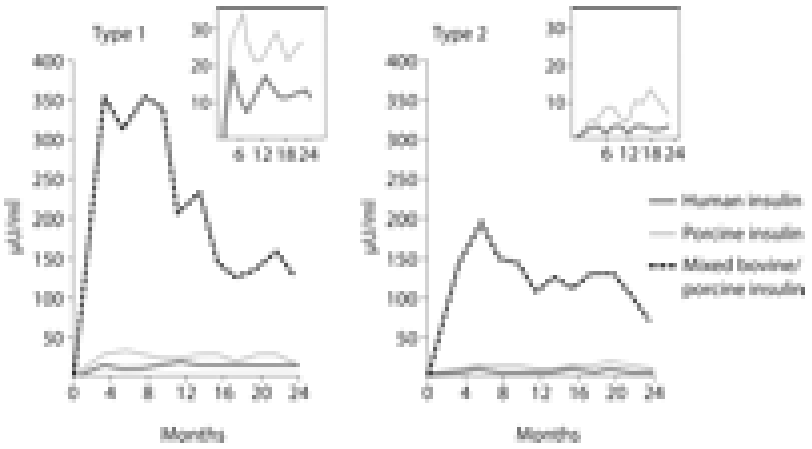

FIG. 1.2: In 1988, Fineberg *et al* [40] reported antibody-bound insulin in previously insulin-naive individuals with type 1 or type 2 diabetes treated with rDNA human insulin, purified porcine insulin, or mixed bovine-porcine insulin over 2 yr. On the *left*, patients with type 1 diabetes reach a peak response within 6 months, with the greatest response to mixed bovine-porcine insulin. On the *right*, antibody responses for patients with type 2 diabetes are diminished for all the insulins. The *insets* compare antibody response to human and porcine insulin.

1.4 Formulation and aggregates

Insulin formulations can also affect immunogenic potential. Soluble forms of insulin, such as regular and semilente, are less allergenic than intermediate- or long-acting preparations, such as NPH, lente, protamine zinc, and ultralente [32,46]. Acid preparations are more immunogenic than neutral ones [32]. Allergic reactions to both protamine and zinc have been reported and require special testing to distinguish them from insulin allergy[47,48].

Aggregation can clearly affect insulin immunogenicity. This is also true for other biological therapeutics and has resulted in strict limitation on aggregates in final drug products. It is thought that insulin aggregates may remain for longer periods at the injection site or be more readily taken up by Antigen Presenting Cells (APC). Insulin aggregates are present in the circulation of patients with diabetes being treated with insulin [49], and antibodies that bind to insulin dimers have been demonstrated [49]. Lymphocyte transformation studies suggest that insulin aggregates, rather than monomers, are the cause of persistent cutaneous allergy [50]. Insulin aggregation and degradation products in a glycerol-based formulation of insulin for use in Intra Peritoneal Pumps (IPPs) have been shown to be associated with increased immunogenicity [51].

Studies in animal models also indicate that aggregates are more immunogenic. Insulin samples taken directly from an insulin vial (material that contained no aggregates) and insulin from an IPPs pump reservoir (material with a high level of aggregates) were administered to rats, and IAs levels were subsequently measured [52]. The percentage binding in sera from rats immunized with insulin from the pump reservoir was approximately 2-fold higher than that obtained with insulin directly from the vial, suggesting that insulin aggregates formed in the reservoir were slightly more immunogenic. This may be relevant to elevated IAs levels that have been observed in patients with implanted IPPs.

1.5 Route of delivery

Animal and human studies suggest that the site of antigen delivery can affect antibody responses. Insulin-specific tolerance induction by intravenous injection in mice is antigen-specific and dose-dependent. The same dose of insulin given ip results in an augmented rather than an inhibited antibody response [53]. Intraperitoneal injection of insulin in mice has been found to enhance the immune responses to multiple antigens, including insulin[30,54,55]. Oral,

parenteral, and aerosol insulin administration in diabetes-prone animal models can induce immune tolerance [54,55].

Intraperitoneal insulin delivery via IPPs has been shown to increase levels of IAs in patients with type 1 diabetes [56], but not in those with type 2 [56,57]. Intravenous insulin delivery via IPPs, as well as oral insulin delivery, does not result in detectable IAs [58-61]. Pulmonary delivery of human insulin, as discussed above, has been shown to be associated with higher levels of IAs than subcutaneous delivery [62,63].

1.6 Dose-response effects

In humans, evidence that the development of IAs secondary to exogenous insulin is related to dose has not been directly tested; however, clinical trials shown no significant relationship between insulin dose and the development of antibodies. In a study of 46 newly diagnosed patients with type 1 diabetes, Karjalainen *et al* [64] found that 35% of them were positive for IAAs before therapy. After 3 months of therapy, the IAA-positive patients had higher antibody levels than those who were initially IAA-negative, but this difference in IA levels did not persist with further duration of therapy. At no time was insulin dosage found to be related to the development of IAs[62,65,66]. Additional prospective treatment trials of animal and human insulins, insulin analogs, and inhaled human insulin in insulin-naive and insulin-treated patients have shown no significant relationship between insulin dose and level of IAs [62,65,66].

1.7 Patient-related factors

Known genetic determinants influence susceptibility to antibody formation. The presence of histocompatibility leukocyte antigen (HLA)-B15, -DR4, and -DR7 has been shown to increase the rate of IA formation, whereas HLA-B8 and HLA-DR3 are associated with decreased formation rates [9,67-70]. However, Asplin *et al* [71] assessed the development of IAs in 54 newly treated patients with type 1 diabetes and found no effects of HLA-DR3, HLA-DR4, or combinations on insulin-binding antibody formation. Most of these studies were conducted during the late 1970s through the 1980s and evaluated antibody responses in patients treated with bovine or porcine insulin preparations; however, Fineberg *et al* [40] studied patients treated with human insulin and demonstrated no difference in IA levels based on DR4 status, whereas significant differences in IA levels were observed in DR4-positive *vs*. DR4-negative patients treated with porcine and mixed bovine-porcine insulin (Fig. 1.3). This is not

unexpected because the peptide sequences can determine the binding affinity to different alleles of MHC class II molecules. The IgG heavy-chain gene complex has also been shown to modulate susceptibility to IA formation [72].

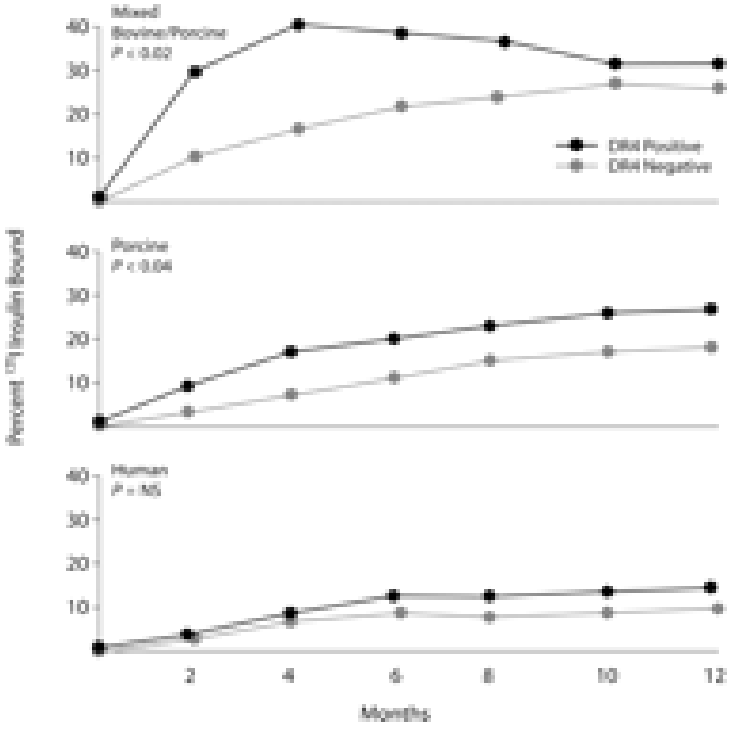

FIG. 1.3: In a 1-yr trial by Fineberg *et al* [40], insulin-naive patients received porcine insulin, mixed bovine-porcine, or rDNA human insulin. Patients who were DR4-positive had greater antibody responses, in terms of percent binding, than DR4-negative subjects to mixed bovine-porcine and porcine insulins but not for human insulin, suggesting an interplay among HLA type, antigen structure, and possibly other patient-related factors affecting antibody responses. The researchers concluded that HLA type affects the magnitude of the immune response.

The presence of IAA at baseline may predict a greater IA response to exogenous insulin. In one study, IAA-positive individuals developed a greater IA level than IAA-negative patients throughout 1 yr of therapy [73]. However, the increased levels could be attributed to the sum of IAA and IA[62].

The findings from studies with inhaled insulin suggest that patients with type 1 diabetes develop greater levels of IAs than those with type 2 diabetes [62]. Studies with insulin lispro also demonstrated that patients with type 1 diabetes had higher IA levels than those with type

2 diabetes [66]. The mechanisms for this difference in response is not clear, but it may be due to defects in regulatory T cell function, as reported in nonobese diabetic (NOD) mice—a model of type 1 autoimmune diabetes [27]. This defect may lead to enhanced immune responses to human insulin in patients with type 1 diabetes. However, it is not clear whether exogenous antibody formation can be attributed to further expansion of autoreactive antiinsulin T cells and B cells that produce IAAs. There are conflicting data on whether the presence of IAAs predicts the IA response to exogenous insulin [64,73,74].

Exogenous insulin administration may also result in antibody formation in individuals who do not have diabetes. Antibody formation induced by as few as six injections of human insulin is comparable to that seen in chronically treated patients with type 1 diabetes [75]. IAAs are also found more commonly in individuals who have endocrine autoimmune syndromes, such as Graves' disease, Hashimoto's thyroiditis, and nonimmune thyroid disease [76].

Age may also play an important role in the IA response with exogenous insulin administration. Immunological competence declines as an individual ages, causing decreased ability to form high-affinity antibodies, decreased ability to generate long-lasting memory cells, and delayed hypersensitivity responses [77-79]. The age of the individual also affects the presence of IAAs before the development of type 1 diabetes and whether an individual is more or less likely to develop a significant antibody response to exogenous insulin [80].

In the studies of Fineberg *et al* [40,66,81], the development of significant levels of antibodies to exogenous insulin has been shown to be inversely related to C-peptide levels and age. The two factors age and C-peptide level are independently correlated, although there is more evidence for a correlation to age than to C-peptide level. A logistic regression analysis of antibody development in 744 individuals who were insulin-naive at the beginning of therapy revealed a 3% decrease in the chances of antibody development for every 1-yr increase in age (odds ratio, 0.97; 95% confidence interval, 0.96–0.99; $P < 0.001$) [82]. Additional analysis showed a 46% decrease for every 1-nmol/liter increase in C-peptide levels (odds ratio, 0.54; 95% confidence interval, 0.38–0.78; $P < 0.001$)[82].

1.8 Characterization of Insulin Antibodies

1.8.1 Measurement of insulin antibodies

Initial studies conducted to characterize IAs showed that Insulin-Insulin Antibody immune complexes do not precipitate and therefore could not be measured using standard immunoprecipitation/agglutination analytical methods [83]. Therefore, the Radio- Labeled Binding (RLB) assay was used, and it remains the most common format in the measurement of IAs and IAAs. Recently, filter plate separation of insulin/IA complexes from unbound insulin has been incorporated into RLB assay protocols allowing for smaller sample volume requirements and higher throughput [84]. In general, RLB assays for total insulin-binding capacity involve coincubating aliquots of serum, buffer, and trace amounts of monoiodinated ^{125}I-insulin. Unbound and bound insulin in the serum is often removed before incubation by using an acidification process followed by treatment with dextran-activated charcoal. Nonspecific binding can be assessed by incubating a duplicate aliquot of the test serum with a high concentration of unlabeled insulin and trace levels of ^{125}I-labeled insulin[84]. Immune complexes are precipitated by the addition of antihuman globulin, protein A/G (conjugated to sepharose beads), polyethylene glycol, or other precipitants. The pellets of immune complexes are separated by filtration or centrifugation, washed to remove unbound radiolabeled insulin, and measured on a gamma counter. The mean nonspecific binding disintegrations per minute (dpm) are subtracted from total binding dpm to determine specific binding. The specific binding dpm are converted to microunits or nanograms of IA-bound insulin, based on the specific activity of the labeled insulin. Data may be expressed as insulin-binding capacity (nanograms or microunits of insulin bound per milliliter of serum). Other laboratories express the data as percentage binding (counts in the pellet relative to the total counts of ^{125}I- insulin added).

The ELISA for IAs involves adsorbing insulin to plastic wells of 96-well plates with subsequent addition of test sera. Insulin-specific antibodies in the test sera bind to the insulin on the plate, and the remaining antibody is washed away. Anti-Ig labeled with horseradish peroxidase or alkaline phosphatase is added and binds to the IA. Substrate for horseradish peroxidase or alkaline phosphatase is added to each well, and a color product is formed and monitored by measuring light absorbance. The amount of color

product formed increases with the amount of antibody bound to the plate. Data for ELISAs are often expressed as end point titers (minimally detectable) or titers that produce 50% maximal signal.

In a few studies of serum from patients with diabetes being treated with insulin, the ELISA was found to correlate with the RLB assay [56,85-87]. However, other studies have demonstrated a poor correlation between the two assays [88-90]. Serum samples were found to have very high antibody levels/titers by the ELISA but very low titers when using the RLB assay and vice versa. The RLB has been shown to be more sensitive than the ELISA for detection of IAA. Furthermore, a positive RLB assay for IAA has been found to be a better predictor for the development of diabetes than a similar ELISA result [91].

The differences between the RLB and ELISA findings have been attributed to inherent differences between assay formats. The RLB is a solution-based assay in which low levels of ^{125}I-labeled insulin are used.Thus, high affinity IAs are primarily measured in this assay [89,90]. In contrast, as serum is progressively diluted, the ELISA reaches the stage of excess antigen and thus has the capacity for measuring IAs of varying affinities [89,90,92]. In addition, it is believed that the binding of insulin to solid phase (plastic wells) in the ELISA results in antibody-binding epitopes that are different from those available with ^{125}I-labeled insulin in solution phase.

Although ELISA assays can be processed rapidly and in larger batch sizes and do not require radiolabeled insulin, most laboratories that measure IAs use the RLB assay for the reasons described above. Insulin autoantibody assays are designed with high sensitivity in mind because autoantibodies are found at significantly lower concentrations than are IAs that develop in response to exogenous insulin therapy; therefore, autoantibody assays may need further modification and validation to accurately measure higher IA concentrations.

Progress has been made toward standardizing assays for the measurement of IAAs [93,94], but there is no standardization for assays designed to quantify insulin-therapy-induced IAs, particularly when IAs are present at high concentrations. Because of the large volume of samples generated in the Diabetes Prevention Trial Type 1 study, micro methods were developed to analyze combined IAA glutamic acid decarboxylase

65 and ICA512 (splice variant of islet antigen-2 autoantigen, also referred to as IA-2A) autoantibodies in less than 3 h [95]. Even slight differences in the concentration of labeled insulin used, washing conditions, precipitating agent (polyethylene glycol or protein A/G beads), or reagents can yield significantly different results, so the absolute concentration of antibodies determined by one assay cannot be assumed to have a quantitative correspondence with the results of another. Comparison of assays used by different laboratories would require bridging studies between the two methods using a set of laboratory standards of varying antibody levels. This has been accomplished with regard to IAA, using multiple laboratory quality controls, and expressing insulin binding as Juvenile Diabetes Foundation units [93]. However, the lack of standardization for the IA assay in patients treated with insulin hampers the ability to correlate titers with clinical findings when IAs are measured by different laboratories using different assay conditions and sometimes different assay formats.

1.8.2 Immunoglobulin classes

IA responses consisting of virtually all Ig classes and IgG subclasses have been reported. Insulin-specific antibodies are primarily composed of IgG1–4 antibodies [96], but IgM, IgA, and IgE have been reported. Antiinsulin IgM has been detected during early insulin treatment [97-99], and both Andersen [96] and Reisman *et al* [99] reported the presence of that class in patients with immunological insulin resistance. However, Patterson *et al* [100] failed to detect IgM in patients with diabetes who were treated with insulin. Faulk *et al* [98] reported detectable IgA in patients, and Kniker *et al* [101] associated IgA with allergic reactions in patients with diabetes.

Most reports regarding allergic reactions implicate IgE alone or in combination with IgG [102-107]. However, correlations have not always been found between patients exhibiting allergic reactions and assays for insulin-specific IgE [108], and patients with detectable antiinsulin IgE have not always exhibited allergic reactions [105,108-110]. IgG antibodies have been associated with cases of severe insulin resistance [103,106]; however, most studies show no correlation between IAs and increasing insulin dose requirements. Antibodies to insulin—both sc and inhaled—are predominantly of the IgG class [62]. In a study of patients receiving either inhaled or sc insulin, distributions of IgG subclasses were similar for both therapies [111]. In general, IgG1 levels were greater than those of IgG4, which were slightly greater than IgG2 and IgG3 levels in

both study groups[112]. Similarly, Fuchtenbusch *et al* [113] demonstrated that after 12 months of sc insulin treatment, patients with type 1 diabetes had predominantly IgG1 and IgG4 antibodies to insulin and that IgG2 and IgG3 were lower in concentration. These authors also showed that IgG1 levels tend to decline, whereas IgG4 levels rise with increased duration of sc insulin treatment.

Immunoglobulin (Ig) antibody class distributions resulting from inhaled insulin therapy have been reported in two articles describing immunogenicity data for Exubera[62,113]. Although a wide range of antibody levels were found in the Exubera studies, only IgG antibodies could be identified; IgA, IgE, and IgM antibodies were undetectable [62]. In 54 patients treated with AERx-iDMS, percentage binding levels rose substantially from a baseline of 6 to 39% after 12 wk [113]. This rise in antibodies was accounted for primarily by an increase in IgG antibodies, with small increases in IgE antibodies in four patients. These data are consistent with antibody development reported in studies of sc insulins and suggest that pulmonary antibody responses result from similar immunological mechanisms [62].

1.8.3 Antibody affinity

The RLB assay format has been used to assess relative affinity of IAs. In the classic equilibrium-binding assay, multiple radioligand binding incubations are set up for a single test serum. In this way, a fixed concentration of antibody in the test serum is incubated with increasing concentrations of unlabeled insulin and a fixed amount of ^{125}I-insulin. Using higher concentrations of unlabeled insulin allows IAs with lower binding affinities to bind the labeled insulin. The amount of ^{125}I-insulin bound in each tube with different concentrations of insulin is determined, and a Scatchard analysis of the data has been performed in several studies by plotting the bound/free *vs.* bound insulin concentration [89,90].

Using this method, Goldman *et al* [40], and Brooks-Worrel *et al* [114] found curvilinear Scatchard plots when serum samples from patients with diabetes treated with insulin were analyzed. Two populations of IAs were identified: high-affinity antibodies with low binding capacity and low-affinity antibodies with high binding capacity. These two subpopulations were typically described by reporting the binding affinity and binding capacity determined from the apparent linear regions of curvilinear Scatchard

plots. The binding capacities, association constant (Ka), and calculated dissociation constant (Kd) of each of these two antibody subpopulations were determined. A mean Kd for the high-affinity and low-affinity antibody populations were 1.9 x 10^{-10} M and 1.6 x 10^{-6} M, respectively. Similar findings were also observed in an earlier study in which a gel-filtration method was used to measure bound and unbound insulin [115]. Scatchard analysis has also been extensively used to evaluate the relationship between antibody affinity and hypoglycemia [116-121].

Concerns have been raised with regard to the use of Scatchard analysis to determine the binding affinities and binding capacities of polyclonal antibody responses [122,123]. Antibody responses to most antigens, including insulin, are polyclonal and comprise antibodies with a wide range of affinities that recognize different epitopes on the insulin molecule [43]. Determining antibody affinity and binding capacity of two selected subpopulations in Scatchard analyses excludes information about many other potentially relevant subpopulations of antibodies. Scatchard analyses are best suited for investigations of monoclonal antibodies to determine binding affinity characteristics. When evaluating polyclonal antibody responses, Scatchard analyses are best suited to determine the variance of affinities [122].

A simpler way to describe data from the equilibrium binding assay was reported by Heise *et al* [124]. Insulin-binding capacities determined from the RLB assay and expressed as microunits of insulin bound per milliliter of serum at specific insulin concentrations were reported. Binding capacities at corresponding insulin concentrations can be compared and analyzed directly. Reporting binding data in this way is not dependent upon applying a two-site binding model to determine the binding/dissociation constants of arbitrarily selected antibody subpopulations.

Heise *et al* [124]. Used this reporting method to evaluate the clinical significance of low-affinity and high-affinity antibodies in patients with type 1 diabetes treated with inhaled insulin (Exubera). The researchers employed a parallel group design in which 23 patients were treated with sc insulin and 24 patients were treated with inhaled insulin for 24 wks, Mean (±SD) IA levels for the sc and inhaled insulin groups were 4.3 ± 9.4 and 101.4 ± 140.4 µU/ml, respectively. The binding capacities of samples measured with 10^{-10} M (high-affinity) and 10^{-8} M (low-affinity) insulin were determined. For the Exubera group, the binding capacities of lower affinity antibodies

ranged from 667 to 3360 µU/ml and from 41 to 387 µU/ml for the higher-affinity antibodies. The greater binding capacities of the low-affinity antibodies are similar to those reported with Scatchard analysis in previous studies with sc insulin. The binding capacities of the low-affinity and high-affinity antibodies were compared with postprandial glucose area under the curve (AUC), duration of insulin action, hypoglycemic events, and fasting plasma glucose. No correlation was observed between these pharmacodynamic markers and binding capacities of high or low affinities.

1.8.4 Insulin epitope and insulin antibody idiotype analyses

Numerous mouse monoclonal antibodies against human insulin have been generated to identify potential binding sites within the insulin molecule [124-128]. These studies have shown that insulin administration could result in antibodies capable of recognizing many different epitopes. Monoclonal antibodies have been used in competition assays to determine the major sites recognized by naturally occurring polyclonal IAs. This approach has been used to evaluate epitopes recognized by IAAs [129], but not by IAs.

Phage display libraries have also been used to investigate insulin epitopes. The displayed random hexapeptide phagotopes recognized by IAA and IA from different sources have been identified and sequenced [130-132], and consensus sequences have been determined and compared against the amino acid sequence of human insulin. These comparisons may lead to the identification of new immune markers of diabetes disease states based on insulin epitopes recognized by IAA and may be used to identify patients for type 1 diabetes prevention therapy. Phagotype analysis may allow IAAs to be distinguished from IAs [130].

1.8.5 Characterization of insulin antibody immune complexes

It was discovered early on that IAs do not form precipitable immune complexes as observed with immune complexes against larger proteins [83]. Using ultracentrifugation methods to analyze the nature of the IA immune complexes, it was determined that insulin may bind IAs and form monomers and dimers. The dimers consisted of two IgG molecules bound to chains of insulin [133]. Two different epitopes on insulin are recognized (bivalent) and act as a bridge between two IgG molecules. Larger immune complexes are not formed. It is known that small immune complexes do not activate

complement (C1q binding) and are not rapidly cleared [134]. This is consistent with the finding that insulin-antibody(IA) immune complexes remain in circulation and are not readily cleared. IA immune complexes circulate and thereby act as a "sink" for insulin in the circulation. It was also determined that C1q binding to immune complexes of serum samples taken from patients with type 1 diabetes with IAs was not greater than normal controls [135]. Immune complex size may also explain the rarity of immune complex hypersensitivity reactions or immune complex diseases in patients with high levels of IAs who receive daily exogenous insulin.

1.9 Clinical Significance of Insulin Antibodies

The incidence and severity of immunological complications of insulin therapy have dramatically decreased with the use of highly purified porcine or rDNA human insulin. Although the new preparations still produce IAs, the titers are lower, and they are rarely associated with clinical events. This section will review previous investigations into the relationships of IA and the following clinical issues: hypersensitivity reactions, hyper- or hypoglycemia, pregnancy,autoimmune markers, immune complexes,glycemic variability, and diabetes risk. It should be noted that interpretation of these investigations is often limited by uncontrolled observations, small sample sizes usually consisting of individual case reports, and nonstandardized antibody measurement methods.

1.9.1 Hypersensitivity reactions

Before the 1980s, local hypersensitivity reactions were common complications of insulin therapy. Since the introduction of highly purified insulin in clinical practice, the incidence of allergic complications has markedly decreased [33]. Local and systemic reactions with insulin administration may be mediated by insulin-specific IgE (type 1, immediate hypersensitivity reactions) or IgG (type 3, intermediate immune–complex-mediated reactions) antibodies. In addition, nonantibody-mediated type 4 delayed hypersensitivity reactions have been reported[137]. Surveys of these published cases and reviews reveal that the exact type of local reaction is difficult to identify, and in-depth evaluation of such patients using skin tests and skin biopsies have not been commonly reported; thus, the frequency of insulin reactions described in the literature may not be entirely accurate. Furthermore, local allergic reactions to insulin may be of mixed types.

1.9.1.1 Type 1 hypersensitivity reactions

Local, immediate reactions are the least common cutaneous insulin reaction, occurring in less than 1% of patients [136]. These reactions begin within minutes of an insulin injection, peak from 12 to 24 hrs later, and can be followed by generalized anaphylaxis [109]. Type 1 reactions can occur at any time with respect to the initiation of insulin, particularly when there is a history of interrupted insulin therapy [33].

Anaphylactic reactions are often preceded by a series of local, immediate reactions [137]. They are initiated by the binding of antiinsulin IgE to insulin. These complexes bind to mast cells, releasing into the circulation a variety of vasoactive substances that mediate the syndrome.

Patients with generalized reactions have higher insulin-specific IgE an levels than do patients with only local immediate reactions [138]. Antiinsulin antibody IgE levels can be 10-fold to 20-fold higher in patients with allergic disease than in patients without clinical allergies [106,139]. However, the demonstration of circulating antiinsulin IgE does not establish the diagnosis of insulin allergy because IgE can be found in patients with no apparent allergy [110,140]. Patients with antiinsulin antibodys IgEs can have concomitant insulin-specific IgG antibodies [45,106].

1.9.1.2 Type 3 hypersensitivity

Local, intermediate reactions appear 4 to 8 h after insulin injections, peak at 12 h, and generally subside within a day [141]. These phenomena are believed to be a vasculitic response to soluble antigen-antibody complexes (Arthus reactions) [136, 138,142]. Lipoatrophy—or loss of fat at insulin injection sites—may represent a persistent, localized Arthus reaction. In biopsies from affected sites, IgM, IgE, and C3 have been demonstrated in dermal vessel walls [143]. Lipoatrophy was found to occur in up to 20% of patients before the wide availability of highly purified forms of insulin [141], but it has become virtually nonexistent in patients treated with purified porcine or human insulin. It is possible that lipoatrophy is mediated by contaminant-specific immune responses rather than by IAs. In recent years, the

more commonly observed lipohypertrophy at insulin injection sites has been linked to immune responses to insulin as well [144].

1.9.1.3 Type 4 hypersensitivity reactions

Local, delayed reactions are the most common hypersensitivity reaction and usually occur at the start of insulin therapy [136,142,145,146]. They generally begin 8 to 12 h after an insulin injection and peak at 24 to 48 h. Most local delayed reactions are mild and self-limited. Although histological analyses suggest that some of these reactions may be mediated by sensitized T lymphocytes [136,138], it is not known how many of these delayed reactions are truly type 4 reactions. Occasionally, local reactions follow a biphasic course, with temporary improvement occurring between an immediate and a delayed reaction. Type 4 hypersensitivity reactions may also be attributed to contaminants in the older insulin preparations.

1.9.2 Effects of insulin antibodies on Glycemic control

A number of cross-sectional studies involving small numbers of patients have suggested that IAs can be associated with alterations in a variety of insulin pharmacokinetic parameters. Some of these observed alterations might predict a clinical tendency to hyperglycemia or insulin resistance by neutralizing the biological effect of circulating bioactive insulin, whereas other observed alterations might predict a predisposition to clinical hypoglycemia by prolonging duration of insulin action. Most clinical trial data do not show pharmacodynamic consequences on glucose control corresponding to the pharmacokinetic observations, but case reports of immunological insulin resistance or hypoglycemia syndromes attributed to IAs continue to be published.

1.9.2.1 Antibodies and insulin pharmacokinetics and pharmacodynamics

Insulin-IA interactions like insulin-insulin receptor interactions are reversible and follow the principles of equilibrium binding. Thus, the amount of insulin bound is dependent on antibody affinity, insulin concentration, and antibody concentration (insulin-binding capacity). Hypothetically, the amount and time course of

bioavailable insulin in plasma (insulin pharmacokinetics) could be influenced by IAs and may be mediated by antibody affinity and binding capacity. However, evaluating the pharmacokinetic effects of IAs presents several challenges, not the least of which is validation of the accurate and reproducible measurement of insulin concentrations in the presence of IAs [147,148]. Free insulin results may not always be true reflections of bioactive insulin in the presence of IAs [149].

With an increase in IA binding, there is an apparent increase in the volume of distribution of insulin [150,151]. By acting as a "sink" for exogenously administered insulin, an increase in the apparent volume of distribution of free insulin related to IAs could in theory result in alterations in the disposition kinetics of free insulin.

It has been reported that patients with elevated levels of IAs or antibodies with higher binding capacity experience reduced initial rates of increase and delayed time to peak (120 to 180 min *vs*. 90 min), and prolonged return to baseline of plasma-free insulin levels after sc insulin injection in comparison with antibody-negative patients or patients with lower antibody-binding capacity [151-154]. Although time peak of insulin level after administration of insulin appears to be delayed in most reports, the absolute magnitude of the peak does not appear to be markedly different in patients with high and low levels of IAs[119,152,154]. The presence of IAs has also been associated with lower maximal (C_{max}) free insulin concentrations and AUCs after sc injection of NPH insulin and iv infusion of porcine insulin [119,153]. Prolonged half-life (up to 8-fold greater), increased distribution space (up to 10-fold greater), and faster metabolic clearance rates have been documented after iv infusion of recombinant human and/or bovine insulin in patients with type 1 diabetes [150,151,155]. Alterations in pharmacokinetic parameters appeared to be correlated with various measures of antibody characteristics, such as insulin-binding affinity, insulin-binding capacity, or percentage of insulin binding.

Waldhausl *et al* [155] found antibody responses to iv insulin administration in subjects with more than 25 μg/liter insulin-binding capacity. Their data suggest a shift in the time course of the plasma free insulin profile but not in the overall extent of the plasma free insulin exposure.

Measurements of the pharmacodynamic responses to insulin are more clinically relevant than pharmacokinetic responses and not subject to questions regarding the validity of insulin measurements in the presence of IAs. Higher postprandial plasma glucose concentrations in subjects with IAs have been described in two pharmacodynamic studies [152,154]. In the first report[152], the effects of IAs appeared to vary with the insulin species under study. Postprandial plasma glucose concentrations after injection with rDNA human insulin did not differ among patients with low (<10 U/liter) or moderate (>10 U/liter) insulin-binding capacity, but postprandial plasma glucose concentrations were significantly greater in the moderate binding group when bovine insulin was administered.

In the other study, Van Haeften *et al* [154] found no differences in postprandial insulin or glucose when comparing porcine to human insulin injections, although postprandial glucose excursions in response to a standard meal did appear to be correlated with the IA binding. Correspondingly, increases in plasma free insulin levels after injections of both insulins were negatively correlated with IA binding. The effect of IAs on postprandial insulin and glucose was largely accounted for by the association constant of the high-affinity IA binding sites (K_1). Peak postprandial glucose was 237 ± 10 mg/dl in patients in the upper quartile, compared with 166 ± 12 mg/dl in patients in the lowest association constant quartile.

In contrast, a prospective, open-label, parallel-group trial of 47 patients with type 1 diabetes randomized to receive inhaled insulin (Exubera) or sc regular human insulin, was designed to evaluate whether IA development with inhaled insulin is associated with the loss of postprandial glucose control [124]. Mean IA levels increased from baseline after inhaled insulin treatment but not after sc regular insulin treatment. The researchers found no significant differences in postprandial plasma glucose profiles between treatment arms, and no correlation between postprandial blood glucose exposure and antibody-binding affinity was apparent.

A body of literature suggests antibody-mediated prolongation of elevated insulin levels after sc injection [150-152,155-157]; however, pharmacodynamic consequences of this finding have not been consistently demonstrated. Importantly, increased rates of hypoglycemia have not been confirmed in clinical trials. Although IAs

may not cause hypoglycemia, Bolli *et al*. [156,157] reported prolonged recovery time from experimentally induced hypoglycemia in patients with IAs. The researchers concluded that patients with insulin-dependent diabetes can have impaired glucose counterregulatory hormone reserve, which can be compounded by a prolongation of the half-life of insulin by IAs. IAs have not consistently been shown to have the property of prolonging the duration of insulin action. Euglycemic clamp studies have been conducted to explore the effect of IAs on the pharmacodynamic response to exogenously administered insulin. In one study, glucose infusion rates did not vary between subjects with high and low insulin-binding capacity but were lower than in subjects without diabetes (lower glucose infusion rates indicate decreased insulin action) [155].

Peters *et al* [153] noted that exposure (AUC) to free insulin and glucose infusion rates were lower in patients with greater than 10% antibody binding compared with subjects with less than 1.5% antibody binding. Gardner *et al* [158] found no correlation between antibody status and the onset of action of insulin administered sc. Furthermore, the peak effects of insulin action as well as the duration of insulin action were similar both in patients who were antibody positive and in those who were antibody negative. Similarly, Heise *et al* [124] were not able to detect a significant difference in duration of inhaled insulin action in subjects with or without IAs. They reported no correlation between IA-binding affinity and the duration of insulin action.

It seems possible that pharmacokinetic differences attributable to IAs may be observed, but the effects are small in the context of the myriad of other factors that influence glycemic response. The inconsistency between pharmacokinetic and pharmacodynamic observations may lie in the incomplete understanding of the *in vivo* conditions that control insulin bioactivity in the presence of IAs. This is further complicated by the imprecise relationship between insulin pharmacokinetics and pharmacodynamics that exists in the absence of IAs [159,160].

1.9.2.2 IAs and hyperglycemia/interference with insulin action (Immunological insulin resistance).

IAs are determined to be present when binding of labeled insulin is demonstrated *in vitro*. Whether *in vitro* binding quantitatively reflects the binding of circulating insulin *in vivo* is difficult to directly determine. If antibody binding did occur *in vivo* to significant levels, one might expect to observe increasing insulin dose requirements and, possibly, worsening glycemic control in patients with diabetes. Although a rare syndrome of severe insulin resistance has been described in patients with high IA levels, a mechanism for a causal relationship between the antibodies and the syndrome has not been clearly established. Furthermore, IAs do not correlate with measures of glycemic control or insulin dose requirements in most large population studies.

Insulin resistance can be defined as a daily insulin dose requirement that exceeds the normal daily pancreatic output in the nondiabetic state, *i.e.*, approximately 40 ± 20 U/d [161]. Severe insulin resistance is usually defined as insulin requirement of more than 200 U/d for at least 2 d [161-163].

In a subsequent case series, Davidson and DeBra [163] characterized 35 patients with severe insulin resistance associated with the presence of high levels of circulating IAs. Patients studied in this series had mean daily insulin requirement of 550 U/d (range, 200 to 2000 U/d) and maximum insulin-binding capacity greater than 10,000 μU/ml. In addition, the following underlying explanations for large insulin requirements were ruled out: diabetic ketoacidosis, significant infection, significant dietary indiscretion, lipoatrophic diabetes, significant complicating endocrine disease, insulin receptor defects, antibodies to insulin receptors, or factitious claim of insulin dose greater than 200 U/d.

At least 60% of patients diagnosed with immunological insulin resistance had a history of diabetes with onset after age 30 yr, suggesting that the syndrome is more commonly observed in patients with type 2 diabetes [163,164]. The onset and subsequent course of insulin resistance is often associated with symptomatic hyperglycemia, including episodes of ketoacidosis and hyperosmolar coma [161,163]. The duration of insulin therapy before the onset of severe insulin

resistance has been reported to range from 1 month to 15 yr [161,163] although 50–85% of patients with insulin resistance received insulin for less than 1 yr, and 10–25% received insulin for less than 1 month before the onset of severe insulin resistance [161,163]. Insulin allergy may coexist with immune insulin resistance in 10–35% of cases [161,164]. Some cases of insulin resistance that occurred as insulin allergy spontaneously subsided, prompting speculation that increasing concentrations of IgG can inhibit IgE-mediated clinical events [106,164,165]. Although the dominant feature of this syndrome is decreased insulin action, episodes of hypoglycemia can occur. Furthermore, the frequency of hypoglycemia may diminish after resolution of the insulin-resistant state [163].

Immunological insulin resistance is thought to occur less commonly now than it did before the 1980s, but cases of at least partial resistance attributed to IAs continue to be reported—even in patients treated only with human insulin or insulin analogs [103,166-173]. Severe insulin resistance has also rarely been described in the setting of high levels of serum insulin-binding activity associated with underlying chronic lymphocytic leukemia, lymphoma, multiple myeloma, and macroglobulinemia [174]. Possibly, the severe resistance in these cases was due at least in part to the production of monoclonal paraproteins with significant insulin-binding activity, although monoclonal insulin binding was demonstrated in only two cases. Insulin resistance occurring in patients with hematological malignancy is not always associated with an insulin-binding monoclonal protein [44].

1.9.2.3 Overall metabolic control and insulin dose requirements

Some pharmacodynamic studies that examined the relationship between IAs and postprandial glycemia have suggested that IAs can be associated with relative hyperglycemia after meals [116,152,154].

A prospective study evaluating postprandial glucose tolerance during the development of IAs with inhaled insulin therapy showed no loss of postprandial glucose control [124].

Some investigators have reported IA levels that correlated with higher average glucose in populations of patients [175-177], but most showed no correlation between IA and glucose control—usually measured as glycated hemoglobin [178-194].

Researchers have postulated that IAs can be associated with mild degrees of insulin resistance, which would be detected clinically as mild to moderate increases in insulin dose requirements. Additionally, some studies have suggested that increasing insulin dose requirements correlate with IA levels [195-197]; however, most studies have showed no relationship or correlations between IAs and decreasing dose requirements [198-202]. Evidence suggests that IA-positive patients who switch insulins to a less immunogenic preparation can experience reduced dose requirements in concert with declining IA levels [203,204], or that switching insulin preparations can result in declining IA levels without decreased insulin dose requirements [205-209].

1.9.2.4 IAs and hypoglycemia

Rare syndromes in which recurrent or prolonged hypoglycemia is the dominant feature have been attributed causally to IAs. Most frequently, this situation is encountered in insulin autoimmune syndrome (IAS; also called Hirata's disease), in which nondiabetic patients with no history of insulin exposure spontaneously develop IAAs and hypoglycemia. Evidence that IAs induced by exogenous insulin therapy can also cause hypoglycemia is limited to case reports. Although hypoglycemia is the most notable feature of these syndromes, some affected patients have also been reported to have clinical evidence of attenuated insulin action (including severe insulin resistance). It remains to be determined whether antibodies associated with pathological hypoglycemia can be distinguished *in vitro* from the far more commonly occurring IAs that are not linked to clinical hypoglycemia.

1.9.2.4.1 Hirata's disease

IAS consists of high levels of IAs with or without concomitant Graves' disease associated with fasting hypoglycemia in insulin-naive patients. The disease was first described in 1970 by Hirata *et al*. [210]. This disorder is HLA-linked [211]

and is the third most common cause of hypoglycemia in Japan, but has been sporadically reported outside Japan [212,213].

In most patients, remission occurs within 6 months; however, life-threatening hypoglycemia may necessitate measures such as plasmapheresis [214,215]. Impairment in glucose tolerance is reported for some of these patients [212]. Patients with IAS often have late postprandial hypoglycemia, possibly due to late release of endogenous insulin from the autoantibody pool [215] . It is also possible that some instances of hypoglycemia associated with IAS can be attributed to the development of antiidiotypic antibodies that have insulin agonistic properties. Among white patients in the United States, IAS manifests primarily as postprandial hypoglycemia; as in Japanese patients, the syndrome can be associated with polyclonal or monoclonal IgG insulin-binding antibodies [216].

1.9.2.4.2 Hypoglycemia and antibodies to exogenous insulin

Published case reports have attributed unusually prolonged episodes of hypoglycemia to high levels of antibodies to exogenous animal insulin [217,218]. The first case, reported in 1960 by Harwood [217], described a 44-yr-old woman with type 1 diabetes who for 9 yr experienced periods in which she would need to discontinue insulin therapy for up to 23 days because of prolonged hypoglycemia. She was found to have 106,000 μU/ml insulin-binding capacity with an unusually slow rate of dissociation of the insulin-antiboy complex.

Seven case reports have been published of hypoglycemia occurring in patients with antibodies found in the setting of human insulin therapy [219,222]. Interestingly, these cases were all reported from Japan, where IAS is thought to be more prevalent than in the rest of the world. These cases also occurred predominantly in patients over 70 yr of age, as is the case with IAS.

Hypoglycemia has also been reported in up to 54% of patients receiving a combined pancreas and kidney transplant [223]. Tran *et al* [224] compared patients who had repeated episodes of hypoglycemia or hypoglycemic symptoms after a pancreatic transplant matched with patients who did not have hypoglycemia

after pancreatic transplant. They found a decrease in the ratio of fasting free insulin to total insulin in the patients who had a hyperglycemic response to a liquid meal challenge. This subgroup had a substantial increase in total, but not free, insulin concentrations. Although IAs were not directly measured, these data are consistent with the hypothesis that hypoglycemia was associated with high levels of circulating IAs [224].

Although anecdotal information from these case reports suggests that very high levels of IAs can be associated with unusual hypoglycemia syndromes, studies of large populations have failed to establish a relationship between IAs and hypoglycemia event rates [225, 226].

1.9.3 Pregnancy

Maternofetal IgG transfer begins early in the second trimester, with most antibodies transferring to the fetus during the third trimester [227]. Because organogenesis occurs during the first trimester, a lack of correlation between congenital malformations and maternal IAs is not surprising [228]. Recently, it has been shown that transmission of maternal antibodies to exogenous insulin does not affect diabetes risk in offspring [229].

Pregnancy-related risks from IAs have not been clearly demonstrated. early studies reported an associations between IAs and neonatal hypoglycemia [230-232]. However, these reports did not adequately describe maternal glycemic control in the patients studied. Maternal glycemic control is important because it is known to influence many fetal and neonatal risks, including neonatal hypoglycemia and macrosomia. Most notably, despite the strong association between neonatal hypoglycemia and increased birth weight in the infant of a diabetic mother, no consistent linkage was found between IAs and birth weight, even in the early studies that linked IAs to neonatal hypoglycemia. Furthermore, hypoglycemia remains a common complication in neonates born to mothers with diabetes, despite the fact that insulin preparations of low immunogenicity are now in routine use [233].

The potential for increased incidences of neonatal hypoglycemia, respiratory distress syndrome, and hypocalcemia has also been suggested in small number of studies published between 1980 to 1990 [234, 235]; however, these reports lacked adequate

documentation of maternal glycemic control. Subsequent studies, performed in the modern era of prenatal care for women with diabetes, argue against a connection between IAs and fetal morbidities [236]. Additionally, many studies have failed to show a relationship between maternal IAs and birth weight. A randomized trial comparing human insulin with animal insulin during pregnancy found that improved glycemic control, not IAs, influenced infant birth weight [237]. Wellik *et al*. [238] showed no correlation between IAs and neonatal glucose level or birth weight. Three recent studies with substantially larger sample sizes reported no relationships between IAs and birth weight [239-, 241]. A recent study found similar birth weights in offspring of 138 mothers with type 1 diabetes across a range of maternal IA levels and cord blood insulin levels [239]. Islet autoantibody concentrations also were found to have no influence on birth weight.

Two hypotheses have been put forward to explain a possible relationship between maternal IAs and neonatal hypoglycemia [34], both of which would predict clear correlations between IA levels, birth weight, and neonatal hypoglycemia risk. In the first, maternally derived antibody interference with insulin action in the fetal circulation could result in compensatory fetal hyperinsulinemia. In this scenario, fetal hyperinsulinemia results in increased birth weight and neonatal hypoglycemia. Although neonatal cord blood C-peptide levels (reflecting endogenous insulin secretion) were found to correlate with IAs in one study [231], the finding was not reproducible [228, 240]. Recently, no relationship was found between cord blood insulin levels and birth weight [239]. Furthermore, multiple studies, including the only randomized prospective study, have found no relationship between IAs and fetal birth weight [237].

A second hypothesis suggests that insulin is transferred to the fetus via IA complexes. These complexes could then dissociate in the fetal circulation, releasing bioactive insulin. Were this to happen during fetal life, increased birth weight would be expected. As discussed, multiple studies have failed to show relationships between maternal IAs and macrosomia. If IA complex dissociation occurred after birth, unusually prolonged neonatal hypoglycemia syndromes might be expected, given that the biological half-life of circulating IgG is approximately 23 d. No distinguishing

clinical characteristics of neonatal hypoglycemia associated with IAs, such as unusual prolongation of what have been described in babies born to mothers with IAs.

1.9.4 Autoimmune diseases

Antibody responses to exogenous insulin have not been shown to cause generalized immune activation resulting in autoimmune disease states. Lassmann-Vague *et al* [242] measured a panel of autoantibodies before implantation with IPPs and then subsequently every year in 28 patients with type 1 diabetes. At baseline, 19 of 28 patients were negative for all tested autoantibodies (antithyroglobulin, antithyroperoxidase, gastric parietal cell, smooth muscle, mitochondrial, liver-kidney microsome, antinuclear, antiendomysium, and antigliadin antibodies). During 2 yr of IPPs with insulin treatment, the sera of the patients negative at baseline remained negative throughout the study, despite the expected IA response. Nine patients with preexisting autoantibodies had no change in most autoantibody titers. Two of these patients had increases in antithyroperoxidase titers, whereas three patients had decreases in these titers. No difference was seen in IA responses to IPPs in the nine patients with preexisting autoantibodies relative to those who did not have preexisting antibodies.

1.9.5 Immune complexes

A series of reports published from the 1960s through the 1980s yielded conflicting results regarding the role of insulin-antiinsulin immune complexes in the development of long-term diabetic complications in animal models [243]. Studies in humans have not shown consistent links between IAs and long term diabetic complications. Although some studies have shown increased levels of immune complex formation in patients with diabetic microangiopathic complications compared with patients without complications [244- 246], these immune complexes often do not contain insulin or IA [245-248]. Furthermore, insulin administration does not contribute to this immune complex formation [249]. The nonspecific immune complexes observed in patients with diabetes may reflect general inflammatory reactions associated with angiopathies [245].

Overall, no direct evidence has shown that immune complexes, insulin immune complexes, or IAs are capable of mediating vascular or glomerular damage. Associations have been reported, but they have been largely based on the prevalence

of immune complex detectability rather than on quantitative measurements of the amount of immune complex present [250]. Andersen [251] showed that high porcine insulin-binding levels were more frequent in patients with long term diabetes complications than in patients without complications but found no significant differences in mean antibody levels between groups. Virella *et al* [252] found correlations between the presence or absence of detectable insulin-antiinsulin immune complexes and the presence or absence of some diabetic complications.

Although immune complexes in insulin-treated patients have been associated with procoagulant markers in some studies [247, 253, 254], the majority of studies have shown no relationship between IA and diabetic microangiopathic complications. Specifically, no relation has been found between IAs and histological findings of nephropathy [255], muscle basement membrane thickness [256], clinical nephropathy [257, 258], clinical retinopathy [256- 260], clinical autonomic neuropathy [261], clinical peripheral neuropathy [262], or complement activation in patients with diabetes [263].

The absence of a pathogenic effect may be related to the properties of the immune complex. It has been observed that insulin and IA immune complexes do not precipitate (at antigen or antibody excess) and cannot be detected by standard immunodiffusion methods [264]. In addition, insulin-insulinantibody immune complexes are monomers or dimers, and large immune complexes are not formed. Small immune complexes do not readily bind to C1q complement and are, therefore, not readily cleared by the reticuloendothelial system of the liver or spleen [134]. Small immune complexes have a similar half-life to circulating Ig molecules, which explains the high levels of circulating total insulin (antibody bound) in patients with high levels of IAs.

1.9.6 Buffering effect of insulin antibodies

Both glycemic stability and instability have been attributed to the presence of IAs. Instability has been described only in case reports and is characterized either by both hyperglycemia and hypoglycemia occurring within a single 24-h interval or by periods lasting days to weeks of hyperglycemia alternating with periods of hypoglycemia of similar duration [219, 220, 265-267]. Some of these case reports describe patients with relative overnight hypoglycemia and daytime hyperglycemia, although there is no

clear mechanistic explanation for such diurnal patterns. Because these glycemic instability syndromes are rare and variable in nature and because in vitro parameters are not predictive of clinical findings, it is difficult to establish cause and effect relationships between the antibodies and such glucose variability syndromes.

Conversely, because studies in patients being treated with insulin—as well as those in individuals with insulin autoimmune-hypoglycemia syndrome—have suggested that the presence of high levels of IAs is associated with a retarded disappearance rate of insulin, some authors have suggested that antibodies may serve as a "buffer" to glucose variability [268, 269]. Limited studies demonstrated that this slowed rate of insulin disappearance appeared to decrease the likelihood of diabetic ketoacidosis, contributing to stability of glycemic control [270- 272]. However, for the majority of patients, the levels of antibodies seen with insulin therapy are unlikely to result in significant effects on glycemic variability [273].

1.9.7 Insulin antibodies and risk for diabetes

1.9.7.1 Insulin antibodies IAS and ß-cell loss

Although the spontaneous appearance of IAA in nondiabetic patients is known to be predictive of type 1 diabetes development [274, 275], there is no evidence that IAAs or IAs themselves causally mediate ß-cell destruction. Support for this comes from studies in which nondiabetic patients with circulating antibodies to insulin were followed for the onset of diabetes [276- 279]. Bock *et al* [276] investigated whether insulin treatment of patients without diabetes who were undergoing insulin shock therapy for psychiatric disorders would be at increased risk for the development of diabetes. In their retrospective analysis of 481 patients observed for an average of 22 yr, one patient developed type 1 diabetes, and 12 developed type 2 diabetes. These instances did not differ from the background population. Only two of the 27 patients samples examined were positive for IAs, and none was positive for islet cell antibodies. Based on this study the researchers concluded that exogenous insulin used in diabetes prevention trials was safe and would not increase the risk for diabetes.

1.9.7.2 Cellular immune response to exogenous insulin

Most of the reports on insulin-specific T cell responses have focused on the autoreactive T cells involved in the pathogenesis of type 1 diabetes. Studies using NOD mice have demonstrated the presence of CD4+ and CD8+ cells that recognize insulin and lead to the destruction of ß-cells in this model of spontaneous autoimmune diabetes [(280)]. However, multiple interventions, including parenteral, oral, or aerosolized insulin treatment have been shown to delay the onset of diabetes in these mice[(280)].

T cell clones have been generated from the draining pancreatic lymph nodes of patients with type 1 diabetes that were found to be responsive to insulin [(281)]. These data indicate that insulin may be one of the initial antigens recognized by autoreactive T cells before the onset of clinical type 1 diabetes and exogenous insulin treatment.

Few studies have evaluated insulin-specific T cell responses in patients with diabetes being treated with insulin. The most commonly used assay to measure insulin-responsive T cells involves coincubating peripheral blood mononuclear cells with or without insulin for 6 to 10 d. The proliferative response is measured by the incorporation of radio-labeled nucleotide [(282)]. T cell responses in patients with type 1 diabetes treated with porcine and bovine insulin or porcine-bovine mixtures were first reported in 1975 [(283, 284)]. Patients with recent-onset diabetes (32%) and those with long-standing illness (47%) were found to have positive T cell responses to human insulin [(285)]. Since T cell help is required for B cell development and antibody production, IA levels might be expected to correlate with T cell responsiveness. However, poor correlations between IA and/or IAA levels and T cell responsiveness have been observed [(285- 287)]. Patients with high IAs or IAAs had very low T cell responses, and patients with low IA or IAA levels had high T cell responses. The relatively infrequent finding of a strong cellular immune response to insulin in patients with type 1 diabetes treated with exogenous insulin may be partly explained by the activation of regulatory T cells [(29)].

Additional investigations suggest that T cells respond to different regions (epitopes) of the insulin molecule. T cell responses are greater with the B chain than the A chain of insulin [288, 289]. Theoretically, insulin-specific T cells measured in these studies may be autoreactive T cells generated by endogenous insulin, autoreactive T cells expanded by exogenous insulin, and/or T cells initially generated by exogenous insulin; however, based on the assays used, it is not possible to differentiate among these possibilities. T cell responses with exogenous insulin treatment likely will be further investigated if insulin treatment is found to prevent the onset of type 1 diabetes in humans, as observed in NOD mice [289].

1.9.7.3 Exogenous insulin and prevention of type 1 diabetes in clinical studies.

Results from the NOD mice studies led to the Diabetes Prevention Trial Type 1, which was designed to test the ability of sc and orally administered insulin to prevent type 1 diabetes in subjects known to be at risk based on the presence of autoimmune markers (e.g., IAAs and islet cell antibodies) [290]. The sc insulin administration arm of the trial showed no acceleration or delay of type 1 diabetes onset. The same was true for the oral insulin treatment arm when all patients were included [291]. A subset analysis of the oral insulin treatment group, however, demonstrated a significantly lower annualized rate of diabetes onset in patients with baseline IAA levels of at least 80 nU/ml treated with oral insulin compared with patients treated with placebo.

Additional studies showed no effect of oral insulin on residual ß-cell function in patients with new onset type 1 diabetes [292,293]. More recently, 38 children at risk for type 1 diabetes were treated with intranasal insulin and showed no evidence for accelerated loss of ß-cell function [294]. Furthermore, IA responses to intranasal insulin were demonstrated, as were immune changes consistent with mucosal tolerance to insulin. A small trial suggested that low-dose sc insulin may have favorable immunomodulatory effects in adult patients with latent autoimmune diabetes [295].

1.10 Influence of IAs on urine microalbumin

A link between circulating anti-insulin antibodies and diabetic glomerulopathy has been suggested. Brun JF(1), Fédou C, Orsetti A confirmed preliminary reports indicating a statistical relationship between anti-insulin antibodies and microalbuminuria. They hypothesized that anti-insulin antibodies may be an additional factor of risk in the pathogenesis of early (reversible) stages of diabetic nephropathy[296].

CHAPTER 2

AIM OF STUDY

Aim of the study

1. To determine prevalence of anti insulin antibodies in Libyan type 2 diabetic patients at BDC.
2. To correlate the clinical data of these patients with anti insulin antibodies.
3. To evaluate the effects of anti-insulin antibodies on glycemic control.

CHAPTER 3

MATERIELS & METHODS

Materials and Methods

3.1 Subjects

This study composed of two parts:

- Part 1 was carried out on 200 type 2 diabetic patients followed up at BDC during the period of 2 months.

  Patients were subjected to full history taking according to attached questionnaire (appendix I). The patients were divided into 2 groups:

  - Group 1- Insulin treated group(140 patient)
  - Group 2- Oral Hypoglycemic Drugs treated group (60 patient)

- Part 2 was carried out on 95 type 2 diabetic patients followed up at BDC during the period of 2 months.

  Patients were subjected to full history taking according to attached questionnaire (appendix I).

3.2 Data collection

Data from each patient included :
Age, sex, weight, diet control, regular exercise, type of treatment, metformin intake, insulin dose and duration.

3.3 Blood Collection

Blood samples were obtained through venous puncture.
Each sample was centrifuged, serum was separated and stored at-70 c until processing.

3.4 Materials

- The following kit were made available:
  Anti-Insulin ELISA kit from DRG Diagnostics (USA).
  Anti-Insulin ELISA kit from Medipan GMBH (Germany).
- Micro plate reader, automatic plate washer (mark) vortex mixer, pipets for 10 ul, 100ul and 1000 ul, laboratory timing device, data reduction soft ware, distilled water , graduated cylinder for the wash solution.

3.5 Determination of anti-insulin antibodies by ELISA.

3.5.1 Principle of Anti-Insulin IgG Test

A mixture of highly purified preparations of bovine, porcine and recombinant human insulin is bound to microwells. Antibodies against these antigens, if present in diluted serum or plasma, bind to the respective antigen. Washing of the microwells removes unbound serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG immunologically detects the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to from a blue color. The addition of an acid stops the reaction forming a yellow end- product. The intensity of this yellow color is measured photometrically at 450nm and 620 as reference filter. The amount of colour is directly proportional to the concentration of IgG anti-bodies present in the original sample.

3.5.2 Method

patient sera against anti- insulin qualitative and quantitative evaluation of IgG antibodies were done according to the instruction provided by the manufacturer. After measurement of optical density, the quality control was checked according to values given by the manufacturer. Standard curve prepared using values of parameter gave either high positive, Low positive and negative control or international standards, and the values for sample read from the curve in IU/ml.

3.6 Determination of serum insulin

Serum insulin was measured according to HS-INSULIN-CHECK-1 procedure test (VEDA.LAB)[297].

3.7 Determination of urea

Serum urea was measured according to Fluitest procedure test (analyticon)[298].

3.8 Determination of creatinine

Serum creatinine was measured according to Fluitest procedure test (analyticon)[299].

3.9 Determination of AST

Serum AST was measured according to HSUV-Method recommended by the international federation of clinical chemistry (analyticon)[[300].

3.10 Determination of ALT

Serum ALT was measured according to HSUV-Method recommended by the international federation of clinical chemistry (analyticon)[301].

3.11 Determination of ALP

Serum ALP was measured according to Fluitest procedure test (analyticon)[302].

3.12 Determination of BIL

Serum BIL was measured according to Fluitest procedure test (analyticon)[303].

3.13 Determination of cholesterol

Serum cholesterol was measured according to Fluitest procedure test (analyticon)[304].

3.14 Determination of TG

Serum triglycerides was measured according to Fluitest procedure test (analyticon) [305].

3.15 Determination of HDL

Serum HDL was measured according to Fluitest procedure test (analyticon)[306].

3.16 Determination of LDL

Serum LDL was measured according to Fluitest procedure test (analyticon)[307].

3.17 Determination of HbA1c

Blood HbA1c was measured according to the competition immune-detection method done by i-CHROMATM HbA1c immunoassay system[308].

3.18 Determination of urine MAU

Urine microalbmin was measured according to the sandwich immune-detection method done by i-CHROMATM HbA1c immunoassay system[309].

3.19 Determination of Glucose

Serum Glucose was determined by enzymatic colorimetric test on basis of Trinder-Reaction[310].

3.20 Statistical Analysis

T-test as well as confidence intervals were measured using an SPSS (Version 11).

CHAPTER 4

RESULTS

Results

4.1 Demographic and Risk factors for group (A)

(Figures 4.1, 4.2, 4.3)

Out of the 200 patients recruited in this study; 52.5% were females. The majority (59%) aged 50-69 yrs compared to only 20.5% in the age group below 50 yrs. Nearly half of them weighed 70-89 kgm, however, extreme obesity with body weight greater than 100 Kg was less common being found in 6% only. (figure 4.1).

Poor diet control was found in the majority of these patients (65.5%) and 70% were not adherent to regular exercise program. Insulin was the treatment in 70% and similarly 70% were on metformin therapy. On the other hand, 30% of patients were on OHD only (figure 4.1).

Among the 140 patients on insulin treatment, 82(58.6%) were on less than 60 u/day. The duration of insulin treatment was found to be less than 10 yrs in 32.9% of patients and greater than 20 yrs in 24.2%. (figure 4.2).

Glycemic good control as assessed by FPG < 126 mg/dl was only found in 3(16%) of patients, poor control as reflected by FPG > 126 mg/dl was found in the rest of the patients. When the post-lunch plasma glucose of less than 200 mg/dl was used to assess the degree of control only 31 patients (15.5%) achieved the target. (figure 4.3).

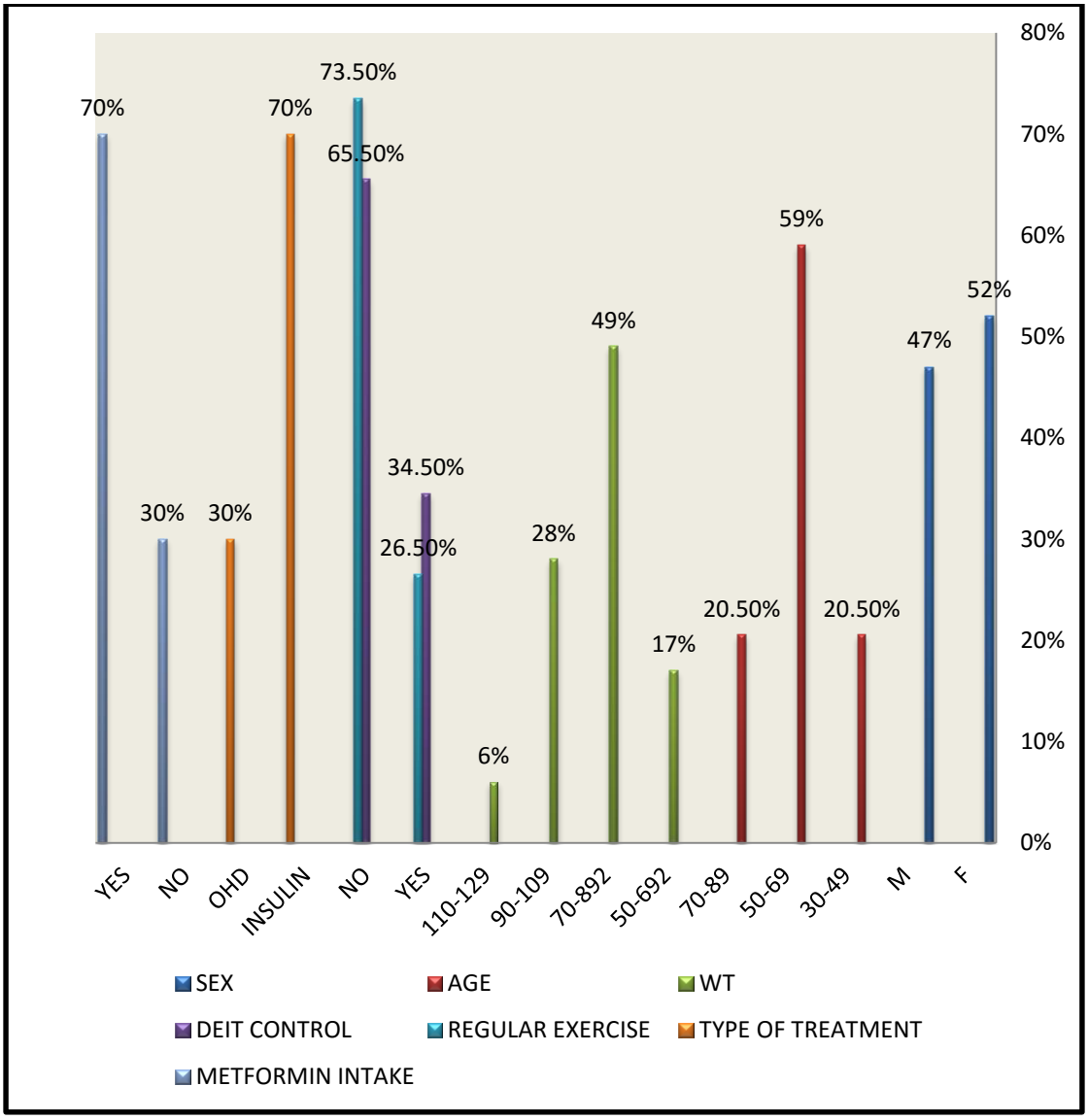

Figure 4.1: Demographic characteristic of group (A) patients

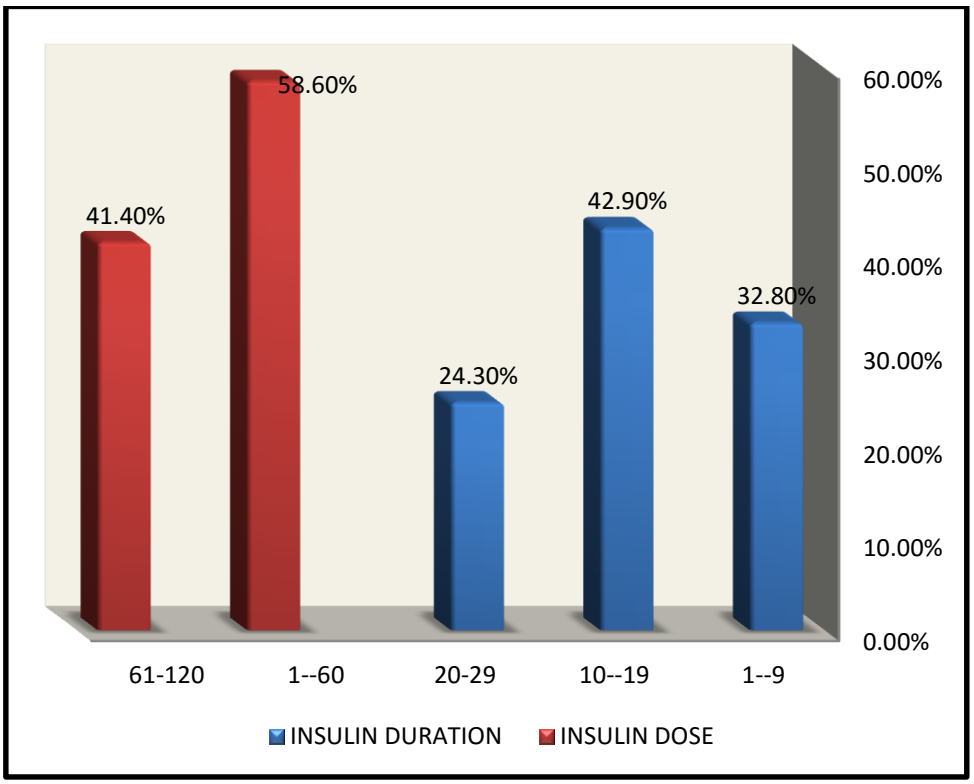

Figure 4.2: Risk Factors for development of IAs to group (A) patients

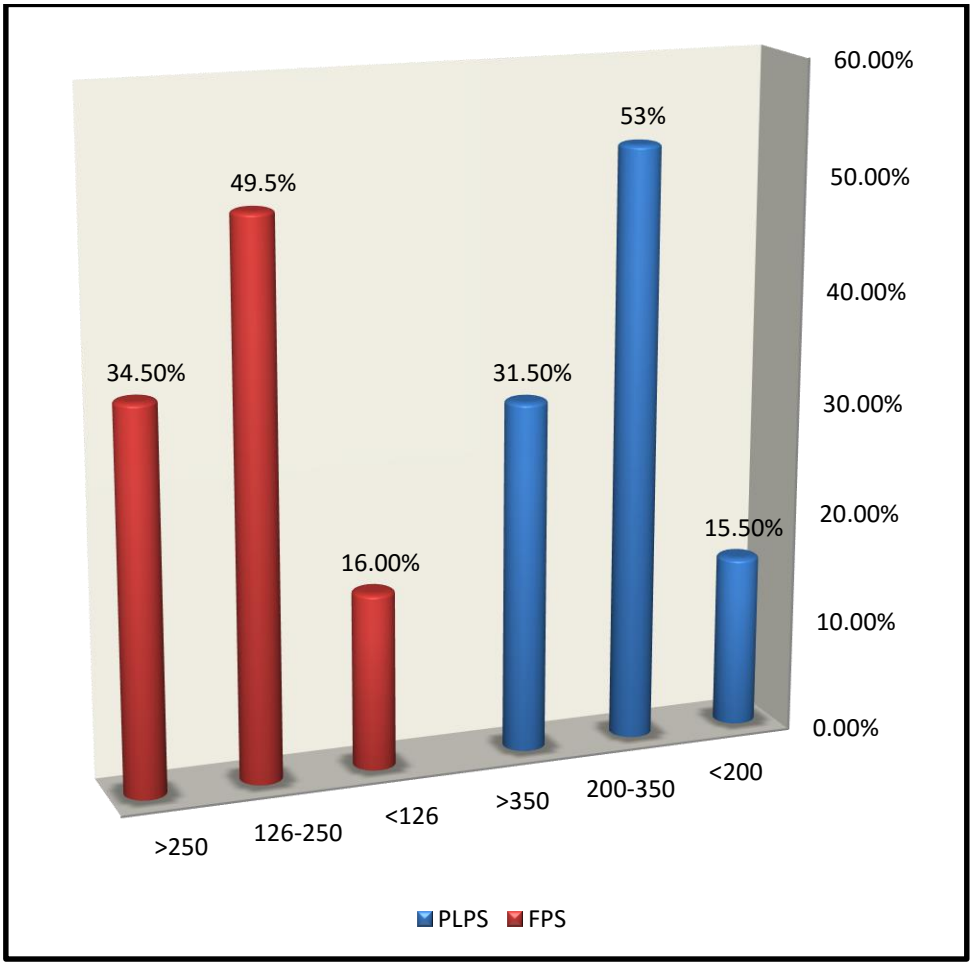

Figure 4.3: Glycemic control of group (A) patients

4.2 IAs seroprevalence for group (A) (figure 4.4)

A total of 200 diabetic patients who attended Benghazi diabetic centre over a period of 2 mths were recruited in this cross sectional study to estimate seroprevalence of IAs. serum samples were collected from them and assayed using ELISA techniques. Overall seroprevalence to IAs out of 200 serum samples 22(11%) were positive for IAs by ELISA. Titre was (10.11-71.05 u /ml).

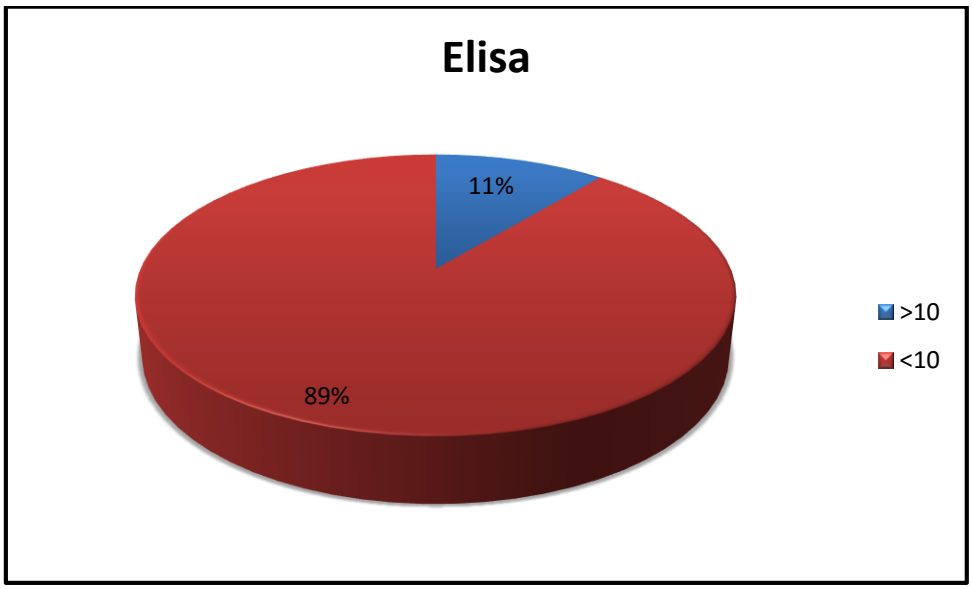

Figure 4.4: Overall seroprevalence to IAs for group (A)

4.3 IAs and Demographic characteristics of group (A)

(figure 4.5, 4.6)

4.3.1 IAs and Sex

Out of 105 female patients 10 (9.5 %) were seropositive compared to 12(12.6 %) of 95 male patients. Differences in IAs seropositivity in relation to sex were statistically significant (P< 0.05).

4.3.2 IAs and Age

In the 30-49 years age group, 2(4.9%) of 41 patients were seropositive .In the 50-69 years age group, 15(12.7%) of 118 patients were seropositive. Whereas among the 70-89 years age group, 5(12.2%) of 41 patients were seropositive. Differences in IAs seropositivity with respect to age were statistically significant (p< 0.05).

4.3.3 IAs and Weight

In the 50-69 kgm Wt group, 5(14.7%) of 34 patients were seropositive .In the 70-89 kgm wt group, 10 (10.2%) of 98 patients were seropositive. In the 90-109 kgm wt group, 6(10.7%) of 56 patients were seropositive. whereas In the 110-129 kgm wt group, 1(8.3%) of 12 patients were seropositive. Differences in IAs seropositivity with respect to weight were statistically insignificant (p> 0 .05).

4.3.4 IAs and Diet Control

Out of 69 patients controlled diet, 12(17.4%) were seropositive compared to10(7.6%) of 131 patients uncontrolled diet were seropositive. Differences in IAs seropositivity in relation to diet control were statistically insignificant (p>0.05).

4.3.5 IAs and Regular Exercise

Out of 53 patients performing regular exercise, 10(18.9%) were seropositive, However in the nonactive 147 patients only 12(8.2%) were seropositive.

Differences in IAs seropositivity in relation to regular exercise were statistically significant (p< 0 .05).

4.3.6 *IAs and Type of Treatment*

Of 140 patients on insulin, 20(14.3%) were seropositive compared to 2(3.3%) of 60 patients on OHD. Differences in IAs seropositivity in relation to type of Treatment were statistically significant (p< 0.05).

4.3.7 IAs and Metformin Intake

Out of 140 patients on metformin, 15(10.7%) were seropositive compared to 7(11.7%) of 60 patients not on metformin were seropositive. Differences in IAs seropositivity in relation to metformin Intake were statistically insignificant (p> 0.05).

4.3.8 IAs and Duration of Insulin therapy

In those with <10 years duration group, 5(10.9%) of 46 patients were seropositive .In the 10-19 years duration group, 9(15%) of 60 patients were seropositive. Whereas in the 20-29 years duration group, 6(17.6%) of 34 patients were seropositive. Differences in IAs seropositivity with respect to duration were statistically significant (p< 0 .05).

4.3.9 IAs and Insulin Dose

Out of 82 patients with insulin dosage (1-60 U/day) 13(15.8 %) were seropositive compared to 7(12.1 %) of 58 patients their dosage (61-120 U)/day). Differences in IAs seropositivity in relation to insulin dose were statistically insignificant (P> 0.05).

4.3.10 IAs and FPG

Out of 32 patients with FPG (<126 mg/dl), 3(9.4%) were seropositive. Out of 99 patients with FPG (126-200mg/dl), 13(13.1%) were seropositive. Whereas out of 69 patients with FPG (>200 mg/dl), 6(8.7%) were seropositive. Differences in IAs seropositivity with respect to FPG were statistically significant (p< 0 .05).

4.3.11 IAs and PLPG

Out of 31 patients with PLPG (<200 mg/dl), 3(9.7%) were seropositive. Out of 106 patients with PLPG (200-300mg/dl), 11 (10.4%) were seropositive. Whereas out of 63 patients with PLPG (>300mg/dl), 8(12.7%) were seropositive. Differences in IAs seropositivity with respect to PLPG were statistically significant (p< 0 .05).

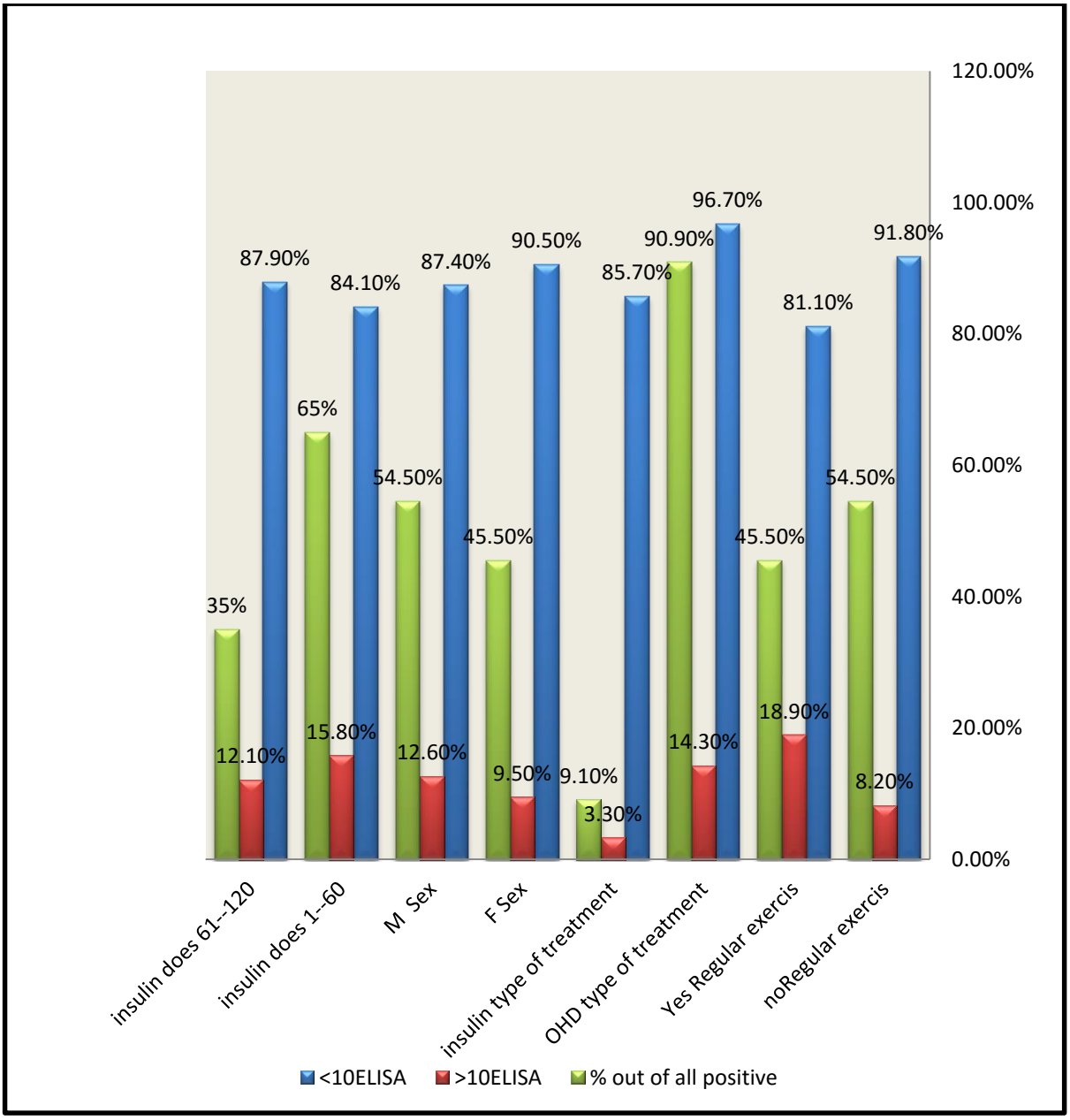

Figure 4.5: ELISA, Demographic characteristic of group (A) patients statistically significant (p< 0 .05)

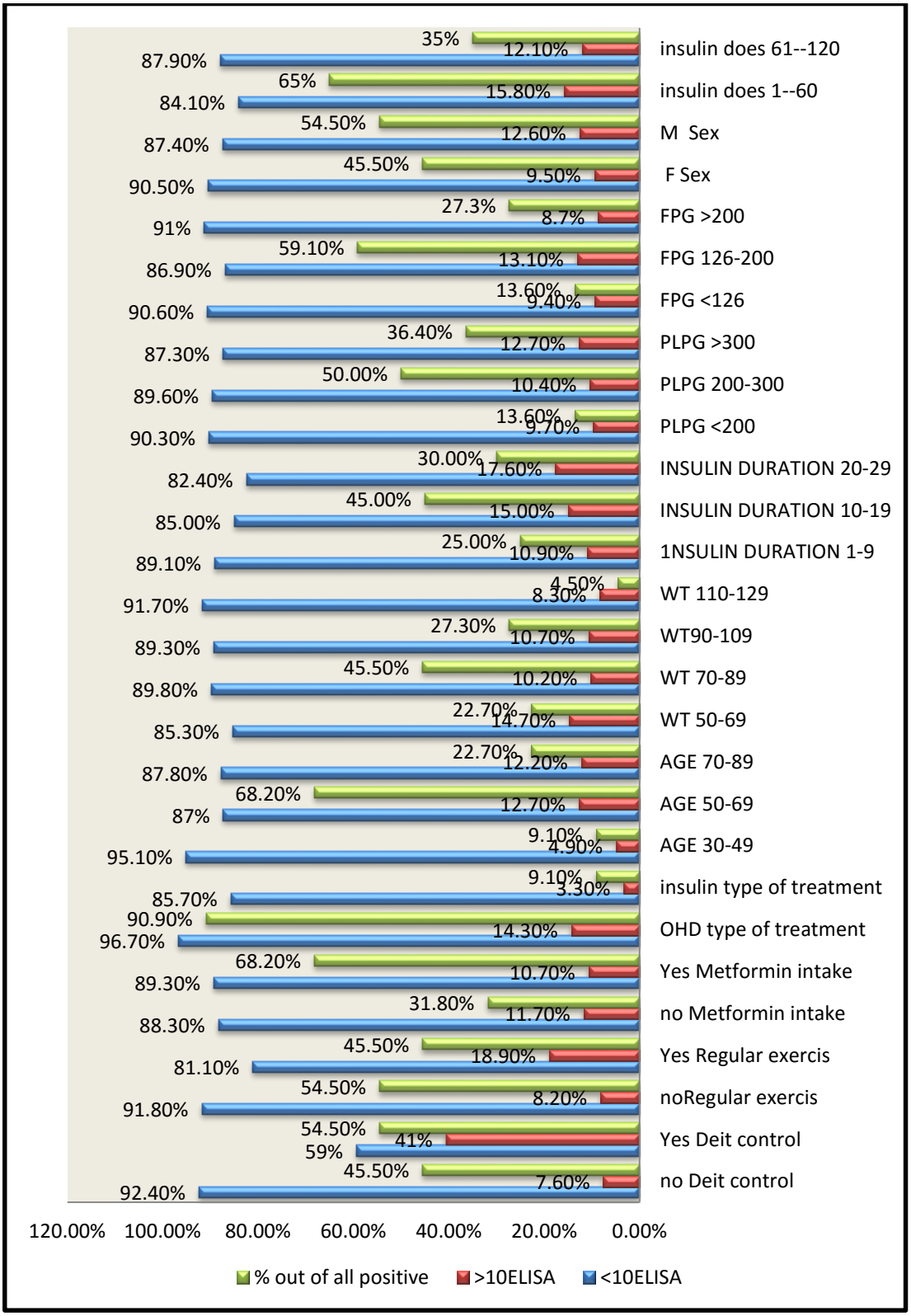

Figure 4.6: ELISA, Demographic characteristic of group (B) patients statistically insignificant (p> 0 .05).

4.4 Demographic and Risk factors for group (B) (figure 4.7, 4.8, 4.9, 4.10, 4.11)

This part of the study included 95 patients and the majority (74.75) were females.

In this group, 57 patients (60%) aged 50-69 yrs, however, only 20% aged less than 50 yrs.

Less than quarter of the patients (23.2%) had body weight between 50-69 Kg and 33.7% had a body weight of 70-89 Kg, however, only 14.7% of the patients had body weight greater than 110 Kg.

Adherence to diet control was very poor with only 25 patients (26.3%) were on diet control. Exercise was not performed in the majority of the patients, 69 (72.6).

All the patient were on insulin treatment, and in 59 (62.1%) the insulin dose was between 1-60 U/day. The duration of insulin treatment was 1-9 yrs in 22 (23.2%) of patients, but the majority 46 (48.4%) of patients were on insulin treatment for 10-19 yrs. Metformin therapy was reported by only 25 patients (26.3%).

Serum electrolytes (Sodium and Potassium) were within the normal range of 135-145 and 3.5-5.0 in all patients respectively.

The majority of these patients 90.5% had blood urea within the normal range of 20-45 mg/dl and only about 10% had higher levels. Similarly 86 patients (90.5%) had their serum creatinine in the normal range less than 1.5 mg/dl.

Liver function tests, AST, ALT, ALP and BIL were normal in over than 90% of patients.

A cholesterol level < 200 mg/dl was achieved in 58 patients (61.6%). The majority of the patients 92.6% had their TG < 140 mg/dl. The HDL levelswere 25-45 mg/dl in 83 patients (87.4%). The majority 92.6% of the patients achieved the LDL target level of 70-100 mg/dl.

The degree of control was assessed using HbA1c and an HbA1c < 6.5% was found n only 23 (24.2%) of patients, the rest of the patients were poorly controlled as reflected by an HbA1c > 6.5%.

All patients had serum insulin within the normal range of 2-200 iu/ml.

Evidence of diabetic nephropathy as assessed by urine albumin excretion. In 87 patients (91.6%) urine microalbumin was in the normal range 0-20 mg/dl, higher levels were noticed in 8 patients (8.4%).

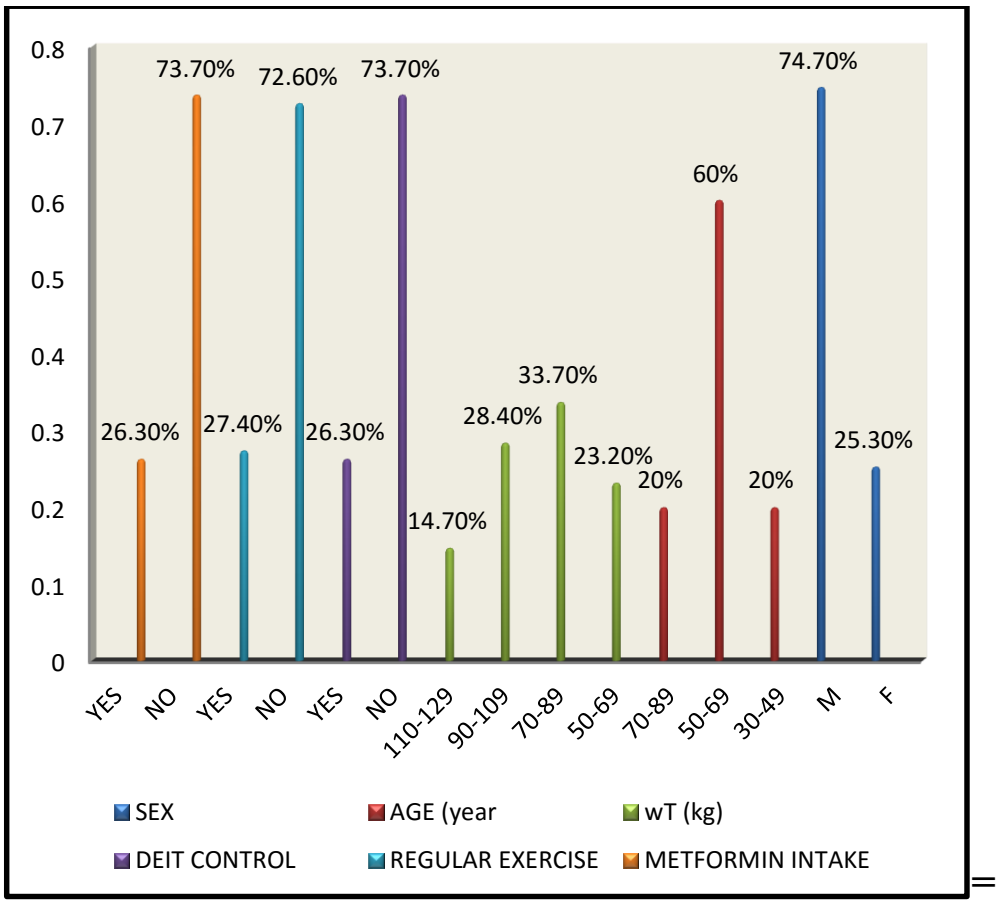

Figure 4.7 Demographic characteristic of group B patients

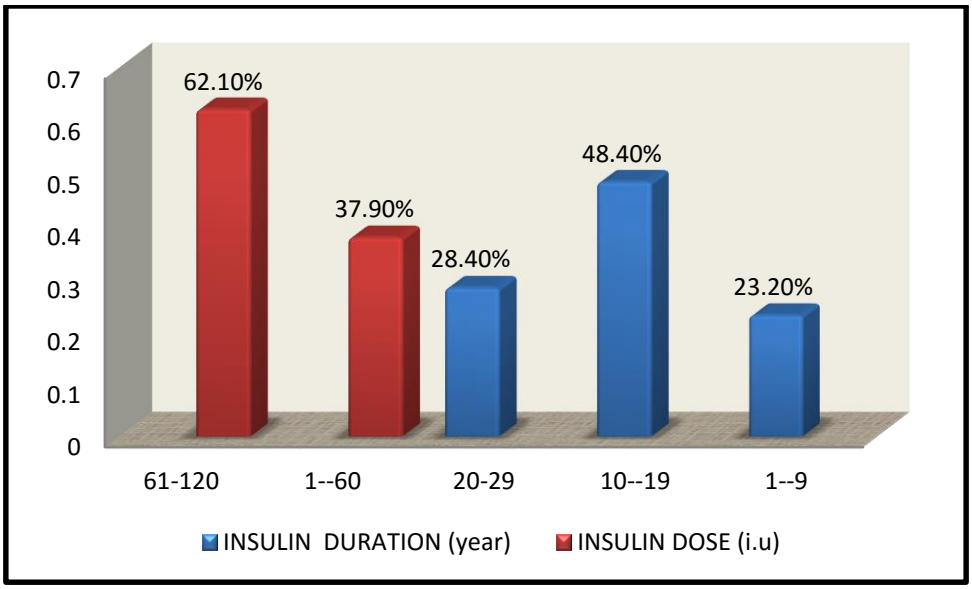

Figure 4.8 Insulin as a risk Factor of group B patients

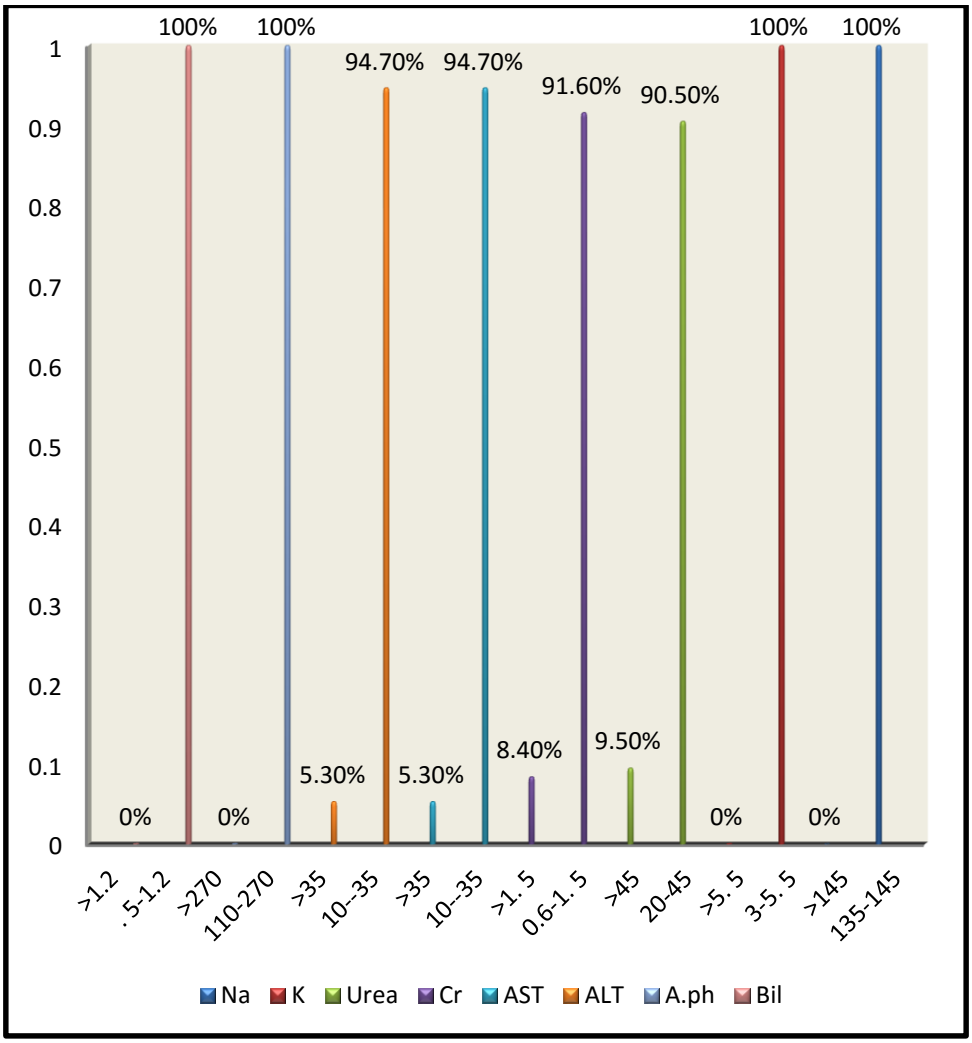

Figure 4.9 Associated risk factors of group B patients

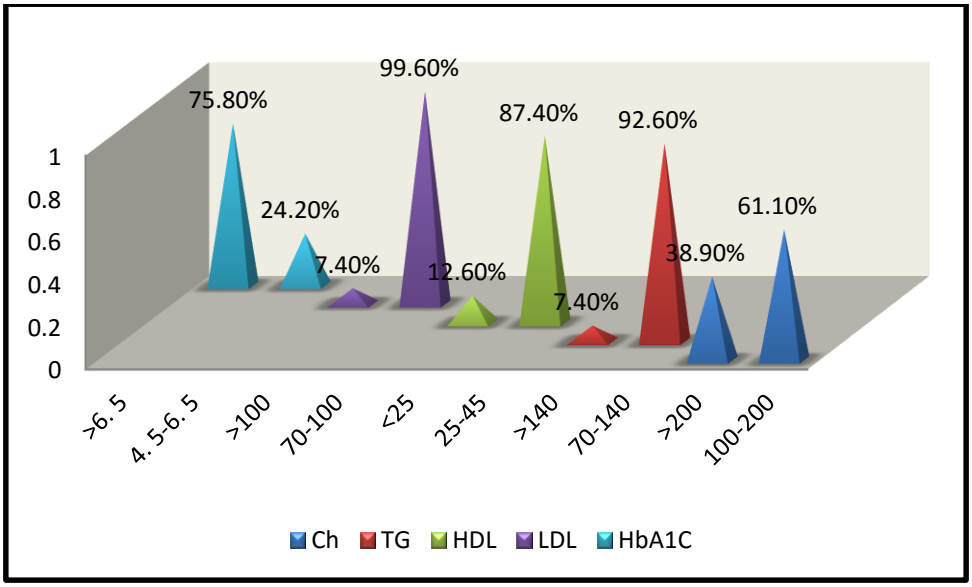

Figure 4.10 Associated risk factors of group B patients

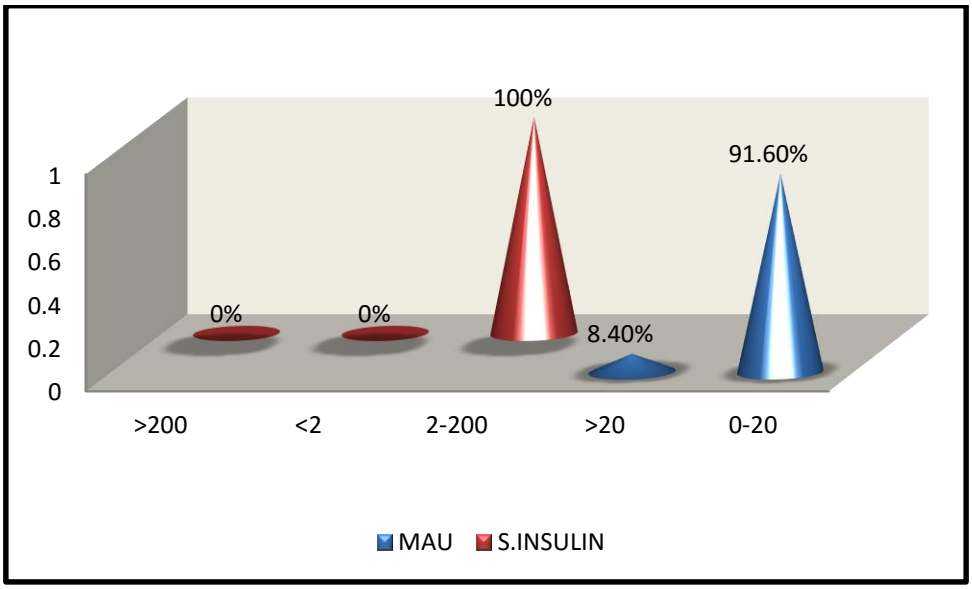

Figure 4.11 Associated risk factors of group B patients

4.5 IAs seroprevalence for group (B)(figure 4.12)

A total of 95 diabetic patients who attended Benghazi diabetic centre over a period of 3mths were recruited in this cross sectional study to estimate seroprevalence of IAs. serum samples were collected from them and assayed using ELISA techniques. Overall seroprevalence to IAs out of 95 serum samples 25(26.3%) were positive for IAs by ELISA. Titre was (3.1-60.5 u/ ml).

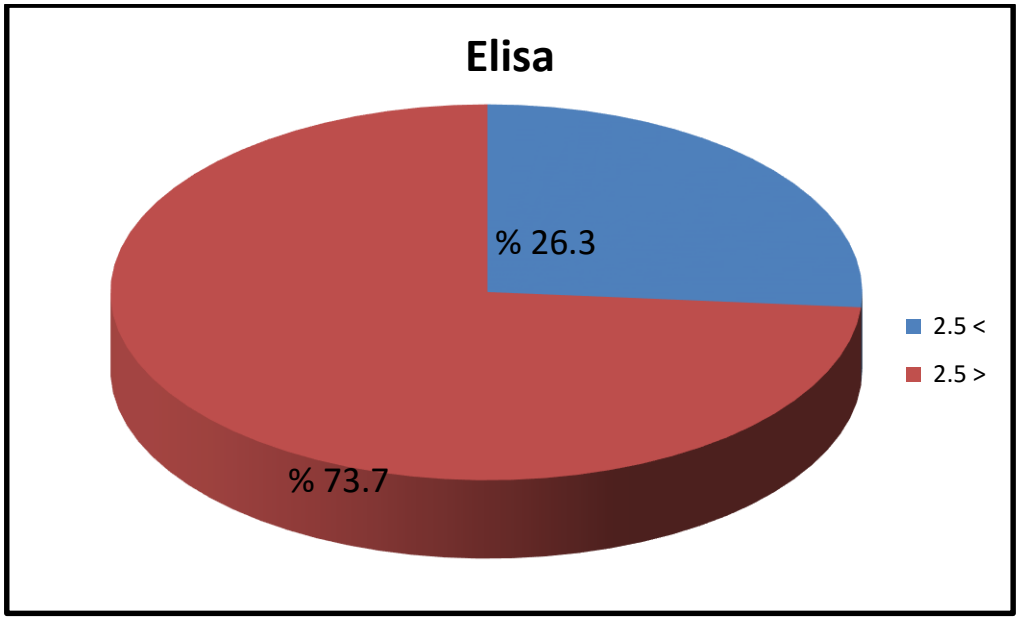

Figure 4.12 Overall seroprevalence to IAs for group (B)

4.6 IAs and Demographic characteristics of group B (figure 4.13, 4.14, 4.15)

4.6.1 IAs and sex

Out of 71 female patients 18(25.4 %) were seropositive compared to 7(29.2 %) of 24 male patients. Differences in IAs seropositivity in relation to sex were statistically significant (P< 0.05).

4.6.2 IAs and Age

In the 30-49 years age group, 3(15.8%) of 19 patients were seropositive. In the 50-69 years age group, 18(31.6%) of 57patients were seropositive. Whereas In the 70-89 years age group, 4(21%) of 19 patients were seropositive. Differences in IAs seropositivity with respect to age were statistically significant (p< 0.05).

4.6.3 IAs and Weight

In the 50-69 kgm Wt group, 5(22.7%) of 22 patients were seropositive .In the 70-89 kgm wt group, 9 (28%) of 32 patients were seropositive. In the 90-109 kgm wt group, 7(26%) of 27 patients were seropositive. whereas In the 110-129 kgm wt group, 4(29%) of 14 patients were seropositive. Differences in IAs seropositivity with respect to weight were statistically significant (p< 0 .05).

4.6.4 IAs and diet control

Out of 25 patients controlled diet, 4(16%) were seropositive compared to21(30%) of 70 patients uncontrolled diet were seropositive. Differences in IAs seropositivity in relation to diet control were statistically significant (p<0.05).

4.6.5 IAs and regular exercise

Out of 26 active patients, 5(19%) were seropositive compared to 20(29%) of 69 inactive patients were seropositive. Differences in IAs seropositivity in relation to regular exercise were statistically significant (p< 0 .05).

4.6.6 IAs and metformin Intake

Out of 25 patients on metformin, 6(24%) were seropositive compared to 19(27%) of 70 patients not on metformin were seropositive. Differences in IAs seropositivity in relation to metformin Intake were statistically significant ($p< 0.05$).

4.6.7 IAs and Duration of insulin treatment

In the 1-9 years duration group, 5(23%) of 22 patients were seropositive. In the 10-19 years duration group, 13(28%) of 46 patients were seropositive. Whereas in the 20-29 years duration group, 7(26%) of 27 patients were seropositive. Differences in IAs seropositivity with respect to duration were statistically significant ($p< 0 .05$).

4.6.8 IAs and insulin dose

Out of 36 patients their dosage (1-60 i.u) 8(22.2 %) were seropositive compared to 17(29 %) of 59 patients their dosage (61-120 i.u). Differences in IAs seropositivity in relation to insulin dose were statistically significant ($P<0.05$).

4.6.9 IAs and RFT

Out of 86 patients who had urea and Cr within normal levels, 20(23.3%) were seropositive .However out of 9 patients with high urea and Cr, 5(55.5%) were seropositive. Differences in IAs seropositivity with respect to RFT were statistically insignificant ($p> 0 .05$).

4.6.10 IAs and LFT

Out of 90 patients who had AST and ALT within normal levels, 22(24.4%) were seropositive .However out of 5 patients with high AST and ALT, 3 (60%) were seropositive.. Differences in IAs seropositivity with respect to LFT were statistically insignificant ($p> 0 .05$).

4.6.11 IAs and Lipid profile

Out of 58 patients their CHOL were within normal levels, 13(22.4%) were seropositive .out of 37 patients their CHOL were high, 12 (32.4%) were seropositive($P< 0.05$). Out of 88 patients their TG were within normal levels, 23(26.1%) were seropositive .out of 7 patients their TG were high, 2 (28.6%) were seropositive($P< 0.05$). Out of 83 patients their HDL were within normal levels, 20(24.1%) were seropositive .out of 12 patients their HDL were low, 5 (41.7%) were seropositive($P< 0.05$). Out of 88 patients their LDL were within normal levels, 24(27.2%) were seropositive .out of 7 patients their LDL were high, 1 (14.3%) were seropositive($P< 0.05$). Differences in IAs seropositivity with respect to Lipid profile were statistically significant ($p< 0.05$).

4.6.12 IAs and Serum Insulin

Out of 95 patients who had serun insulin within normal levels, 25(100%) were seropositive. Differences in IAs seropositivity with respect to Insulin were statistically insignificant ($p> 0 .05$).

4.6.13 IAs and urine Microalbumin

Out of 87patients who had MAU within normal levels, 20(23%) were seropositive. However out of 8patients with high MAU, 5 (62.5%) were seropositive. Differences in IAs seropositivity with respect to MAU were statistically insignificant ($p> 0 .05$).

4.6.14 IAs and HbA1C

Out of 23 patients who had HbA1C within normal levels, 6(26%) were seropositive. However out of 72 patients with high HbA1C, 19 (26%) were seropositive. Differences in IAs seropositivity with respect to HbA1C were statistically significant ($p< 0 .05$).

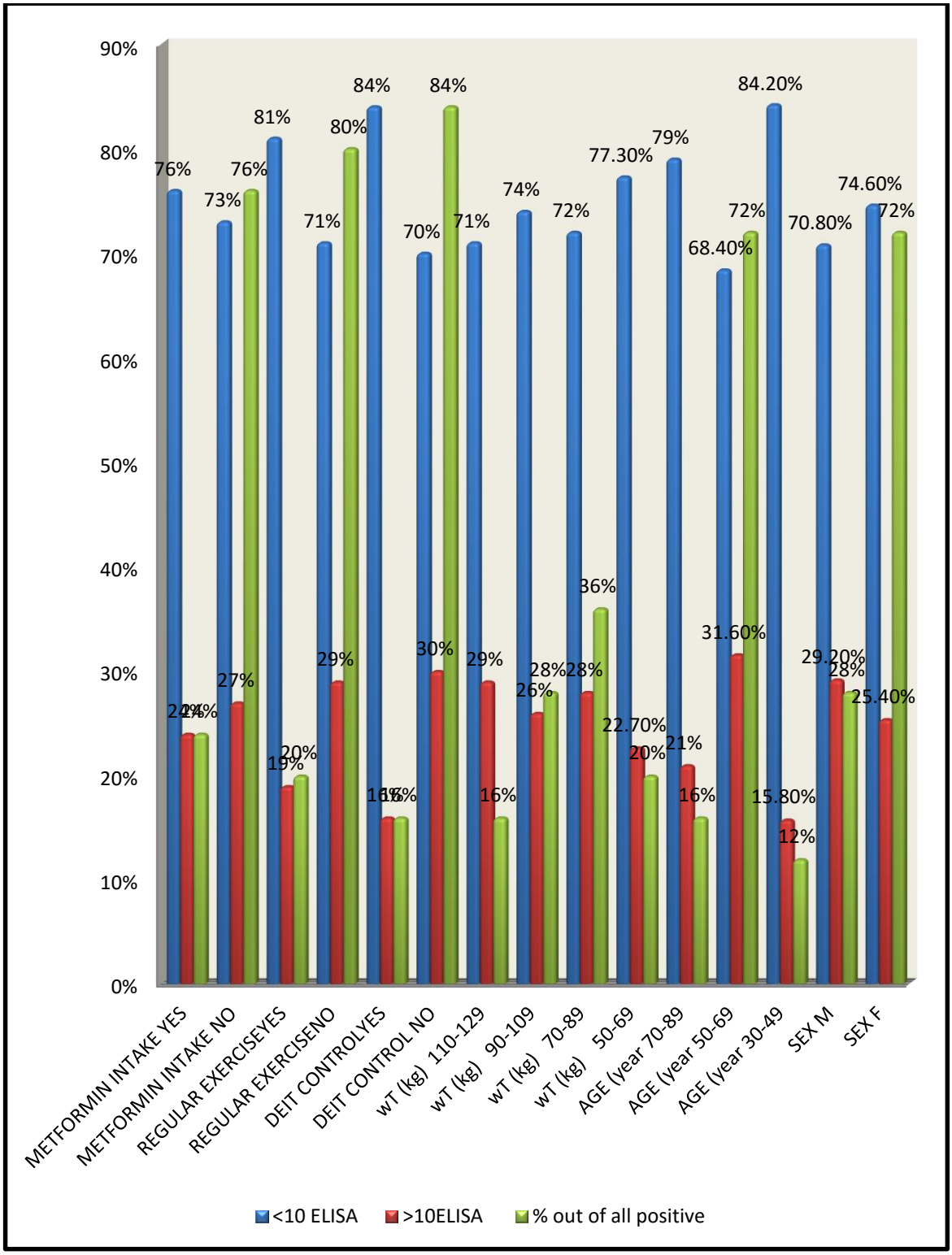

Figure 4.13 ELISA, Risk Factors and Demographic characteristic of group B patients statistically significant (p< 0 .05)

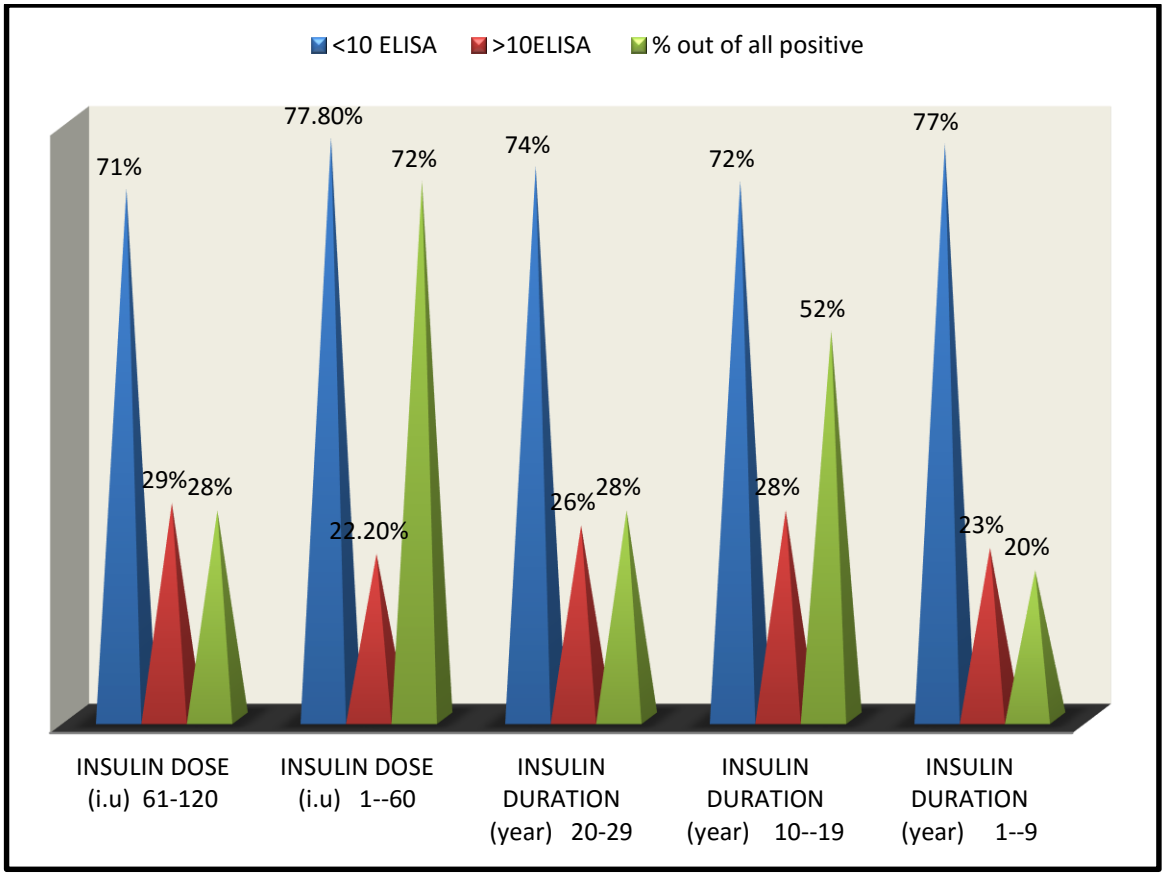

Figure 4.14 ELISA, Risk Factors and Demographic characteristic of group B patients statistically significant (p< 0 .05)

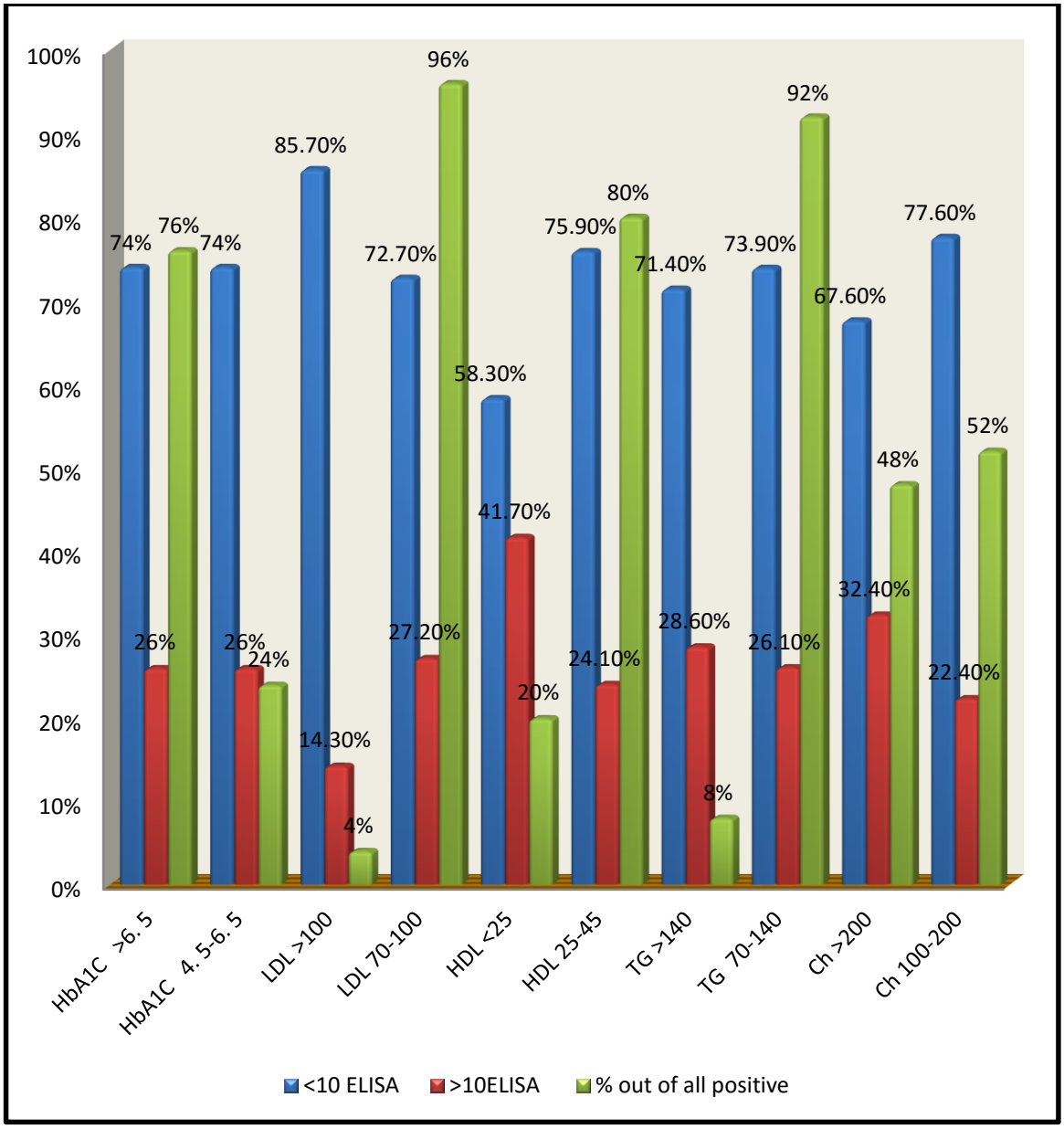

Figure 4.15 Laboratory parameters affected by IAs in group B

statistically significant (P< 0.05)

CHAPTER 5

DISCUSSION

Discussion

Normal insulin secretary function is essential for the maintenance of normal glucose tolerance and abnormal insulin secretion is invariably present in type 2 diabetes mellitus patients. Insulin antibodies could also be one of the causes for decreased insulin function and therefore uncontrolled blood glucose (Insulin Resistance). Exogenous insulin may induce production of IAs, but IAs may also be detectable in insulin-naïve patients[(2)].

Although in recent years the titers and prevalence of insulin antibodies in insulin-treated patients have decreased due to the availability of highly purified insulins and the use of human insulin, many patients still produce levels of insulin antibodies that may be clinically significant with respect to optimization of glycemic control due to alterations in insulin pharmacokinetics[(9)].

This study was performed to evaluate the prevalence of insulin antibodies in type 2 diabetic patients in Benghazi. Those treated with exogenous insulin and those treated with oral hypoglycemic drugs, in order to determine effect of insulin and oral hypoglycemic drugs on induction of IAs, and to detect any association with other factors related to the patient (sex, age, weight,diet control,FPG,PLPG) and production of IAs, and to evaluate the effect of IAs on glycemic control of the patients and on laboratory parameters (RFT, LFT, lipid profile, HbA1c and urine microalbumine).

In this study two groups of type 2 diabetic patients were evaluated :
Group (A) includes 200 patients, group (B) includes 95 patients.

Group (A):

In the present study, the overall IAs seroprevalence for group (A) was 11% among type 2 diabetic patients. This percentage is clinically significant and it was not reported before in Benghazi.

Concerning factors that may play role in appearance of IAs, the present study revealed that prevalence of IAs were more prevalent among males. This result is very important as insulin autoantibodies appears in type 1 diabetes mellitus that is more common in females.

The present study revealed that no relationship between increasing age and appearance of IAs and were different to studies of Fineberg et al.which proved that development of significant levels of antibodies to exogenous insulin has been shown to be inversely related to age (Immunological competence declines as an individual ages)[40,66,81].

Reducing weight is an important measure in reducing insulin resistance. The present study showed no association between IAs prevalence and weight of the patients.

Regular exercise improves the body's sensitivity to insulin by increasing the muscles' uptake of glucose from the bloodstream, by increasing the efficiency of the circulatory system and glucose transport, and by reducing the amount of fat around the patient's abdomen. The present study revealed that prevalence of IAs increases in nonactive patients (do not attend regular exercise). This result reflect the importance of exercise in controlling high blood glucose and reducing incidence of IAs production.

Metformin is a drug known to decrease insulin resistance by increasing body sensitivity to insulin. The present study showed no association between IAs prevalence and metformin intake.

The present study clearly showed that IAs are prevalent among insulin users rather than OHD users. statistical differences in seropositivity in relation to insulin use were significant. This result is anticipated because exogenous insulin is the antigen that inhance immune system to produce IAs.

Regarding the group used insulin, all patients used same insulin (recombinant human insulin) and the same route of delivery (subcutaneous injection). seroprevalence was of 10 %. These results were comparable to these represented by Schernthaner et al [9] who found IAs of the IgG class in 14% of patients receiving human insulins. However our results were lower than 44% which represented by Fineberg etal [11], and further lower than 89% represented Takeyuki et al [12], this large difference is probably due to use of highly sensitive sandwich enzyme immunoassay technique.

Researchers have postulated that IAs can be associated with mild degrees of insulin resistance, which would be detected clinically as mild to moderate increases in insulin dose requirements. Additionally, some studies have suggested that increasing insulin dose requirements correlate with IA levels [195-197]; however, most studies have shown no relationship or correlations between IAs and increasing dose requirements [198-202]. The present study showed no effect of insulin dose on prevalence of IAs. Evidence suggests that IA-positive patients who switch insulins to a less immunogenic preparation can experience reduced dose requirements in concert with declining IA levels [203,204], or that switching insulin preparations can result in declining IA levels without decreased insulin dose requirements [205-209].

The duration of insulin therapy before the onset of severe insulin resistance has been reported to range from 1 month to 15 yr [161,163] although 50–85% of patients with insulin resistance received insulin for less than 1 yr, and 10–25% received insulin for less than 1 month before the onset of severe insulin resistance [161,163]. The present study showed more prevalence of IAs with chronic use of insulin.

Regarding the second group (Oral hypoglycemic drugs) the present study showed that IAs can be elicited even in non insulin user diabetic patients. Among 60 patients only 2 patients were seropositive for IAs (3.3 % prevalence).This suggests that under certain circumstances, immune tolerance to insulin can be overcome.

The present study showed obvious effect of IAs on post lunch plasma glucose levels, these results are comparable to study represented by Francis etal and Van Haeften etal who suggested that IAs can be associated with relative hyperglycemia after meals [152,154].

Group (B):

In the present study, the overall IAs seroprevalence for group (B) was 26.3% among type 2 diabetic patients. This result differ from that of group (A) due to difference of sensitivity test of ELISA kit used and size of sample subjected to study.

Concerning factors that may play role in appearance of IAs, the present study revealed that IAs were more prevalent among males, inactive individuals and with chronic use of insulin treatment. This result coincided with that of group (A). However, prevalence of IAs were more prevalent by aging, increase weight, uncontrolling diet, untaking metformin and increasing dose of insulin, which differ from that of group (A) due to difference of sensitivity test of ELISA kit used and size of sample subjected to study.

A link between circulating anti-insulin antibodies and diabetic glomerulopathy has been suggested. The present study showed no influence of IAs on urine microalbumin. This result was different from study represented by Brun et al [296] who confirmed preliminary reports indicating a statistical relationship between anti-insulin antibodies and microalbuminuria [296].

CHAPTER 6

CONCLUSION

Conclusion

1. IAs are significantly prevalent in type 2 diabetic patients attending BDC.

2. IAs may be detectable in insulin-naïve diabetic patients.

3. IAs were more prevalent in males, inactive subjects, and with chronic use of insulin treatment.

4. Prevalence of IAs and its correlation with patient's age, weight, diet control, metformin intake and insulin dose was affected by difference in sensitivity test of ELISA used and by size of sample subjected to the study.

5. IAs obviously correlated with high blood levels of HbA1c and lipid profile.

CHAPTER 7

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Appendix

Proforma

Personal details:

Case number: ….. file number: …..

Name: ….. age: …..

Gender: ….. weight: …..

Medical history:

Type of treatment: ….. metformin intake: …..

Duration of treatment: ….. dose: …..

Diet control: ….. regular exercise: …..

الملخص العربى

المقدمة: النوع الثانى من داء السكرى يتميز بنقصان الانسولين أو زيادة مقاومته. الاجسام المضادة للأنسولين هى أحد اسباب مقاومة الانسولين و التى يمكنها التأثير على ضبط سكر الدم للمرضى.

الأهداف: هذه الدراسة أجريت لتحديد معدل انتشار الاجسام المضادة للأنسولين بين مرضى السكرى من النوع الثانى فى مركز بنغازى للسكرى و تقييم تأثير استعمال الانسولين و خافضات السكر الفموية على انتاج الاجسام المضادة للأنسولين و ربط البيانات الطبية للمرضى مع النتائج المعملية و لتقدير مدى تأثير الاجسام المضادة للأنسولين على ضبط سكر الدم للمرضى.

مواد و طرق البحث: أجريت الدراسة على مجموعتين من المرضى، المجموعة (ا) أجريت على 200 مريض سكرى متابع لمركز بنغازى للسكرى (140 يعالجون بالأنسولين و 60 يعالجون بخافضات السكر الفموية). أخذت من المرضى البيانات الطبية و سحبت منهم عينات الدم و تم حساب معدلات انتشار الاجسام المضادة للأنسولين فى الامصال نوعاً و كماً بواسطة إختبار اليزا (طاقم ادوات د.ر.ج)، قيس معدل السكر فى الدم مرتين (صائم و بعد الغداء). المجموعة (ب) أجريت على 95 مريض سكرى متابع لمركز بنغازى للسكرى كلهم يعالجون بالأنسولين أخذت من المرضى البيانات الطبية و سحبت منهم عينات الدم و تم حساب معدلات انتشار الاجسام المضادة للأنسولين فى الامصال نوعاً و كماً بواسطة إختبار اليزا (طاقم ادوات ميديبان)، قيست مستويات وظائف الكلى و الكبد والدهون و الانسولين و السكر التراكمى بالدم بالاضافة الى مستوى الزلال الصغير بالبول. و قد تم التحليل الاحصائى باستخدام (student T test).

النتائج: معدل الانتشار الكلى للمجموعة (ا) كان 11% (10% بين مستخدمى الانسولين و 3.3% بين مستخدمى خافضات السكر الفموية)، معيار الاجسام المضادة للأنسولين (10.11-71.05 و/مل). لم تظهر النتائج أى تأثير لعمر المريض، الوزن، تنظيم الغذاء، تناول عقار الميتفورمين، أو جرعة الأنسولين على معدل انتشار الاجسام المضادة للأنسولين، فى حين أن الاجسام المضادة للأنسولين كانت منتشرة بين الذكور و الغير ممارسى الرياضة، مستخدمى الانسولين، و مع طول مدة العلاج بالانسولين. أظهرت النتائج تأثير هام للأجسام المضادة للأنسولين على ضبط سكر الدم. معدل الانتشار الكلى للمجموعة (ب) كان26.3 %، معيار الاجسام المضادة للأنسولين (3.1-60.5 و/مل). .قد أظهرت النتائج وجود تأثير لزيادة عمر المريض، زيادة الوزن، عدم تنظيم الغذاء، عدم ممارسة الرياضة، عدم تناول عقار الميتفورمين،

زيادة الجرعة و طول مدة العلاج بالانسولين على معدل انتشار الاجسام المضادة للانسولين. لم تظهر النتائج أى تأثير للأجسام المضادة للانسولين على مستويات وظائف الكلى و الكبد و الانسولين بالدم و الزلال الصغير بالبول، فى حين أظهرت النتائج وجود تأثير للاجسام المضادة للانسولين على مستويات الدهون و السكر التراكمى بالدم.

الاستنتاج: الأجسام المضادة للأنسولين منتشرة بين مرضى السكرى من النوع الثانى المتابعين لمركز بنغازى للسكرى. الاجسام المضادة للأنسولين تم كشفها فى المرضى الغير مستخدمى الانسولين. الاجسام المضادة للانسولين أكثر انتشارا بين الذكور و الغير ممارسى الرياضة و مع طول مدة العلاج بالانسولين. معدل اننتشار الاجسام المضادة للانسولين و ارتباطة بعمر المريض و الوزن و تنظيم الغذاء و تناول عقار الميتفورمين وجرعة الأنسولين تأثرت باختلاف حساسية إختبارالأليزا المستخدم و حجم العينة الخاضعة للدراسة. يوجد تـأثير هام للاجسام المضادة للانسولين على ارتفاع مستويات الدهون و السكر التراكمى بالدم.

جامعة بنغازي

كلية الطب

قسم الجراثيم والطفيليات

وزارة التعليم

بنغازي – ليبيا

إدارة الدراسات العليا

معدل انتشار الاجسام المضادة للأنسولين بين مرضى السكرى من النوع الثانى فى بنغازى

مقدم من

حاتم عبد الجواد الزوى

تحت اشراف

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استاذ علم الأحياء الدقيقة

ا.د رجب الرعيض

استاذ مشارك علم الباطنة

رسالة لغرض استكمال متطلبات الحصول على درجة الاجازة العالية فى علم الاحياء الدقيقة

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