# Early Marker for Renal Impairment and Angiopathy in Diabetic Egyptian Children

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**Abstract:** Background: Since diabetes mellitus is a significant risk factor for early onset of many complications (atherosclerotic vascular disease, coronary heart disease, retinopathy, nephropathy, and neuropathy), there is therefore an essential need to know and understand more about the early detection of these complications in order to develop effective prevention strategies.**Objective**: The current work was carried out on type 1 diabetic children to investigate the level of retinol-binding-protein (RBP4) and endothelin-1 (ET-1) as an early marker for renal impairment and angiopathy. **Methods**: Forty-eight children (7-15 years) with type diabetes, 18 children with micro-albuminuria ($MA^+$ group) and 30 children were normoalbuminuria ($MA^-$ group) and these two groups are compared with 24 apparently healthy non-diabetic children. A comparative study was performed for these groups as regarded to the levels of $RBP_4$, insulin, ET-1 and testosterone. **Results:** The high levels of serum and urine RBP4 in $MA^-$ (normoalbuminuria) type 1 diabetic children group, indicates that RBP4 could be an early marker for renal impairment even in the absence of renal impairment (MA). The significantly higher level of plasma ET-1 in $MA^-$ than in $MA^+$ diabetic group, may indicate that endothelial dysfunction, precedes the appearance of microabuminuria in type 1 diabetic patients, and could be used as an early marker for diabetic microangiopathy. Level of serum testosterone was significantly reduced in male diabetic children and showed direct correlation with age and insulin dose.**Conclusion:** (1) High levels of serum and urine RBP4 in $MA^+$ type 1 diabetic children groups, indicates that RBP4 (retinol binding protein) is an early marker for renal impairment even in the absence of MA; (2) The significantly higher level of plasma ET-1 (Endothelin-1) in $MA^+$ (normalalbuminurea) than in $MA^+$ (microalbuminurea) group, may indicate that endothelial dysfunction, precedes the appearance of microalbuminurea in type 1 diabetic patients, and could be used as an early marker for diabetic microangiopathy. In addition, no correlation was found between plasma ET-1 and both of serum insulin and insulin dose in the diabetic children; (3) Level of serum testosterone is reduced significantly in the diabetic children males and showed direct correlation with age and insulin dose.

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**Key words:** Diabetes children, angiopathy albuminuria

## 1. Introduction

Diabetes is one of the fastest growing disease in the world. The World Health Organization ***(WHO, 2008)*** estimates that more than 180 million people worldwide have diabetes. Type I diabetes mellitus is one of the most common chronic diseases in childhood, caused by insulin deficiency resulting from the destruction of insulin-producing pancreatic beta cells. Early functional and structural abnormalities may be present a few years after the onset of the disease in children and adolescents. Therefore, a regular screening for diabetic microvascular disease particularly retinopathy and nephropathy, early detection of diabetic microangiopathy and treatment of early signs of these complications have a vital role in prevention of blindness and end-stage renal failure in children and adolescents with diabetes ***(Chiarelli et al., 2002; Akbay et al.,2010).***

Children with diabetes mellitus type I (IDDM) that is not under strict control tend to develop hypercholesterolemia, micro and macroangiopathy and thus are at great risk of cardiovascular diseases.

Microalbuminurea (MA) is a predictor of future diabetic nephropathy and cardiovascular disease in both type 1 and type 2 diabetes. In type 1 diabetes urinary albumin excretion is an index of glomerular function whereas retinol binding protein "RBP" (RBP coded by the gene $RBP_4$) is an index of renal tubular function. The increased urinary excretion of $RBP_4$ in these patients suggests slightly impaired proximal tubular function in early stages of diabetic nephropathy.

A low level of testosterone might be related to the development of premature coronary artery disease in men. Increased testosterone levels in patients with diabetes could be related to increased risk for cardiovascular complication later in life ***(Meyer et al., 2000).***

Endothelin-1 (ET-1) has a role in type 1 and type 2 diabetes mellitus and the development of diabetic complications. As insulin stimulates endothelin-1 expression in a dose response relationship. Increased insulin exposure (hyperinsulinemia and hypertriglyceridemia in patients with diabetes may have long term effects on vascular wall structure through its stimulation of ET-1 expression ***(Sarafidis and Ruilope, 2006).***

**Aim of the Work:**

The present work was designed to evaluate and introduce more sensitive and specific biochemical early markers such as RBP4 (as an indicator of tubular disorder) and ET-1 (as a sensitive marker for angiopathy) in children with type 1 diabetes. In addition, testosterone was measured in these diabetic children to find out whether there is a correlation between daily insulin dose, duration of diabetes, metabolic control, age, and body mass index versus their levels.

## 2. Patients and Methods

**Subjects:**

Seventy-two children with age range of (7-17 years) were included in this study. They were 48 diabetic children with type 1 diabetes mellitus and 24 apparently healthy children as control (36 were males and 36 females). The duration of the illness varied from 1-10 years and their daily total insulin dose varied from 14-68 unit/day/ all patients were taken from those attending the diabetes program at the Diabetic Center, Kasr El-Eany Egypt. Medical history examination and measurement of weight and height for calculating the body mass index (BMI) were performed.

Under complete septic technique, 10 mL of blood were collected from each child and placed in three test tubes.

1st tube: contain EDTA for determination of glycohemoglobin ($HbA_{IC}$) in the whole blood at the same day ***(Abraham et al., 1978).***

2nd tube: contain EDTA and immediately immersed in ice then centrifuged within one hour in a cooling centrifuge at 0°C the plasma were stored at -20°C for later determination of endothelin-1 (ET-1) with ELISA Kits according to ***Tiyssen (1985).***

3rd tube: the collected blood allowed to clot naturally, then centrifuged at 4000 r.p.m. for 15 minutes to separate the serum. The fresh serum used for the estimation of the glucose concentrations, lipid profile (Total cholesterol, triglyceride and total lipid), kidney function (urea and creatinine), liver function (AST and ALT), insulin, testosterone, sex hormone binding globulin (SHBG) and retinol-binding protein (RBP4).

**Methods:**

Using laboratory and enzyme immunoassay ELISA Kits.

Part of the fresh serum was used for estimation of the glucose concentration the other part was stored at -20°C for further analysis of lipid profile total lipid, total cholesterol and triglyceride using the enzymatic colorimetric method described by ***Fossati and Prencipe (1982)*** kidney function (urea and creatinine) were performed according to ***Fabiny and Eringhausen (1971)***, liver function (AST and ALT) were determined according to the colorimetric method described by ***Reitman and Frankel (1957).***

**Determination of total serum bilirubin by Randox Laboratory Kit**

Insulin, testosterone, sex-hormone binding globulin (SHBG) and retinol binding protein (RBP4) by enzyme immunoassay (ELISA) Kits

In addition urine was collected from each child, part of it was stored at -20°C for later measurement of microalbuminurea (MA), urea and creatinine. The other part was adjusted to a pH (6-8) with NaOH then stored at -20°C for later urine $RBP_4$ measurement. According to the values of microalbuminurea, the diabetic children were classified into two groups.

Positive microalbuminurea children: $MA^+$ (+ve MA) group.

Negative microalbuminurea children: $MA^-$ (-ve MA) group.

**Statistical Analysis:**

The data obtained in the present work were analyzed IBM compatible computer. Quantitatives variables were expressed by mean $\pm$ standard error (S.E.). In all tests, P.value was considered significant at level less than 0.05 ($P<0.05$).

## 3. Results

The current study was carried out on "48" randomized type 1 diabetic children which were divided into two groups:

Normoalbuminurea with negative microalbuminurea: $MA^-$ group.

Microalbuminurea with positive microalbuminurea: $MA^+$ group.

Comparative data of the diabetics children with the data of apparently healthy children are give in (Tables 1-8), and correlation coefficient are shown in the graves.

**Table (1): Comparison of the mean values of the parameters of children with type 1 diabetes ($MA^-$ and $MA^+$) and control group**

| Groups / Parameters | Control children (n= 24) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 30) | $MA^+$ (n = 18) |
| **Age** (y) | **A** 12.21 ± 0.37 | **A** 11.83 ± 0.30 | **A** 12.22 ± 0.45 |
| **BMI** ($Kg/m^2$) | **A** 20.96 ± 0.57 | **B** 19.23 ± 0.66 | **B** 18.94 ± 0.38 |

**Table (2): Comparison of the mean values of duration of diabetes, insulin dose, serum glucose, and glycosylated hemoglobin ($HbA_1c$) in children with type 1 diabetes ($MA^-$ and $MA^+$) and apparent healthy control children**

| Groups / Parameter | Control children (n= 24) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 30) | $MA^+$ (n= 18) |
| **Duration of diabetes** (y) | – | **A** 3.20 ± 0.44 | **A** 3.31 ± 0.57 |
| **Insulin dose** (U/d) | – | **A** 38.40 ± 2.65 | **A** 36.28 ± 3.29 |
| **Glucose** (mg/dL) | **A** 87.67 ± 1.85 | **B** 216.93 ± 16.43 | **B** 179.50 ± 13.10 |
| **$HbA_1c$ %** | **A** 6.25 ± 0.26 | **B** 8.03 ± 1.05 | **B** 8.46 ± 0.74 |

**Table (3): Comparison of the mean values of lipid profile in children with type 1 diabetes ($MA^-$ and $MA^+$) and apparent healthy control children**

| Groups / Parameters | Control children (n=24) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 30) | $MA^+$ (n= 18) |
| **Cholesterol (CH)** (mg/dl) | **A** 171.00 ± 2.36 | **B** 191.43 ± 6.57 | **B** 188.50 ± 6.04 |
| **Triglycerides (T.G)** (mg/dl) | **A** 53.33 ± 1.84 | **B** 74.23 ± 4.30 | **B** 67.56 ± 3.80 |
| **Total lipids (T.L)** (mg/dl) | **A** 622.13 ± 10.85 | **A** 632.80 ± 23.33 | **A** 598.44 ± 20.77 |

n : number of children

All data are represented as mean ± S.E

Different letters at the same row mean significant difference ($p<0.05$) between groups at the level of (0.05), common letters mean non-significant difference ($p>0.05$).

**Table (4): Comparison of the mean values of serum aspartate aminotransferase (s.AST) and serum alanine aminotransferase (s.ALT) in children with type 1 diabetes ($MA^-$ and $MA^+$) and apparent healthy control children**

| Groups / Parameters | Control children (n = 24) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 30) | $MA^+$ (n= 18) |
| **s. AST** (u/ml) | **A** 27.42 ± 0.83 | **B** 35.70 ± 2.31 | **B** 41.39 ± 3.07 |
| **s. ALT** (u/ml) | **A** 8.46 ± 0.50 | **B** 12.53 ± 1.08 | **AB** 11.28 ± 1.02 |

**Table (5): Comparison of the mean values of kidney function tests in children with type 1 diabetes ($MA^-$ and $MA^+$) and apparent healthy control children.**

| Groups / Parameters | Control children (n= 24) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 30) | $MA^+$ (n= 18) |
| **S.urea** (mg/dL) | **A** 16.58 ± 0.65 | **B** 22.53 ± 1.13 | **B** 22.11± 1.32 |
| **U.urea** (g/L) | **A** 18.10 ± 0.73 | **B** 13.11 ± 1.18 | **B** 13.95 ± 1.04 |
| **S.creatinine** (mg/dl) | **A** 0.33 ± 0.02 | **B** 0.27 ± 0.02 | **B** 0.24 ± 0.02 |
| **U. creatinine** (g/L) | **A** 1.39 ± 0.08 | **B** 0.84±0.05 | **A** 1.27 ± 0.16 |
| **Microalbuminuria** (µg/ml) | **A** 15.38 ± 0.39 | **A** 13.93 ± 0.71 | **B** 67.83 ± 4.73 |

**Table (6): Comparison of the mean values of both serum and urine RBP4 in children with type 1 diabetes ($MA^-$ and $MA^+$) and apparent healthy control children**

| Groups / Parameters | Control children (n= 24) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 30) | $MA^+$ (n= 18) |
| **S.RBP4** (mg/L) | **A** 13.67 ± 0.50 | **B** 16.94 ± 0.60 | **C** 18.94 ± 0.62 |
| **U.RBP4** (mg/L) | **A** 0.016 ± 0.00 | **B** 0.020 ± 0.00 | **C** 0.022 ± 0.00 |

n: number of children

All data are represented as mean ± S.E

Different letters at the same row mean significant difference ($p<0.05$) between groups at the level of (0.05), Common letters mean non-significant difference ($p>0.05$).

**Table (7): Comparison of the mean values of serum insulin and plasma endothelin-1(ET-1) in children with type 1 diabetes ($MA^-$ and $MA^+$) and apparent healthy control children.**

| Groups / parameters | Control children (n= 24) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 30) | $MA^+$ (n= 18) |
| **Insulin** (µIU/ml) | **A** 44.50 ± 1.39 | **B** 34.10 ± 2.57 | **AB** 37.94 ± 2.934 |
| **ET-I** (Fmol/ml) | **AB** 0.79 ± 0.04 | **A** 0.99 ± 0.09 | **B** 0.66 ± 0.11 |

**Table (8): Comparison of the mean values of serum testosterone and sex hormone binding globulin (SHBG) in both males and females children with type 1 diabetes ($MA^-$ and $MA^+$) and apparent healthy control children.**

| Groups / Parameters | Control children (n= 12) | Diabetic children | |
|---|---|---|---|
| | | $MA^-$ (n= 15) | $MA^+$ (n= 9) |
| **Testosterone in males** (ng/ml) | **A** 1.49 ± 0.06 | **B** 0.26 ± 0.08 | **B** 0.05 ± 0.01 |
| **Testosterone in females** (ng/ml) | **A** 0.09 ± 0.02 | **A** 0.15 ± 0.04 | **A** 0.16 ± 0.04 |
| **SHBG in males** (nmol/l) | **A** 27.67 ± 1.04 | **B** 39.93 ± 3.13 | **AB** 35.22 ± 1.42 |
| **SHBG in females** (nmol/l) | **A** 22.92 ± 1.20 | **B** 48.80 ± 2.25 | **C** 32.33 ± 1.73 |

n : number of children

All data are represented as mean ± S.E

Different letters at the same row mean significant difference ($p<0.05$) between groups at the level of (0.05), common letters mean non-significant difference ($p>0.05$).

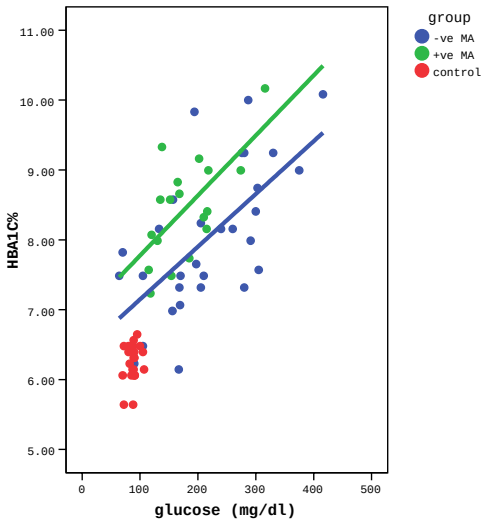


**Figure (1-a)**: Direct correlation between $HbA_1c$ value and serum glucose in $MA^-$ group (r=0.61, P<0.01), and $MA^+$ group (r= 0.57, P<0.05).

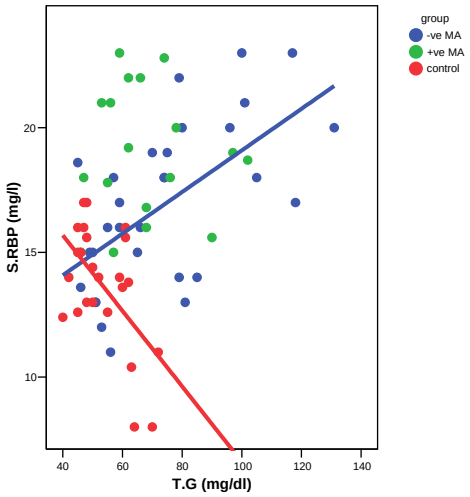


**Figure (1-a)**: Direct correlation between $HbA_1c$ value and ı glucose in $MA^-$ group (r=0.61, P<0.01), and $MA^+$ group (r= 0.57, P<0.05).

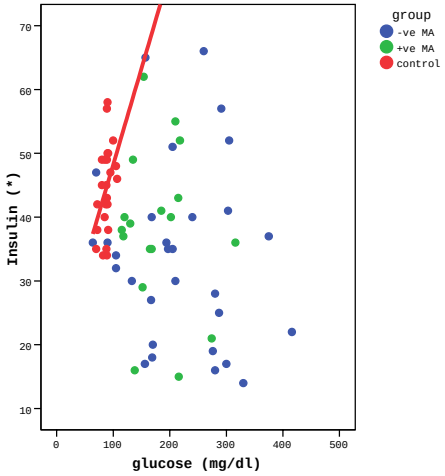


**Figure (2-a)**: Direct correlation between serum insulin levels and serum glucose in healthy control group (r=0.45, P<0.05).

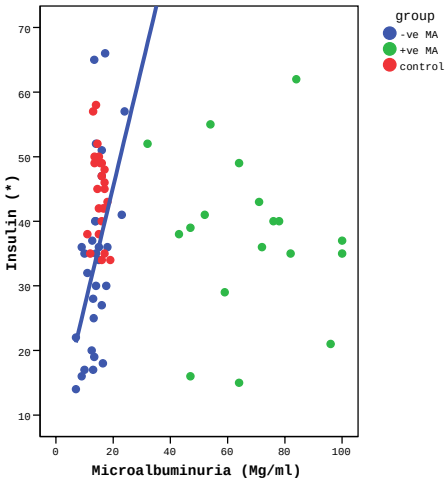


**Figure (2-b)**: Direct correlation between serum insulin levels and microalbuminuria in $MA^-$ diabetic group (r=0.53, P<0.01).

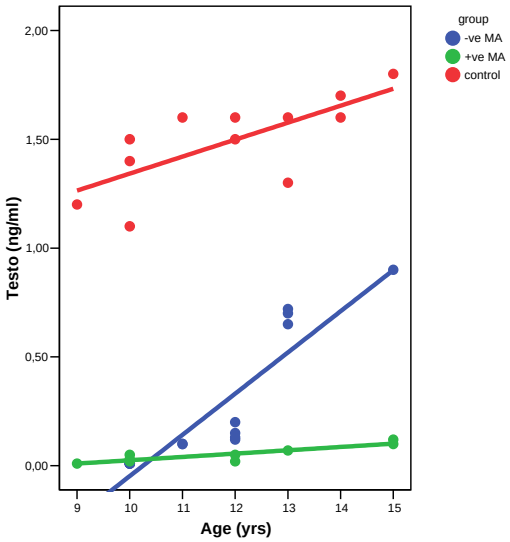


**Figure (3-a)**: Direct correlation between serum testosterone (Testo.) and age in male control, $MA^-$, and $MA^+$ groups (r=0.73, P<0.01; r=0.91<0.01 and r= 0.89, P<0.01 respectively)

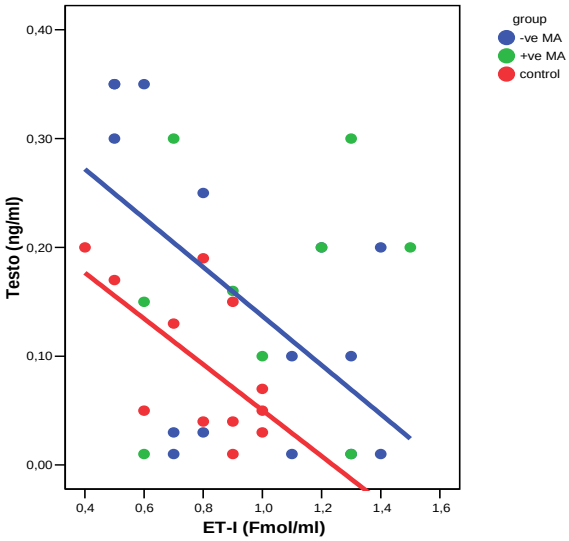


**Figure (3-b)**: Inverse correlation between serum testosterone (Testo.) and plasma ET-1 in control and $MA^-$ females groups (r= -0.62, P<0.05 and r=-0.56, P<0.05 respectively).

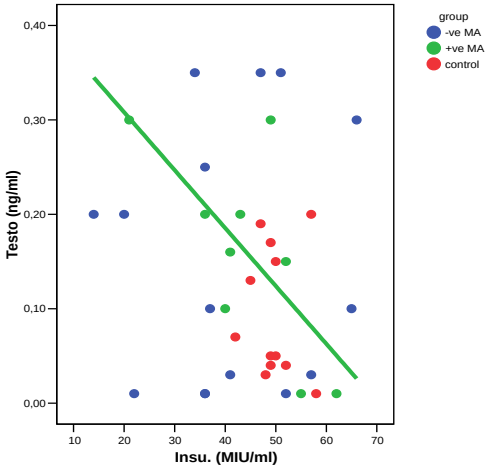


**Figure (4-a)**: Inverse correlation between serum testosterone (Testo.) and serum insulin level in $MA^+$ diabetic female group (r=-0.69, P<0.05)

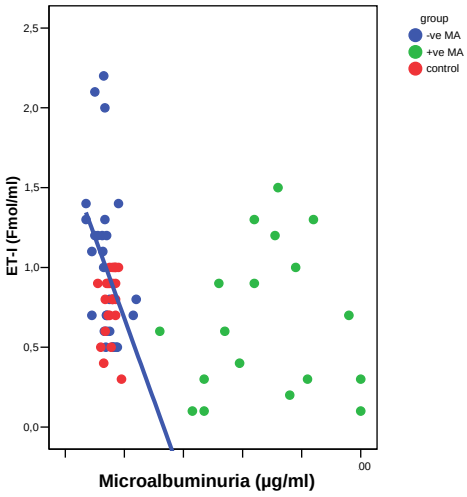


**Figure (4-b)**: Inverse correlation between ET-1 and microalbuminuria in $MA^-$ group (r=-0.41, P< 0.05).

**4. Discussion**

Diabetes is usually accompanied by an increased production of reactive oxygen species and free radicals, or by impaired antioxidant defenses *(Maritim et al., 2003),* which is widely accepted as important in the development and progression of diabetic complications. Research to help counteract these outcomes in diabetic patients has resulted in a number of new and advanced therapies, including novel antidiabetic medications, surgical interventions, islet cell transplantation, hematopoietic stem cell transplantation and gene therapy.

The purpose of this study has been to estimate the effect of diabetes on serum and urine RBP4 as a marker of nephropathy *(Pontuch et al., 1995; Turuner et al., 2011)* plasma ET-1 as a marker of angiopathy *(Schalkwijk and Stehouwer, 2005),* and to investigate the relationship between insulin dose and androgen in type 1 diabetic children. Results of the present work have shown a significant decrease in the body mass index (BMI) of children with diabetes whether $MA^-$ or $MA^+$ compared to their control. A higher BMI was associated with a younger age (0-4.9 year) at diabetes onset and it gradually decreased with increasing age.

Chronic hyperglycemia during diabetes is known to cause glycation of body proteins that in turn leads to secondary complications affecting eyes, kidney, nerves, and arteries. Long-term hyperglycemia, as measured by $HbA_1c$ is further related to cardiovascular mortality in men. Evidence from the current studies has indicated that in both group of type 1 diabetic children ($MA^-$ and $MA^+$) there was a significant increase in the level of serum glucose and $HbA_1c$ values compared to the control. A significant increase in $HbA_{1C}$ concentration was detected in all diabetic patients, which may indicate an increased familial risk of diabetic microvascular disease.

In the present work $HbA_1c$ value was slightly higher in $MA^+$ children diabetic group than in $MA^-$ ones. Improvement of glycemic control declines the incidence of diabetic nephropathy *(Nordwall et al., 2004)* as it reduces hyperfiltration and diminishes albumin excretion rate.

A positive correlation between $HbA_1c$ and serum glucose level has been detected in diabetic children ($MA^-$ and $MA^+$). This finding is supported by many earlier studies.

The dyslipidemia of diabetes is associated with the increased cardiovascular disease found in type "1" diabetes. The similarity between the increased serum cholesterol and triglyceride levels in the current work is a proof that lipid abnormalities remain common in children and adolescents with type 1 diabetes. Abnormally high level of serum lipids is mainly due to the uninhibited action of lipolytic hormones on the fat depots, brought about by the action of insulin. Under normal circumstances, insulin activates the enzyme lipoprotein lipase, which hydrolyses triglycerides. However, in the diabetic state, lipoprotein lipase is not activated due to insulin deficiency, resulting in hypertriglyceridemia, and insulin deficiency is also associated with hypercholesterolemia due to metabolic abnormalities.

Measurement of enzyme activity in serum is of important value because it helps to assess the state of the liver and other organs. Normally serum ALT and AST levels are low, but these enzymes are released into circulation after cellular damage and increased because they are cytoplasmic in location. The liver and heart release Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT), and an elevation in their plasma concentrations are an indicator of liver and heart damage. Our results have shown significant increase in the activity of serum AST and ALT in the diabetic groups as compared to the control one.

Assessment of kidney functions in both children and adolescents with type "1" diabetes is necessary for the early detection of renal lesions and for the application of an adequate therapy. *Amann et al. (2006)* described the potential relation between change in kidney function and the potential consequences for the cardiovascular system as MA reflects impaired vascular function in general and is associated with a higher susceptibility to cardiovascular and renal events. Our results as well as those of former studies have shown that the level of MA was significantly higher in the diabetic children. The present study demonstrates that out of 48 type 1 diabetic children, 30 patients (~62%) had normoalbuminuria ($MA^-$) and 18 (~38%) had microalbuminuria ($MA^+$). Subjects diagnosed with type "1" diabetes before or during puberty are at a higher risk of nephropathy when compared with subjects diagnosed after puberty.

Usually the definition of renal impairment relies upon the serum creatinine and urea levels, or creatinine clearances, which, however, are not sufficient to demonstrate initial renal damage as they are insensitive, nonspecific, and change significantly only after significant kidney injury *(Vaidya et al., 2008).* For that, high and low-molecular weight urinary proteins are good indices of glomerular or tubular function. From these proteins, retinol-binding protein (RBP4), that is a good indicator of initial renal damage, even in the absence of alterations of the routine parameters.

The increase in the urinary excretion of RBP4 in children is highly specific for tubular disease, which occurs earlier than glomerular (albumin) affection, as urinary RBP4 excretion is increased in early diabetic nephropathy and might even be a marker of early renal damage preceding microalbuminuria *(Ziegelmeier et al., 2007; Axelsson et al., 2009).* The results of the present

study have shown a significant increase in the level of both serum and urine RBP4 in the diabetic children ($MA^-$ and $MA^+$). Moreover a significant increase in its level was noticed in the $MA^+$ group compared to the $MA^-$ one. These results are in line with many previous reports, which proved that increased concentrations of RBP4 both in serum and urine have been found in children in the absence of either MA or impaired renal function as assessed by serum creatinine.

Additionally, the absence of correlation between RBP4 and MA in our study may be explained on the basis that RBP4 is not usually correlated with the severity of the disease, but seems to be helpful in identifying a subset of patients with initial renal disorder *(Corso et al., 1999; Henze et al., 2010).* Supporting this concept, the present study indicated that serum RBP4 is the only reliable test for prediction of early renal impairment in the form of microalbuminuria. Moreover, the elevated concentrations of RBP4 in serum reflect that the kidney is the main site for RBP4 catabolism. Owing to its small size, RBP4 filtered at the glomeruli and both reabsorbed and metabolized in the proximal tubule.

As expected, our results showed that serum insulin level was lower in diabetic children as compared with control group. In the $MA^+$ diabetic group, serum insulin level was slightly higher, than in $MA^-$ diabetic ones, and it correlates well with MA in the latter group ($MA^-$).

Children and adolescents with type 1 diabetes mellitus are prone to experience a delay in the onset of pubertal process *(Meyer et al., 2000).* In agreement with this concept, our study showed that serum testosterone level was significantly lower in male diabetic children as compared with the control. Among females, serum testosterone was slightly higher in those with diabetes than in nondiabetic siblings. This result is in line with *Meyer et al. (2000)* who reported that higher serum total and free testosterone were found in females with type I diabetes than in control.

According to the present study, SHBG was significantly higher in the diabetic children comparing with the control. This agrees with *Danielson et al. (2008)* who reported that type 1 diabetes is associated with elevated SHBG concentrations. Serum level of SHBG in the current study was significantly lower in $MA^+$ group as compared with $MA^-$ diabetic females. This result is in accordance with *Rudberg and Persson (1995)* who reported that a hyperandrogenism and low SHBG level in female type 1 diabetic patients has been linked to MA risk.

Male serum testosterone in the current study showed a direct correlation with insulin dose in $MA^-$ diabetic group and not in the $MA^+$ ones. Previously, *Meyer et al. (2000)* found no correlation between daily insulin requirements and serum androgen levels in adolescents with type 1 diabetes. Also male serum testosterone in the present study correlates directly with age in both diabetic and control groups. The present study showed that serum testosterone also correlates positively with BMI in the $MA^+$ diabetic male group.

Reports on plasma ET-1 (Endothelin-1) levels in patients with diabetes mellitus are conflicting because increased, unchanged or decreased *(Smulders et al., 1994)* levels have been reported previously.

In the present study, plasma ET-1 level was insignificantly higher in the diabetic children as compared with the control. Moreover, plasma ET-1 level was significantly higher in $MA^-$ comparing to the $MA^+$. *Stehouwer et al. (1995) and Maier et al. (2009)* reported that endothelial dysfunction precedes the appearance of MA by about 3 years in IDDM patients. Therefore, circulating ET-1 levels could represent an indicator of early diabetes-related renal damage.

The present study recorded also a direct correlation between plasma ET-1 and triglycerides in the diabetic children with $MA^+$.

Insulin resistance has been widely accepted as risk factor for cardiovascular disease, where insulin could promote atherogenesis by direct action on the arterial wall and the enhanced formation of superoxide anion $O_2$ leading to an impaired endothelium dependent arterial relaxation *(Shinozaki et al., 2004).*

Consequently, the present study confirmed that the plasma ET-1 (Endothelin-1) might be considered as an early marker for angiopathy in diabetes (type 1).

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