National and Kapodistrian University of Athens

Medical School of the University of Athens

First Department of Pediatrics

Professor and Chairman: George P. Chrousos

DOCTORAL THESIS

"Early Detection of Renal Dysfunction in Pediatric Patients and Young Adults with Type 1 Diabetes"

Nektaria Papadopoulou, MD Endocrinologist

ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

ΙΑΤΡΙΚΗ ΣΧΟΛΗ

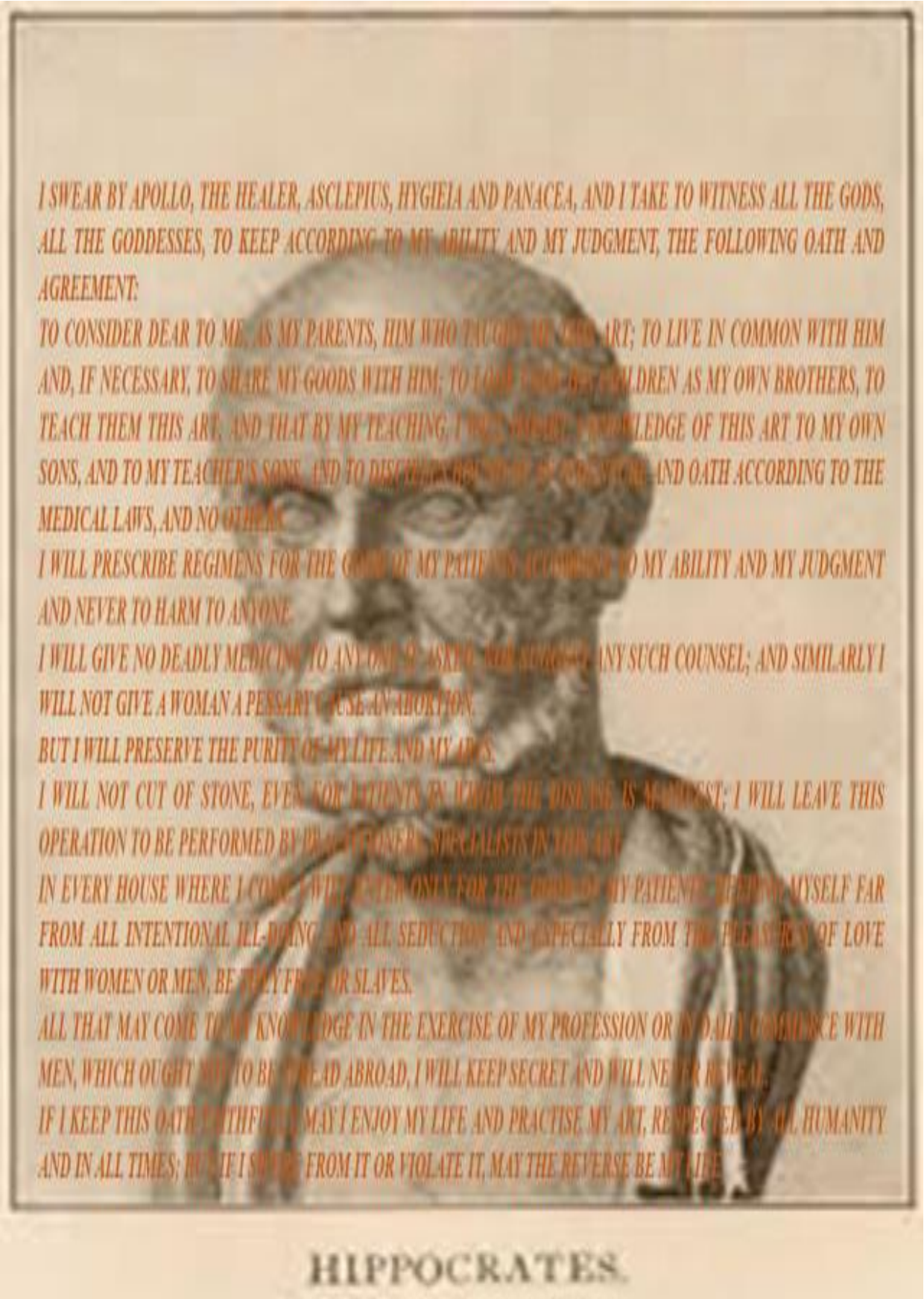
Α΄ ΠΑΙΔΙΑΤΡΙΚΗ ΚΛΙΝΙΚΗ ΠΑΝΕΠΙΣΤΗΜΙΟΥ ΑΘΗΝΩΝ

ΔΙΕΥΘΥΝΤΗΣ-ΚΑΘΗΓΗΤΗΣ: Γ.Π. ΧΡΟΥΣΟΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

«Πρώϊμη ανίχνευση διαταραχών νεφρικής λειτουργίας σε παιδιατρικούς και νεαρούς ασθενείς με Σακχαρώδη Διαβήτη τύπου 1»

ΝΕΚΤΑΡΙΑ ΠΑΠΑΔΟΠΟΥΛΟΥ, ΙΑΤΡΟΣ ΕΝΔΟΚΡΙΝΟΛΟΓΟΣ


I SWEAR BY APOLLO, THE HEALER, ASCLEPIUS, HYGIEIA AND PANACEA, AND I TAKE TO WITNESS ALL THE GODS,
ALL THE GODDESSES, TO KEEP ACCORDING TO MY ABILITY AND MY JUDGMENT, THE FOLLOWING OATH AND
AGREEMENT:
TO CONSIDER DEAR TO ME, AS MY PARENTS, HIM WHO TAUGHT ME THIS ART; TO LIVE IN COMMON WITH HIM
AND, IF NECESSARY, TO SHARE MY GOODS WITH HIM; TO
TEACH THEM THIS ART, AND THAT BY MY TEACHING, I
SONS, AND TO MY TEACHER'S SONS, AND TO
MEDICAL LAWS, AND NO OTHERS.
I WILL PRESCRIBE REGIMENS FOR THE
AND NEVER TO HARM TO ANYONE.
I WILL GIVE NO DEADLY MEDICINE TO ANYONE
WILL NOT GIVE A WOMAN A PESSARY
BUT I WILL PRESERVE THE PURITY OF MY LIFE AND MY ARTS.
I WILL NOT CUT OF STONE, EVEN
OPERATION TO BE PERFORMED BY
IN EVERY HOUSE WHERE I
FROM ALL INTENTIONAL ILL-DOING AND ALL SEDUCTION AND ESPECIALLY FROM
WITH WOMEN OR MEN, BE THEY
ALL THAT MAY COME TO MY KNOWLEDGE IN THE EXERCISE OF MY PROFESSION OR
MEN, WHICH OUGHT NOT TO BE
IF I KEEP THIS OATH FAITHFUL
AND IN ALL TIMES; BUT IF I SWERVE FROM IT OR VIOLATE IT, MAY THE REVERSE BE MY LIFE.
HIPPOCRATES.

ΕΚ ΤΟΥ ΙΠΠΟΚΡΑΤΕΙΟΥ ΟΡΚΟΥ

ΚΕΙΜΕΝΟ

« ΟΜΝΥΜΙ ΤΟΝ ΘΕΟΝ ΕΠΙΤΕΛΕΑ ΠΟΙΗΣΕΙΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ ΟΡΚΟΝ ΤΟΝΔΕ ΚΑΙ ΞΥΓΓΡΑΦΗΝ ΤΗΝΔΕ. ΗΓΗΣΕΣΘΑΙ ΜΕΝ ΤΟΝ ΔΙΔΑΞΑΝΤΑ ΜΕ ΤΗΝ ΤΕΧΝΗΝ ΤΑΥΤΗΝ ΙΣΑ ΓΕΝΕΤΗΣΙΝ ΕΜΟΙΣΙ. ΔΙΑΙΤΗΜΑΣΙ ΤΕ ΧΡΗΣΟΜΑΙ ΕΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ, ΕΠΙ ΔΗΛΗΣΕΙ ΔΕ ΚΑΙ ΑΔΙΚΙΗ ΕΙΡΞΕΙΝ. ΟΥ ΔΩΣΩ ΔΕ ΟΥΔΕ ΦΑΡΜΑΚΟΝ ΟΥΔΕΝΙ ΑΙΤΗΘΕΙΣ ΘΑΝΑΣΙΜΟΝ. ΟΥΔΕ ΥΦΗΓΗΣΟΜΑΙ ΞΥΜΒΟΥΛΙΗΝ ΤΟΙΗΝΔΕ. ΟΜΟΙΩΣ ΔΕ ΟΥΔΕ ΓΥΝΑΙΚΙ ΠΕΣΣΟΝ ΦΘΟΡΙΟΝ ΔΩΣΩ. ΑΓΝΩΣ ΔΕ ΚΑΙ ΟΣΙΩΣ ΔΙΑΤΗΡΗΣΩ ΒΙΟΝ ΤΟΝ ΕΜΟΝ ΚΑΙ ΤΕΧΝΗΝ ΤΗΝ ΕΜΗΝ. ΕΣ ΟΙΚΙΑΣ ΔΕ ΟΚΟΣΑΣ ΑΝ ΕΣΙΩ, ΕΣΕΛΕΥΣΟΜΑΙ ΕΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ, ΕΚΤΟΣ ΕΩΝ ΠΑΣΗΣ ΑΔΙΚΙΗΣ ΕΚΟΥΣΙΗΣ ΚΑΙ ΦΘΟΡΙΗΣ ΤΗΣ ΤΕ ΑΛΛΗΣ ΚΑΙ ΑΦΡΟΔΙΣΙΩΝ ΕΡΓΩΝ. Α Δ' ΑΝ ΕΝ ΘΕΡΑΠΕΙΗ, Η ΙΔΩ Η ΑΚΟΥΣΩ, Η ΚΑΙ ΑΝΕΥ ΘΕΡΑΠΕΙΗΣ ΚΑΤΑ ΒΙΟΝ ΑΝΘΡΩΠΩΝ, Α ΜΗ ΧΡΗ ΠΟΤΕ ΕΚΛΑΛΕΕΣΘΑΙ ΕΞΩ, ΣΙΓΗΣΟΜΑΙ, ΑΡΡΗΤΑ ΗΓΕΥΜΕΝΟΣ ΕΙΝΑΙ ΤΑ ΤΟΙΑΥΤΑ. ΟΡΚΟΝ ΜΕΝ ΟΥΝ ΜΟΙ ΤΟΝΔΕ ΕΠΙΤΕΛΕΑ ΠΟΙΕΟΝΤΙ ΚΑΙ ΜΗ ΞΥΓΧΕΟΝΤΙ ΕΙΗ ΕΠΑΥΡΑΣΘΑΙ ΚΑΙ ΒΙΟΥ ΚΑΙ ΤΕΧΝΗΣ, ΔΟΞΑΖΟΜΕΝΩ ΠΑΡΑ ΠΑΣΙΝ ΑΝΘΡΩΠΟΙΣ ΕΣ ΤΟΝ ΑΙΕΙ ΧΡΟΝΟΝ· ΠΑΡΑΒΑΙΝΟΝΤΙ ΔΕ ΚΑΙ ΕΠΙΟΡΚΕΟΝΤΙ, ΤΑΝΑΝΤΙΑ ΤΟΥΤΕΩΝ. ΤΑΥΤΗΝ ΤΗΝ ΕΠΑΓΓΕΛΙΑΝ ΕΠΙΤΕΛΟΥΝΤΙ ΕΙΗ ΜΟΙ ΤΟΝ ΘΕΟΝ ΑΡΩΓΟΝ ΚΤΗΣΑΣΘΑΙ ΕΝ ΤΩ ΒΙΩ »

ΙΠΠΟΚΡΑΤΗΣ

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The thesis approval from the Medical School of the University of Athens does not constitute acceptance of the opinions of the author (Organization University of Athens, Article 202 of Law 5343)

Αφιερώνεται με αγάπη και εκτίμηση:

Στους γονείς μου, που με αγάπη και θυσίες με έμαθαν να ελπίζω και να κυνηγώ τα όνειρα μου

Στο σύζυγο μου Νίκο, για την ανεκτίμητη υποστήριξη και τις πολύτιμες συμβουλές σχετικά με τη συγγραφή της διατριβής

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Introduction

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GENERAL PART

Introduction

1 Type 1 Diabetes Mellitus

Type 1 Diabetes (T1D), previously encompassed by the terms insulin-dependent diabetes or juvenile-onset diabetes, the most common metabolic disease of the childhood, is a catabolic multisystem disorder, characterized by both biochemical and structural consequences. It is correlated with a chronic and progressive dysfunction of carbohydrate, fat, and protein metabolism caused by relative or absolute circulating insulin deficiency, which results from the marked and progressive inability of the pancreas to secrete insulin in response to all insulin-secretory stimuli.

T1D is due to the progressive autoimmune attack and destruction of pancreatic beta cells which are located in the pancreas in clusters known as the islets of Langerhans.

1.1 Historical review of type 1 Diabetes Mellitus

According to ancient Egyptian Ebers papyrus, Hesy-Ra, an Egyptian physician, the history of diabetes started in approximately 1550BC, where it is referred that an uncommon disease causes the patient to present polyuria and dramatic weight loss. Greek Physician Aretaeus of Cappadocia, gave ‘’diabetes’’ its name, meaning a ‘’flowing through’’ while recording the main symptoms, consisting of polyuria, polydipsia and loss of weight. Galinus, noted the disease and its manifestations as well. In 1675 the word "mellitus," meaning honey, was added to the name "diabetes". It wasn't until the 1800s that chemical tests to detect the presence of sugar in the urine were found. In the 1700s and 1800s, physicians began to realize that dietary changes could help manage diabetes, and only in the late 19th century started a promising research for the treatment with insulin by Oskar Minkowski and Joseph von Mering, at the University of Strasbourg in France. Finally, a new age in the history of diabetes

started in 1923 when Canadian physicians Frederick Banting and John Macleod were awarded the Nobel Prize for their revolutionary discovery of insulin [1].

Picture 1: Aretaeus of Cappadocia

1.2 Epidemiology of type 1 Diabetes Mellitus

1.2.1 Incidence and prevalence of type 1 diabetes mellitus

Worldwide, approximately 78.000 children are diagnosed with type 1 diabetes (T1D) annually. Incidence varies a lot among countries: East Asians and American Indians have the lowest incidence rates (0.1–8 per 100.000/year) as compared with the Finnish who have the highest rates (>64.2 per 100.000/year). In the U.S., the number of patients with type 1 diabetes was estimated to be 166.984 [2]. The increasing incidence of T1D throughout the world is especially marked in young children. Registries in Europe suggest that recent incidence rates of T1D were highest in the youngest age-group (0–4 years) [3]. Usually it can be diagnosed in children aged 4 years or older, fairly abruptly, with the peak incidence of onset at ages 11-13 years, coinciding with early adolescence and puberty. Incidence rates decline after puberty and appear to stabilize in young adulthood (15–29 years). The overall prevalence of type 1 diabetes in the U.S. is ∼0.3%, but if a first-degree relative has diabetes, the empiric risk of being affected is ∼5%, representing a 15-fold increase among family members. Studies evaluating children at risk for developing type 1 diabetes have shown that the presence of more than two autoantibodies was associated with a nearly 70% risk for disease development within 10 years and 84% within 15 years [2]. The incidence of T1D in adults is lower than in children, although approximately one fourth of persons with T1D are diagnosed in adulthood. Clinical presentation occurs at all

ages and as late as the 9th decade of life [4]. Although most common autoimmune diseases disproportionately affect females, on average girls and boys are equally affected with T1D in young populations. A distinctive pattern has been observed such that regions with a high incidence of T1D (populations of European origin) have a male excess, whereas regions with a low incidence (populations of non-European origin) report a female preponderance [5].

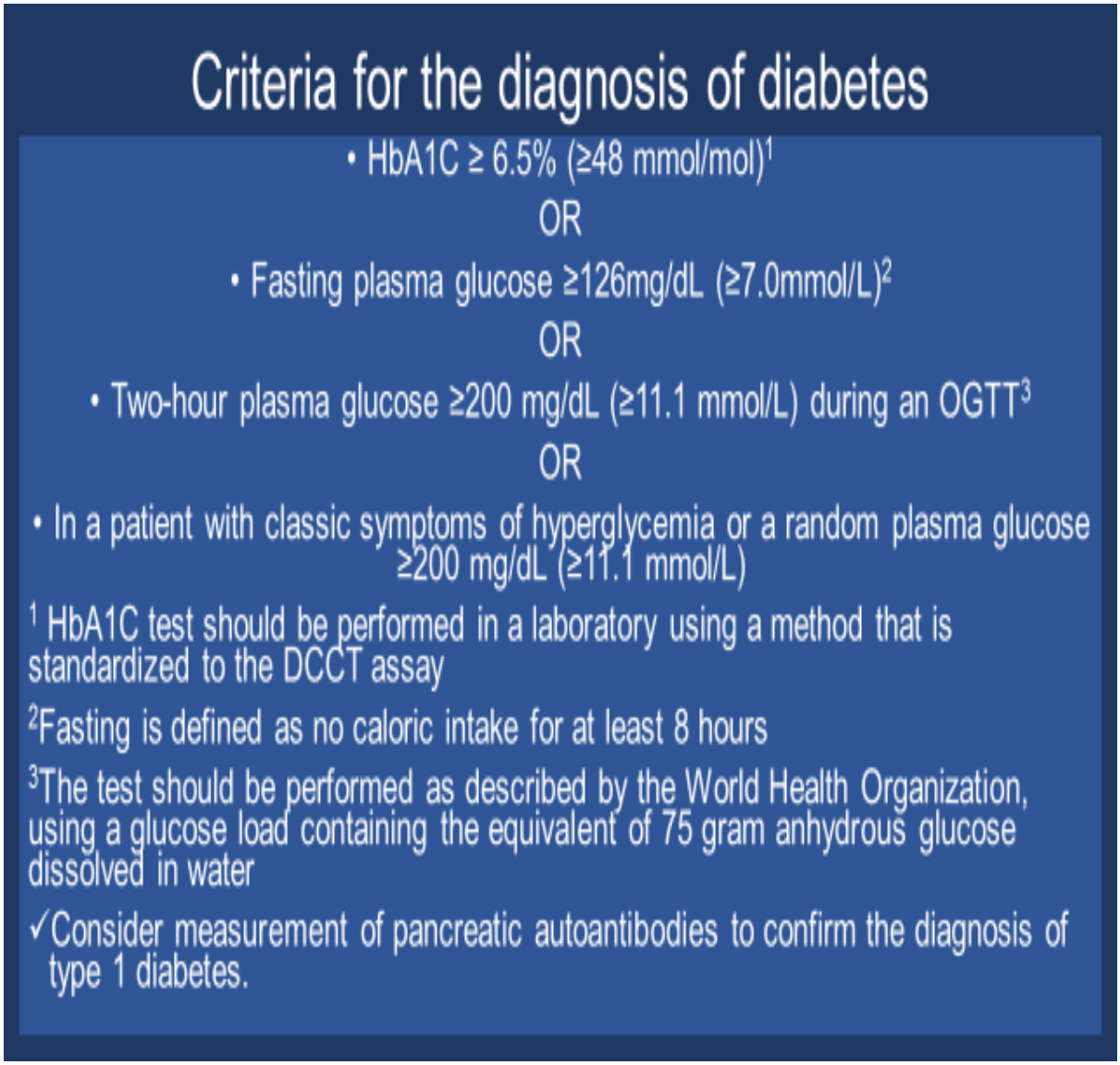
1.3 Diagnosis of type 1 Diabetes Mellitus

According to the American Diabetes Association position statement, the criteria for diagnosis of diabetes 1 are listed at table 1.

Individuals with T1D, usually have one or more positive autoantibodies when fasting hyperglycemia is initially detected. Markers of the autoimmune destruction of the β-cell include autoantibodies, to four islet antigen groups that have so far been identified: insulin or proinsulin (IAA), Glutamate decarboxylase or GAD65 or GAD67(GADA), Islet antigen-2 or ICA512 or IA-2β or PHOGRIN (IA-2) and islet-specific zinc transporter isoform 8 (ZnT8) [6].

One or more of these autoantibodies are present in 85–90% of individuals when fasting hyperglycemia is initially detected or when ketoacidosis presents as the first manifestation of the disease. Others, may have modest fasting hyperglycemia that can lead to severe hyperglycemia and/or ketoacidosis in the presence of infection or other stress. Still others, particularly adults, may retain residual b-cell function sufficient enough to prevent ketoacidosis for a long period; such individuals eventually become insulin dependent with a potential risk for ketoacidosis. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Distinguishing between type 1 and type 2 diabetes mellitus may be challenging. Traditionally, progressive β-cell destruction has been the hallmark of type 1 diabetes, but residual C-peptide may be detected over 40 years after initial diagnosis, regardless of whether the initial diagnosis was made in childhood or in adulthood [2].

Table 1: Criteria for diagnosis of type 1 diabetes mellitus. Adapted from Diabetes Care July 2014 vol. 37 no. 72034-2054 [2]



1.4 Pathogenesis of Type 1 Diabetes Mellitus

The pathophysiologic process behind the destruction of beta cells is mostly autoimmune-mediated while less often it results from idiopathic lesions or progressive failure of beta cells.

The autoimmunity of T1D is T cell–mediated specific for β-cell destruction and is often associated with devastating acute and chronic complications.

The main genetic determinants, responsible for 40% of the genetic susceptibility, map to the major histocompatibility complex (MHC), in particular DR and DQ of the multiple genes implicated in susceptibility of T1D. The most important is the human leukocyte antigen (HLA) complex on chromosome 6, in particular the HLA class II [7]. These HLA-DR/DQ alleles may appear either predisposing or protective [8]. A lot of research has focused on the identification of mediating transcriptional changes providing new hypotheses explaining T1D biology. A recent study suggested that the interferon (IFN) response and IFN response factors were identified as central mediators of the IFN-related transcriptional changes, detected already before the T1D-associated autoantibodies were found positive [9]. The genetic profile of T1D has been a great issue in the last decade. A lot of studies suggest the variety of genotypes in familial and non-familial diabetic patients [10]. It is also shown that the parent-offspring subgroup is characterized by male preponderance, at diagnosis parents were significantly older than their offspring and the descendants were significantly younger than their affected siblings [11]. The genetic contribution is suggested by the relatively high degree of familial clustering among patients with T1D: approximately 10–15% of T1D patients have affected first-degree relatives, either parents, offspring or siblings. The prevalence of T1D among first-degree relatives has been found to be approximately 5%, significantly higher than that in the general population (0.4%) and this risk can be further stratified on the basis of which affected family member has T1D (3%, 5%, and 8% if they have an affected mother, father, or sibling, respectively) [11,12]. T1D with onset before age 5 years is a marker of high familial risk and suggests a major role for genetic factors. The offspring of affected mothers have a 2% to 3% risk, whereas offspring of affected fathers have a 7% risk [12]. The predictive risk for a child who has no family history of T1D at birth increases by a factor of ten if his or her sibling develops

T1D, and if the child has a twin sibling who develops T1D, predictive risk will increase dramatically to around 50% [13]. In addition, several studies showed that type 1 and type 2 diabetes cluster in the same families suggesting a genetic interaction between type 1 and type 2 diabetes [14,15].

Apart from autoantibodies that have been acquired through placental transfer, islet autoantibodies rarely appear prior to age 6 months [16]. Exceptions include cases of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome where neonates can develop insulin or glutamic acid decarboxylase (GAD) antibodies in the first months of life [17].

Environmental factors may also contribute to the pathogenesis of T1D. Potential triggering factors for immunologically mediated destruction of the beta cells include viruses (for example mumps, rubella, coxsackie-virus B1 and B4), toxic chemicals, and exposure to cow's milk in infancy as well as cytotoxins [18]. A recent meta-analysis suggests a significant association between enterovirus infection and T1D [19]. As beta-cell mass declines with ongoing immunologic destruction, insulin secretion decreases until the available insulin is no longer adequate to maintain normal blood glucose levels. After 80-90% of the beta cells are destroyed, hyperglycemia develops and diabetes may be diagnosed. According to the fetal origin of adult diseases hypothesis [20], the intrauterine environment through developmental plasticity may permanently influence long-term health and disease. Therefore, intrauterine environment when exposed to viral and other potential immunomodulatory factors may influence the course of the fetus and the possible future appearance of T1D or other autoimmune disease.

1.5 Type 1 Diabetes Mellitus and clinical manifestations

There is variability in the initial presentation of type 1 diabetes in both children and adults. Children often present acutely, with severe symptoms of polyuria, polydipsia, and ketonemia. However, in adults, T1D appears with a more gradual onset, with a clinical presentation similar with type 2 diabetes. Regarding the clinical manifestations at onset of T1D, diabetic ketoacidosis (DKA) is the most severe. The EURODIAB study

reported on the frequency, severity, and geographical variation of DKA which ranged from 26 to 67% [21,22]. Polyuria has been reported as the most common presenting symptom, followed by weight loss and fatigue [21,23].

Patterns in the seasonality for both the month of birth and the month of diagnosis of T1D have been reported [24,25]. While the seasonality of T1D diagnosis seems intuitively obvious given the well-documented environmental role in T1D's pathogenesis, it is also hypothesized that the seasonal environment during intrauterine life and birth may have an influence on diabetes incidence later in life [25]. According to the SEARCH study, the percentage of observed to expected births differed across the months with a lower incidence of November-February births and a higher incidence in April–July births.

1.6 Comorbidity between type 1 Diabetes Mellitus and other autoimmune disorders

T1D patients are at higher risk to develop another T-cell mediated autoimmune disorder, like Celiac disease (CeD), Autoimmune Thyroid Disease (AITD) or Addison Disease (AD), with the AITD being the most prevalent one. The majority of T1D patients, who present another autoimmune disease, have the other diagnosis after T1D has been diagnosed and only exceptionally the other autoimmune disorder precedes the occurrence of T1D. According to several studies from several European and US diabetes centers, the comorbidity of T1D and AITD is referred to 17%-27%, the co-occurrence between T1D and CeD ranges from 9% to 12% and the association of T1D with Addison disease is reported in approximately 0,5%. In the general population, based both on studies from USA and Europe, the overall incidence of AITD among individuals aged 12-19 years is 3,4- 4,8%. Among T1D children and adolescents, up to 10-30% have positive anti-TPO antibodies, and up to 50% of these patients will develop clinical AITD [26–28].

In Greece, the prevalence of positive anti-thyroid antibodies in the general population has been reported to be 13.9% [29]. In children and adolescents the prevalence has been reported to range 4,3% to 8,2% [30].

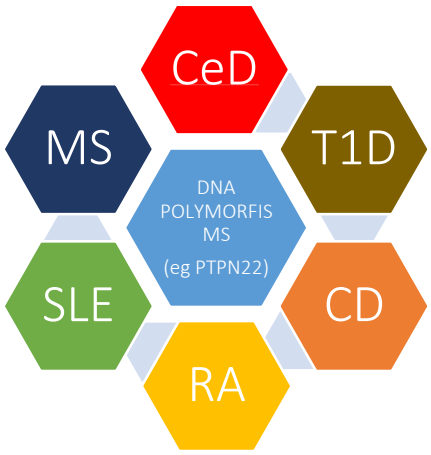
In the general population, the incidence of AITD is significantly higher among females. Among children and adolescents with T1D, females, especially after the onset of puberty, are at higher risk to develop AITD [31]. Analogous to T1D, the trigger factor for autoimmune thyroid disease is thought to be environmental. AITD is characterized by the production of autoantibodies against the thyroid gland, T-lymphocytic infiltration of the gland and subsequent development of various degrees of thyroid dysfunction. These autoantibodies are directed towards thyroglobulin (Tg), a fundamental component of thyroid colloid, and thyroid peroxidase (TPO), an enzyme participating in the production of thyroid hormones [31]. Many studies have focused on the shared genetic susceptibility of both autoimmune diseases [26]. T1D and AITD frequently cluster within the same family [32]. HLA histocompatibility system appears to play a role to this association between autoimmune diseases. The HLA class II genes and especially HLA DR3-DR5 have been shown to have a strong effect on the co-occurrence of T1D and AITD within families and individuals [26]. Several studies have reported some of the predisposing factors for the development of subclinical or even clinical AITD. These are, the younger age at diabetes onset (3-fold higher risk in children with diabetes onset <4yr, vs those diagnosed >9yr), the female gender (2-fold increase) and the concurrent autoimmune thyroid disease [27,33].

The co-occurrence between T1D and CeD is referred to range between 9%-12% [34]. In the majority of cases (approximately 88%) the diagnosis of T1D precedes that of CeD. It consists of a multifactorial, autoimmune disorder that occurs in genetically susceptible individuals and is triggered by a well-identified environmental factor (gluten and related prolamins). The disease primarily affects the small intestine, where it progressively leads to flattening of the small intestinal mucosa. The genetic susceptibility of CeD is conferred by well-identified haplotypes in the human leukocyte antigen (HLA) class II region (ie. DR3 or DR5/DR7 or HLA DR4). Such haplotypes are expressed on the antigen-presenting cells of the mucosa (mostly dendritic cells). HLA DR3-DQ2 is positively correlated with comorbidity of T1D and CeD. CeD is rarely found after 10 years of diabetes duration. It is very important to diagnose celiac disease early and to treat accordingly in order to prevent the risk of gastrointestinal malignancy (lymphomas) and low Bone Mineral Density (BMD). For the diagnosis of

CeD in children and adolescents a regular screening of anti-endomysial (EMA) and tissue Transglutaminase IgA- antibodies (anti-tTG) is recommended. Screening with anti-gliadin (AGA) antibodies is recommended for the diagnosis in T1D children younger than 2 years of age because of higher sensitivity. Total IgA measurement (in order to exclude IgA deficiency) is also recommended because of the often co-morbidity between the CeD and IgA deficiency. CeD is more common in those with IgA deficiency than in the general population (1.7% vs 0.25%). The screening for autoantibodies is recommended upon stabilization of the patient after the initial diagnosis of T1DM and every 1-2 years thereafter or upon symptoms (up to 10 years of T1D duration). Studies have shown that the risk is greater with T1D onset at age younger than 5 years, but after longer diabetes duration the risk is lower [34].

Several studies have shown that there is an overlap in the genetic risk loci for Celiac Disease (CeD), Crohn's Disease (CD), Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), Systemic Lupus Erythematous (SLE) and T1D. The genetic susceptibility with the contribution of environmental factors and endocrine disruptors, constitute the fundamental element leading to the development of autoimmune diseases [26,32]. The genetic contribution is suggested from the relatively high degree of familial clustering among patients with T1D and other autoimmune diseases. It is suggested that the genetic susceptibility is associated to the major histocompatility complex (MHC), mainly to the alleles DR and DQ but even to DNA polymorphisms, unlinked to the MHC region. Recently, genome-wide association studies (GWAS) suggested the possible genetic linkage between the autoimmune diseases and the co-occurrence of diseases within individuals and within families [26,32,35] (Table 3). This clustering of genetic risk factors for many autoimmune diseases suggests that these diseases might share, at least partly, similar underlying causal mechanisms.

Table 2: Overlapping in the genetic risk loci for Celiac Disease (CeD), Crohns Disease (CD), Multiple Sclerosis (MS), Rheumatoid arthritis (RA),* Systemic Lupus Erythematosus (*SLE) and Type 1 Diabetes Mellitus (T1D), which may suggest common mechanisms that lead to the development of autoimmune diseases. Adapted from Hum. Mol. Genet. 17, R116–21 (2008)[35]



1.7 Complications of type 1 diabetes mellitus

1.7.1 Acute complications of type 1 diabetes mellitus

It is noteworthy that especially in adolescents, diabetes-induced microvascular complications' frequency is often observed within 2-5 years' time following initiation of T1D [36].

Acute complications of T1D, that often occur when glycemic control is poor, include hypoglycemia and diabetic ketoacidosis (DKA), a life-threatening condition in which severe insulin deficiency leads to hyperglycemia, excessive lipolysis, and unrestrained fatty acid oxidation, producing the ketone bodies acetone, β-hydroxybutyrate, and acetoacetate. This condition results in metabolic acidosis, dehydration and electrolytes impairment. In parallel, excess secretion of glucagon, catecholamines, glucocorticoids, and growth hormone in combination with insulin deficiency accentuate hyperglycemia by stimulating glycogenolysis and gluconeogenesis.

1.7.2 Chronic complications of type 1 diabetes mellitus

The chronic complications which occur rather often, include development of microvascular lesions in the retina, renal glomerulus, and peripheral nerve. In parallel, T1D is also associated with macrovascular atherosclerotic pathophysiological procedures that resemble macrovascular morbidity in nondiabetic patients, occurring more rapidly, in younger ages.

Early in the course of T1D, specific organs' function decline and tissue abnormalities become evident, while hyperglycemia causes abnormal homeostasis in blood flow and vascular permeability in the retina, glomerulus, and peripheral nerve vasa nervorum. The increased blood flow and intracapillary pressure is thought to reflect hyperglycemia-induced decreased nitric oxide (NO) production on the efferent side of renal capillaries and eventually an increased sensitivity to angiotensin II. As a consequence of increased intracapillary pressure and endothelial cell dysfunction, retinal capillaries exhibit increased leakage of fluorescein and glomerular capillaries have an elevated albumin excretion rate (AER). Likely, changes occur in the peripheral nerves' vasa vasorum. Early in the course of T1D, this increased permeability is reversible but under the continuing trigger effect of hyperglycemia the lesions become irreversible.

The importance of glycemic control in the pathogenesis of diabetic complications is supported by the observation that Glycated hemoglobin (HbA1c) is an independent risk factor for cardiovascular morbidity in T1DM and hyperglycemia is a continuous risk factor for macrovascular disease [37–39]. On the other hand, epidemiological and prospective data have shown that the stressors of diabetic vasculature persist beyond the point when glycemic control has been achieved. This kind of persistent adverse effects of hyperglycemia on the development and progression of complications has been defined as "metabolic memory", and oxidative stress, advanced glycation end-products (AGEs) and epigenetic changes may play a significant role in the process[39,40].

Lower HbA_{1c} has been associated with more favorable renal outcomes after microalbuminuria diagnosis. Overall, a 21% reduction in the risk of the composite primary CVD outcome per 10% lower mean HbA_{1c} has been shown during the DCCT study [41]. Major beneficial effects of intensive insulin treatment on long-term complications, including retinopathy, nephropathy with reduced glomerular filtration rate (GFR), and autonomic manifestations of neuropathy, have been demonstrated. In addition, measurements of atherosclerosis in several macrovascular beds, including carotid intima media thickness and computed tomography–measured coronary artery calcification, have revealed less atherosclerosis in people with diabetes who have a

good glycemic control. The clinical expression of these changes, fatal and non-fatal myocardial infarctions and stroke, were also reduced by intensive insulin-therapy, with a 58% reduction in CVD [41]. Moreover, according to the EURODIAB study, HbA_{1c} was related to all-cause mortality in a non-linear manner after adjustment for age and sex. All-cause mortality risk was increased at both low (5.6%) and high (11.8%) HbA_{1c} compared to the reference (median HbA_{1c}: 8.1%) following a U-shaped association [42].

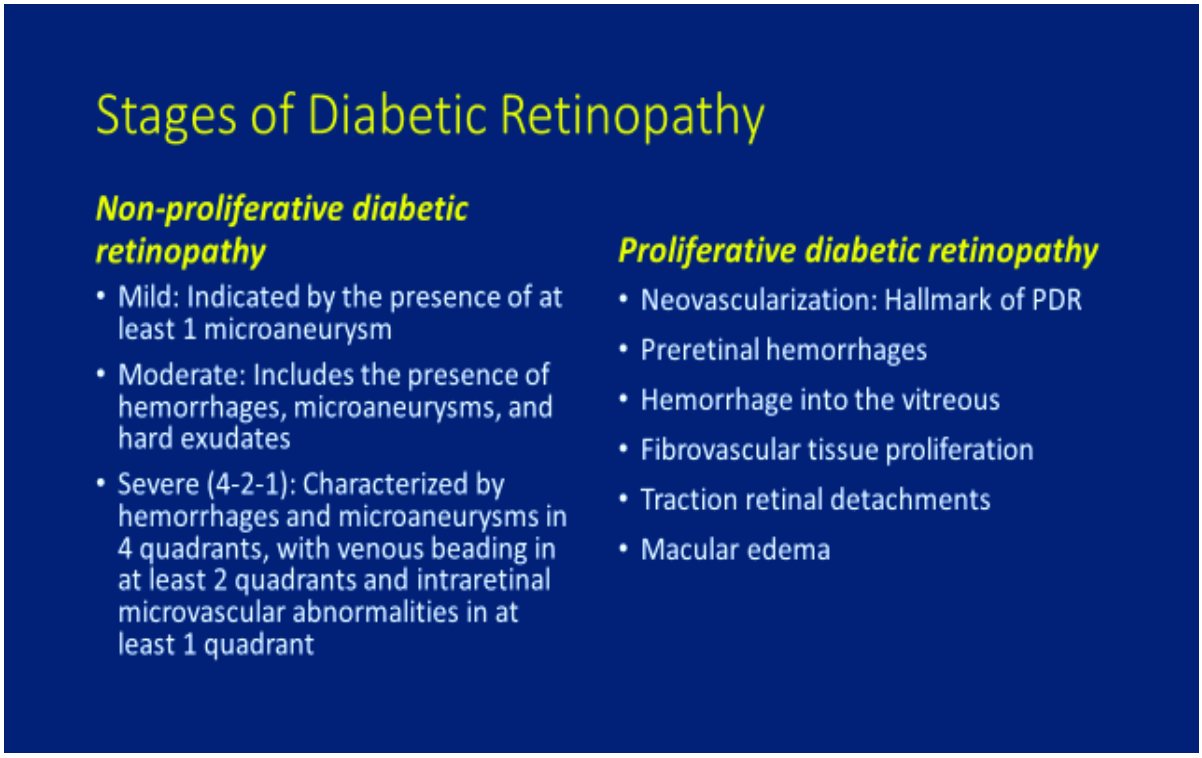
However, a lot of studies have shown that different patients with similar duration and degree of glycemic control differ markedly in their susceptibility to microvascular complications. Such observations suggested that genetic susceptibility may play a rigorous role in affecting pathways which lead to microvascular lesions [43–45]. The role of AGEs is also suggested to be very important in the development of diabetic microvascular complications, especially for diabetic retinopathy [46].

Previous studies have shown strong relationship between smoking and diabetic microvascular complications, despite intensive insulin treatment and sufficient glycemic control [47].

1.7.2.1 Diabetic retinopathy

Diabetic retinopathy is a sight-threatening chronic microvascular complication that is characterized by gradually progressive alterations in the retinal capillaries, leading to areas of retinal hypo- or even non-perfusion, increased vascular permeability termed macular edema, and pathologic uncontrolled intraocular proliferation of retinal vessels, termed proliferative diabetic retinopathy (PDR). In the initial stages of diabetic retinopathy, patients are generally asymptomatic but in the more advanced stages patients' symptomatology may include floaters, blurred vision, distortion, and progressive visual acuity loss. The clinical stages of Diabetic Retinopathy are listed in table 3[48,49].

Table 3: Clinical classification for diabetic retinopathy. Diabetic macular edema is classified as apparently present or apparently absent (Ophthalmology. 2003 Sep;110(9):1677-82).



1.7.2.2 Diabetic neuropathy

Diabetic neuropathy (DNE) include a heterogeneous group of disorders and present a wide range of abnormalities that affect distinct regions of the nervous system, isolated or combined. DNE is one of the most common long-term diabetes-induced complications and highly associated with morbidity and mortality [50,51]. The prevalence of DNE varies substantially, depending on specific diagnostic criteria [51]. Cohort studies reported that prevalence was found to reach approximately 45% after 25 years [50,52]. Clinical signs and symptoms can be non-specific and insidious, and progression can be slow. Neuropathy may be silent for many years or it can manifest with clinical symptoms and signs that mimic those seen in many other diseases. It is, therefore, often diagnosed by exclusion.

The major morbidity associated with somatic neuropathy is foot ulceration, the precursor of gangrene and limb loss. Neuropathy increases the risk of amputation 1.7-fold, 12-fold if there is deformity (itself a consequence of neuropathy), and 36-fold if there is a history of previous ulceration [53]. Autonomic neuropathy, one of the most devastating types of DNE, is often underestimated and misdiagnosed, but once sets in, quality of life can be dramatically reduced, and the mortality rate approximates 25% to 50% within 5 to 10 years [53].

Diabetic neuropathy is not a single entity but a number of different syndromes with subclinical or clinical manifestations depending on the classes of nerve fibers involved. According to San Antonio Convention [54], the main groups of neurologic disturbance in diabetes mellitus are listed in the table 4.

Subclinical neuropathy is diagnosed on the basis of abnormal electrodiagnostic tests with decreased nerve conduction velocity (NCV) or decreased amplitudes; abnormal quantitative sensory tests (QST) for vibration, tactile, thermal warming, and cooling thresholds; and quantitative autonomic function tests (QAFT) revealing decreased heart rate variation with deep breathing, Valsalva maneuver, and postural testing [55].

Table 4: Classification of Diabetic Neuropathy (Adapted from Consensus Panel: Report and recommendations of the San Antonio Conference on Diabetic Neuropathy. Diabetes 1988; 37: 1000)

1.7.2.3 Coronary heart disease

Several studies, have shown an excess morbidity and mortality associated with diabetes and elevated glucose, even after adjustment for traditional coronary heart disease (CHD) risk factors.

According to the results of a large Finnish database, cardiovascular disease (CVD) mortality in patients with type 1 diabetes aged 45 to 64 years at baseline increases by about 50% with every 1% increase in glycated hemoglobin (HbA1c) [56].

1.8 Diabetic nephropathy

1.8.1 Diagnosis of Diabetes Nephropathy

Microalbuminuria has been traditionally considered as the primary predictive marker of risk for progress to the advanced stages of chronic kidney disease. Actually, the origins of this model can be traced to three follow-up studies published in the 1980's [57–59]. Patients with T1D who present with microalbuminuria, defined by persistent urinary albumin excretion approximately in the range of 30–300 micrograms per minute, were followed for 7 to 14 years, and advanced kidney disease developed in 60–90% of them.

The presumed association of renal function impairment with proteinuria inspired a simple model of diabetic nephropathy comprising three sequential stages: Microalbuminuria heralds proteinuria, which plays a leading role for the pathways of the progress of renal dysfunction to end stage renal disease [60]. Following this traditional model, therapy in T1D patients with microalbuminuria has been focused on the prevention of albuminuria through blockade of the Renin-Angiotensin-Aldosterone system (RAAS) [61].

However, in contrary to the outcomes from the studies that showed microalbuminuria was a step toward advanced stage kidney disease, many recent studies have suggested that it may rather be considered as a dynamic process that is more likely to remit to normoalbuminuria than to progress to albuminuria. Indeed, recent results from the Oxford Regional Prospective Study showed that the majority (52%) of

subjects with microalbuminuria had a dynamic process described as “intermittent microalbuminuria” [62] and the 6-year cumulative incidence of remission was approximately 50%. According to other epidemiological studies the risk of remission far outweighs the risk of progression to proteinuria [62–64,65].

Although microalbuminuria plays an essential diagnostic role to the model of early diabetic nephropathy, because of the frequent occurrence of microalbuminuria remission, it seems that it can no longer be considered as the only independent predictive marker for advanced stage of chronic kidney disease [64]. Recently, several authors regarding the early nephropathy literature, have provided major insight into the early natural history of diabetic nephropathy in type 1 diabetes and have identified a new phenotype – early renal function decline (ERFD) [66]. In order to diagnose ERFD, it is important to detect the presence of a progressive loss of GFR over time even if it remains within normal range [67,68].

Indeed, several recent studies showed that renal dysfunction appears to begin prior to the onset of albuminuria. In the 2nd Joslin Kidney Study on the Natural History of Microalbuminuria, over one-third of T1D patients with microalbuminuria at the time of enrollment already had evidence of mild (GFR<90) or moderate (GFR<60 ml/min) renal function impairment [66].

1.8.2 Prevalence and incidence of diabetic nephropathy

According to earlier studies, 25% to 40% of patients with T1D and 5% to 40% of patients with T2D ultimately develop diabetic kidney disease [50]. Approximately 20 to 30 percent of T1D have microalbuminuria after a mean duration of disease of 15 years. The overall incidence of end stage renal disease is reported to be 4-17% at 20-30 years from T1D diagnosis. Up to 20% of patients with T2D already have diabetic kidney disease when they are diagnosed with diabetes [50] and a further 30% to 40% develop diabetic nephropathy, mostly within 10 years of diagnosis [69].

1.8.3 Genetic susceptibility of diabetic nephropathy

Recent studies suggest a role for cell death in the progression of human DN. Gene ontology identified 112 cell-death-related genes that were significantly differentially regulated in the tubulointerstitium of renal biopsies from DN patients[70]. The landmark study by Seaquist et al. in 1989 showed that family history of kidney disease appears to be among the strongest risk factors for initiation of diabetes-associated nephropathy [44]. Researchers have identified genetic associations for susceptibility to diabetic nephropathy for T1D at loci near the FRMD3 and CARS genes[71], the AFF3 gene and an intergenic SNP on chromosome 15q26 between the genes RGMA and MCTP2 [72]. Functional data have suggested that AFF3 influences renal tubule fibrosis via the transforming growth factor-beta (TGF-β1) pathway. Another association with DN as a primary phenotype was seen for an intronic SNP in the ERBB4 gene [72,73]. In addition, it is suggested that genetic variations on chromosome 11q at the MMP-3/MMP-12 locus influence susceptibility for DN in type 1 diabetes [74].

1.8.4 The pathogenesis of diabetic nephropathy

It is evident that hyperglycemia is necessary for the initiation of renal structural injury, since people without diabetes do not develop the same type of nephropathy. Early in the course of T1D, specific organs' function declines and tissue abnormalities become evident, hyperglycemia causes abnormal homeostasis in blood flow and vascular permeability in the glomerulus. The increase in blood flow and intracapillary pressure is thought to reflect hyperglycemia-induced decreased nitric oxide (NO) production on the efferent side of renal capillaries and eventually an increased sensitivity to angiotensin II with profibrotic effects. In vitro studies have demonstrated that hyperglycemia induces mesangial cell matrix production and mesangial cell apoptosis. Early in the course of T1D, this increased permeability is reversible but under the continuous trigger effect of hyperglycemia the lesions become irreversible.

Moreover, intensive antidiabetic treatment may attenuate the development of nephropathy, as assessed by urinary albumin excretion, although it cannot be fully prevented[75]. However, it is now clear that other factors may also be involved, as

continuous hyperglycemia is not necessarily required for diabetic hyperfiltration to occur. Indeed, glomerular hyperfiltration and tubular hypertrophy can persist in patients with T1D even after optimal glycemic control is achieved [76].

Studies performed in endothelial cells demonstrating other pathways that may be involved in diabetic nephropathy, include:

A central role of mitochondrial reactive oxygen species (ROS) which are generated as byproducts of oxygen metabolism in renal mesangial cells [76]. Overproduction of mitochondrial superoxide during hyperglycemia has been postulated as the primary initiating mechanism that activates pathways of diabetic vascular tissue damage, leading to cellular redox imbalance and oxidative stress. A number of in vitro and in vivo studies suggest that oxidative stress is increased in diabetic nephropathy. In addition, overproduction of ROS by high glucose compromises the antioxidant defense mechanisms in diabetic nephropathy such as reduced levels of mitochondrial-specific manganese superoxide dismutase (MnSOD) and further aggravates oxidative stress. Oxidative stress including ROS may damage mitochondrial DNA (mtDNA) and impair electron transport chain, leading to increased ROS production [77].

Accumulation of Advanced Glycated End-products (AGEs). Advanced glycation that occurs at an accelerated rate in T1D is a prominent phenomenon in the kidney. The excretion of AGEs is mostly renal, but also many of the proteins with a long life, such as collagen, are extensively glycated in patients with diabetes [76]. Furthermore, various AGE receptors such as RAGE have been described in the kidney, which appear to play a role in mediating some of the deleterious effects of AGEs, inducing the expression of TGF-beta and other cytokines that are proposed to mediate the transdifferentiation of epithelial cells to form myofibroblasts (it is strongly believed that myofibroblast formation represents a key step in the development of tubulointerstitial fibrosis) [78].

Activation of intracellular signaling molecules such as protein kinase C (PKC)[79]. PKC is a family of enzymes that phosphorylate serine or threonine residues of various intracellular proteins and is thus involved in a lot of cellular functions that may lead to the pathophysiology of diabetic complications including basement membrane

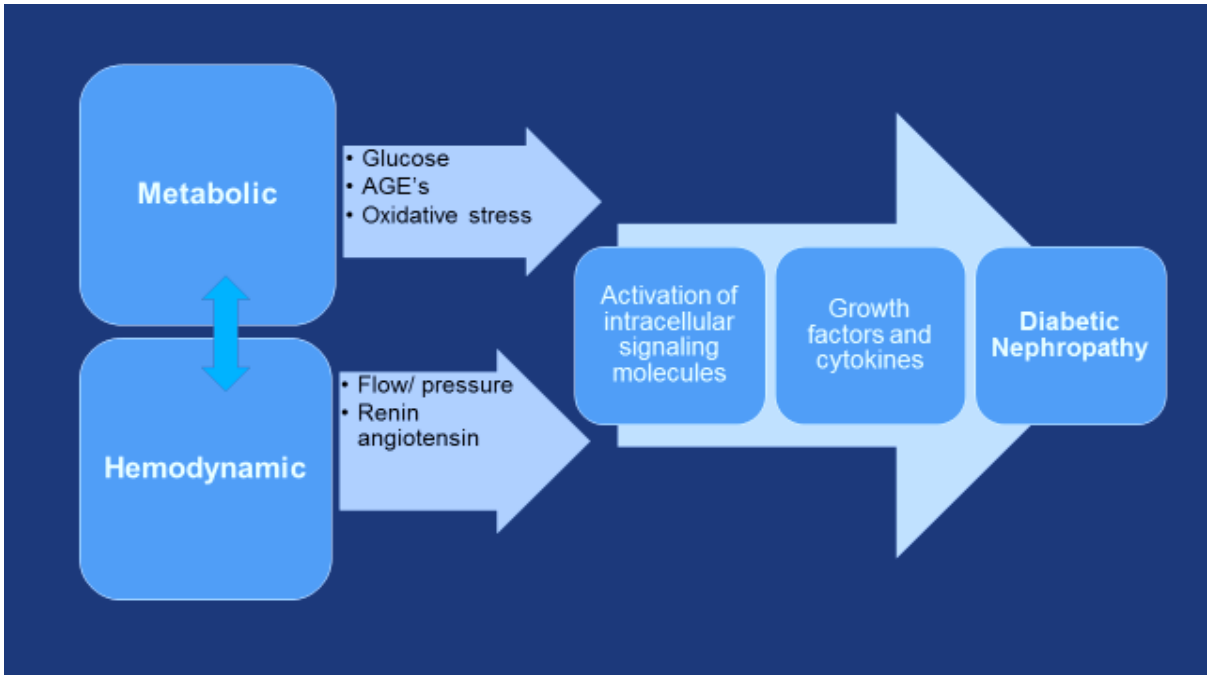
hyperplasia and signal transduction for growth factors. At least 11 isoforms of PKC have been identified. Although hyperglycemia may play an important role in PKC activation, a number of other stimuli have been demonstrated to play a role, including AGEs, AGE receptors, angiotensin II and ROS [79].

Increased prorenin activity in children and adolescents with T1D is suggested to be a risk factor for the development of DN. Prorenin binds to a specific tissue receptor that induces activation of mitogen-activated protein kinases (MAPK).

Activation of several cytokines [i.e. transforming growth factor-beta (TGF-β)], profibrotics and vascular growth factors like vascular endothelial growth factor (VEGF), have been suggested as promoters of matrix accumulation in T1D.

Renal expression of Nephrin (a transmembrane protein expressed by podocytes) may be impaired in T1D, inducing DN by influencing the proper functioning of the renal filtration barrier.

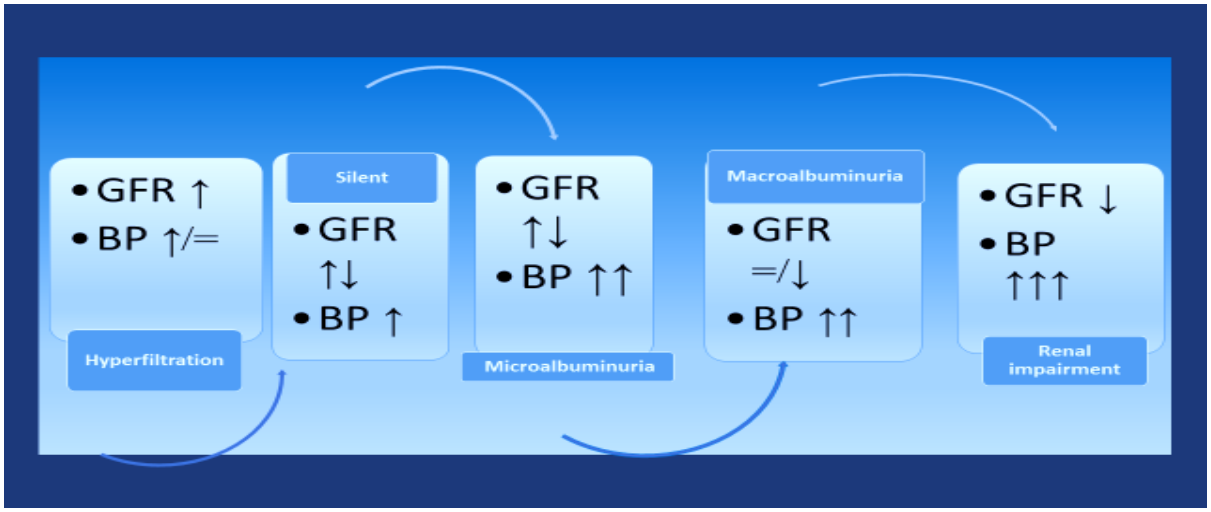
Table 5: Promoting factors in diabetic complications including nephropathy. Adapted from Williams textbook of Endocrinology[76]



1.8.5 Clinical course of diabetic nephropathy

Diabetic nephropathy is often associated with the clinical manifestations of a triad of hypertension, albuminuria, and, ultimately, renal impairment. The classic five stages of diabetic nephropathy, based on functional renal evaluation, as described by Mogensen [80], remain the most acceptable way of describing this condition (table 6).

Table 6: The classic five stages of diabetic nephropathy, based on functional renal evaluation. Adapted from Williams textbook of Endocrinology [81]



1.8.5.1 The stages of diabetic nephropathy

The early natural history of diabetic nephropathy in T1D is traditionally described as following:

Stage 1: Hyperfiltration stage

The initial phase has been termed as the ‘’hyperfiltration stage’’ which is associated with an increase in glomerular filtration rate (GFR) and increased capillary glomerular pressure. Hyperfiltration is considered to appear as a result of renal hypertrophy [82]. However its pathophysiology in T1D remains unexplained and its importance in predicting diabetic nephropathy has been controversial. Past studies have confirmed the relationship between initially elevated GFR and later development of proteinuria[58].

Stage 2: The Silent Stage

The second stage, is known as the ‘’silent stage’’, where, clinically, there is no overt evidence of renal dysfunction. GFR is usually normal with no signs of albuminuria. However, this phase is associated with significant structural changes including basement membrane thickening and mesangial expansion. Only studies of renal morphology can postulate that there will be subsequent renal damage. Indeed, more than a decade ago, it was reported that normoalbuminuric subjects, including prepubertal children with average diabetes duration of 5-8 years, often have GBM thickening and mesangial expansion [83] and the frequency of these findings has been slightly underestimated, while long-standing normoalbuminuric T1D patients have significant diabetic glomerulopathy lesions [84,85,86,69].

Prospective studies have suggested that ambulatory blood pressure monitoring demonstrated modest rises in arterial blood pressure in T1D patients in this silent phase up to 5 years before microalbuminuria supervenes [87].

Stage 3: Microalbuminuria stage

The third phase is known as ‘’microalbuminuria stage’’ or the ‘’stage of incipient nephropathy’’ [69]. At this stage, which is usually apparent 5 to 15 years after the initial diagnosis of T1D, the urinary albumin excretion rate is increased into the microalbuminuric range of 20 to 200 μg/min or 30 to 300 mg/24 hours [38,88]. Recent studies showed evidence that microalbuminuria is related to higher HbA1c levels, dyslipidemia, hypertension, longer diabetes duration and younger age at diagnosis. However, it has been also reported that a significant number of young T1D patients are diagnosed with microalbuminuria in their first 2 years of diabetes. The most prominent risk factors for microalbuminuria are hypertension, higher HbA1c levels, longer diabetes duration, and dyslipidemia [52]. In the past, microalbuminuria was considered to be a predictor rather than a manifestation of renal pathology. Recently several renal-structure studies have demonstrated that when persistent microalbuminuria occurs, there is already widespread evidence of advanced glomerular structural alterations [85,86,89]. GFR during this phase may be increased, normal, or reduced.

Screening for microalbuminuria remains controversial. Traditionally 24-hour or overnight urine sampling methods are used. However, a spot urine albumin-to-creatinine ratio of an early morning urine sample has been assessed and appears to be a practical option for the screening for microalbuminuria during the follow-up of T1D patients [88]. Persistent microalbuminuria (PMA) is defined when MA is confirmed on at least 2 occasions 3-6 months apart. Recent studies reported that in patients with T1D, MA can be transient and may be reversible especially when microalbuminuria has short duration, glycemic control is appropriate (HbA1c <8%), systolic blood pressure is low (<115 mm Hg), cholesterol and triglycerides levels are low [90].

Stage 4: Macroalbuminuria stage

The stage when macroalbuminuria occurs is characterized by overt nephropathy and usually occurs 10 to 15 years after T1D onset. This stage is highly predictive of subsequent progress to renal failure if left untreated. During this phase urinary albumin excretion rate is greater than 300 mg/24 hours (200 µg/min) [69].

In association with macroalbuminuria, more than two thirds of T1D patients have arterial hypertension [91].

Stage 5: Renal impairment

The final stage characterized by uremia and end stage renal disease (ESRD), which can occur in up to 40% of T1D patients, requires renal function replacement therapy. Many patients with diabetes and ESRD are now considered candidates for renal transplantation, which is associated with better outcomes than remaining on dialysis [69]. Moreover, simultaneous pancreas-kidney (SPK) and pancreas-after-kidney (PAK) transplantation have become therapeutic options for patients with T1D and ESRD, and have better success rates than the kidney-alone transplantation. In particular, some studies have provided evidence that diabetes control is dramatically improved after pancreas transplantation which remains the only available treatment that is associated with the reversibility of diabetic nephropathy lesions. These studies also showed that tubulointerstitial remodeling was also possible [92,93].

1.8.5.2 Pathology of diabetic nephropathy

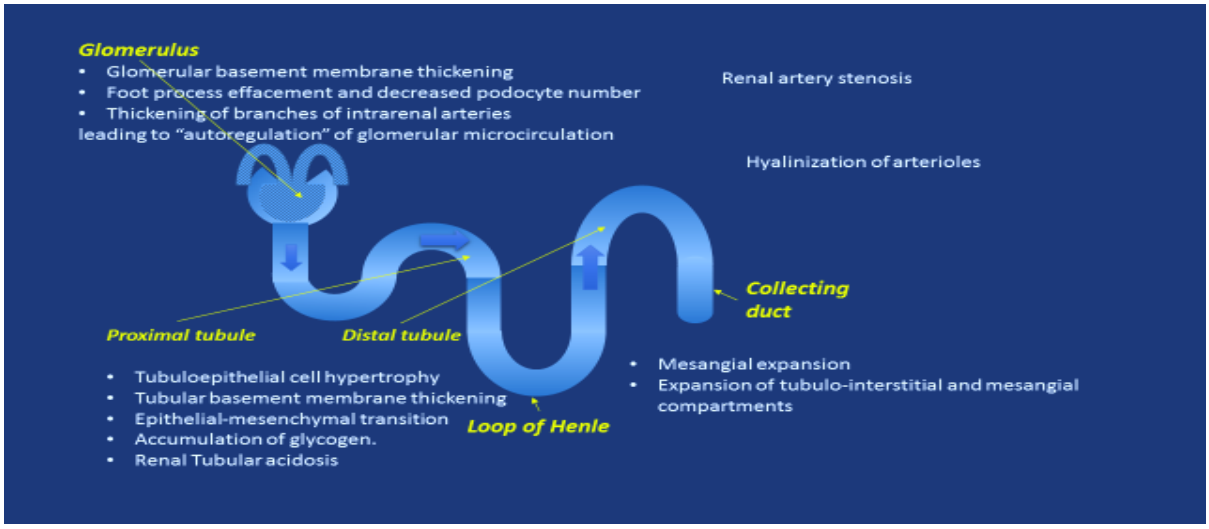
1.8.5.2.1 Glomerular and tubulointerstitial injury

Diabetic nephropathy was originally described as a glomerulopathy associated with diffuse or nodular glomerulosclerosis. However, less than one third of patients with T1D and microalbuminuria have the typical glomerulopathy described by Kimmelsteil and Wilson in 1936. Numerous studies using electron microscopy have shown that glomerular basement membrane thickening and mesangial expansion are prominent glomerular lesions in diabetes [94]. The pathophysiologic changes in diabetic nephropathy include cellular and extracellular derangements in both the glomerular and tubulo-interstitial compartments, such as hyperplasia or hypertrophy of various cell types of the glomerulus and tubules, associated with thickening of glomerular and tubular basement membranes, as well as expansion of tubulo-interstitial and mesangial compartments. Other changes include hyalinization of arterioles and sometimes thickening of branches of intrarenal arteries that lead to impairment in "autoregulation" of glomerular microcirculation, which apparently could amplify the renal damage. More recently, however, interest in the possible role of podocytes in the development and/or progression of diabetic nephropathy has grown. Podocytes (specialized visceral epithelial cells) are important in maintaining glomerular selectivity of permeability, and the development of proteinuria is associated with morphological changes causing dysfunction and subsequent apoptosis ultimately leading to depletion of these cells within the glomerulus including foot process effacement [95]. Foot process effacement and decreased podocyte number and/or density per glomerulus have been reported in patients with type 1 and type 2 diabetes [96,97,98]. It has been recently shown that tuft to Bowman's capsule adhesion (TBCA), known to be associated with GBM pathology of podocytes, is common in albuminuric patients with type 1 diabetes [99].

Although most of the studies have focused on glomerular changes in the diabetic kidney, more recently important changes in other sites of the renal tissue have been reported, including the tubules, interstitium, medulla, and papilla [100]. Diabetic tubulopathy is characterized by a variety of structural and functional alterations (table 7) including tubuloepithelial cell hypertrophy, tubular basement membrane thickening,

epithelial-mesenchymal transition and glycogen accumulation. It is also demonstrated an expansion of the interstitial space with infiltration of various cell types, including myofibroblasts and macrophages [69].

Table 7: The pathology of diabetic nephropathy in T1D varies and different injuries may occur in either glomerular, tubular, vascular or mesangial compartments



These renal tubular alterations occurring in T1D can precede or at least accompany the glomerular changes [101]. Indeed, recent studies have suggested that tubuloglomerular feedback mechanisms can drive hyperfiltration associated with diabetes induced tubular dysfunction and therefore can contribute to albuminuria due to defective uptake and lysosomal processing [100].

A classification of type 1 and 2 diabetic nephropathy has been suggested by the committee of the Renal Pathology Society. It consists of 4 classes of glomerular lesions and severity scores of interstitial and vascular lesions, as listed in table 8 and 9 [102].

Table 8: Classification of pathology of glomerular lesions. Adapted from: J Am Soc Nephrol. 2010 Apr;21(4):556-63

Classification of pathology of glomerular lesions

Class	Description	Characteristics
I	Isolated glomerular basement membrane (GBM) thickening	GBM > 395 nm in females and >430 nm in males, older than 9 years of age
IIa	Mild mesangial expansion	Mild mesangial expansion in >25% of mesangium
IIb	Severe mesangial expansion	Severe mesangial expansion in >25% of mesangium
III	Nodular sclerosis (Kimmelstiel–Wilson lesion)	At least one Kimmelstiel–Wilson lesion (nodular intracapillary glomerulosclerosis)
IV	Advanced diabetic glomerulosclerosis	>50% glomerular sclerosis

Table 9: Interstitial and vascular lesions' score of Diabetic Nephropathy. Adapted from: J Am Soc Nephrol. 2010 Apr; 21(4):556-63

Interstitial and vascular lesions of Diabetic Nephropathy		
Lesion	**Criteria**	**Score**
Interstitial lesions		
Interstitial fibrosis and tubular atrophy (IFTA)	No IFTA	0
	<25%	1
	25% to 50%	2
	>50%	3
Inflammation		
Inflammatory interstitial infiltrates (T lymphocytes and macrophages)	Absent	0
	Infiltration occurs around atrophic tubules	1
	Infiltration in other areas than around IFTA	2
Vascular lesions		
	Absent	0
	One arteriolar hyalinosis	1
	More than one arteriole with hyalinosis	2
Arteriosclerosis (most severely affected artery in the biopsy)	No intimal thickening	0
	Intimal thickening less than thickness of media	1
	Intimal thickening greater than thickness of media	2

1.8.5.2.2 Renal artery stenosis

Patients with T1D have, in general, an increased burden of atherosclerosis, which results to a higher risk of renal artery stenosis. However, although angiographic studies have demonstrated a high prevalence of renal artery stenosis in T1D, these lesions are often of no hemodynamic significance. Nevertheless, a small subgroup will have a hemodynamically significant stenosis enhancing hypertension, increased risk of acute pulmonary edema, and progressive renal impairment, where specific interventions such as surgery or angioplasty are desperately needed [103]. Furthermore, some patients have bilateral renal artery stenosis and in those patients medication with an angiotensin-converting-enzyme inhibitor (ACE inhibitor) should be avoided because it can lead to acute renal failure [104].

1.8.5.2.3 Renal papillary necrosis

Renal papillary necrosis is a severe destructive process, resulting from ischemia to the medulla and papilla [105]. The renal papilla is very sensitive to ischemic changes because even in the normal setting it is exposed to a relatively hypoxic environment. Concomitant exacerbating factors include urinary tract infection and non-steroidal anti-inflammatory drugs (NSAIDs) medication. The impact of ischemia and possibly the role of angiotensin II in this disorder have been suggested in experimental studies in transgenic rats that overexpress renin and angiotensin II in their kidney after diabetes induction [106]. In these rats, diabetes was associated with development of papillary necrosis which could be prevented by blockade of the renin-angiotensin system. Clinically, papillary necrosis is often manifested as flank pain, hematuria, and fever. Urinalysis reveals red and white blood cells, bacteria, and papillary fragments. Ureteric obstruction can occur as a result of these fragments and should be treated properly [107,64].

1.8.5.2.4 Renal Tubular acidosis

Renal tubular acidosis, a condition that is manifested as life-threatening hyperkalemia and hyperchloremic metabolic acidosis, has been associated with hypoaldosteronism linked to diabetes, resulting in proximal tubular ammonia production reduced to levels inadequate to buffer acid in the distal nephron. It has been suggested that a defect in the conversion of prorenin to active renin along with damage of the cells of the tubuli and juxtaglomerular apparatus lead to impaired renin release, possibly due to reduced renal prostaglandin production and elevated vasopressin levels [69,108]. This is an important issue due to the widespread use of ACE inhibitors Angiotensin II receptor blockers (ARBs), potassium-sparing diuretics (such as spironolactone) and β-blockers which are often used as combined treatment, in T1D patients.

1.8.5.2.5 Other renal manifestations

Since diabetes is strongly associated with impaired renal function, there is a higher risk of renal dysfunction caused by certain nephrotoxic agents such as radiocontrast dyes. Where possible, intravenous contrast forms that are an indispensable tool to diagnosis and management, low-osmolality, nonionic or gadolinium-based contrast media which should be the agents of choice because they are less nephrotoxic in patients with diabetes [69].

1.9 Biomarkers associated with renal structural lesions

According to the National Kidney Foundation (NKF) and the Kidney Disease Outcomes Quality Initiative (KDOQI), a patient is considered to be in chronic kidney disease (CKD) if he/ she presents a GFR<60 mL/min per 1.73 m^2 for three months or more. Alternatively, any ongoing (at least three months) structural or functional abnormality of the kidney, regardless of GFR that can be detected by pathological abnormalities or specific markers is considered CKD [68].

Glomerular and renal tubular interstitium injury plays the main role in the pathogenesis of Diabetic nephropathy [82,99,109–111] and in order to diagnose this pathology, several glomerular and tubular damage markers have been recently discovered. Increased levels of these markers are supposed to indicate proximal tubular damage in the case of kidney injury molecule (KIM)-1, neutrophil gelatinase–associated lipocalin (NGAL), N-acetyl-b-D-glucosaminidase (NAG), and Cystatin C (CysC). These tubular damage markers have been extensively investigated in predicting the occurrence of acute kidney injury after various nephrotoxic insults, such as ischemia during cardiac surgery or sepsis [111–114]. The most studied biomarkers associated with diabetic nephropathy are listed below, in table 10.

Table 10: Serum and Urinary markers of diabetic nephropathy

Markers of Diabetic Nephropathy		
Biomarker	**Characteristics**	**Findings**
Urinary Albumin	65 kDa; filtered in glomeruli followed by tubular reabsorption; gold standard for the diagnosis DN	A large number of patients with microalbuminuria develop clinical DN. In some patients microalbuminuria is only temporary with no signs of renal impairment at follow-up.
Urinary Type IV collagen	540 kDa; component of basement membrane	Modest correlation with decline in eGFR in patients with T1D.
Urinary TGF-β	25 kDa; regulates extracellular matrix production	High levels are associated with progression of DN with controversial results.
Urinary Fibronectin	440 kDa; component of extracellular matrix in mesangium	Increased levels in type 1 and 2 diabetic patients with macroalbuminuria.
Urinary Connective tissue growth factor	36–38 kDa; fibrogenic cytokine under regulation by TGF-β	Increased levels have been shown in diabetic patients

		with micro- or macroalbuminuria.
Serum Cystatin C	13 kDa; endogenous cysteine proteinase inhibitor.	It is highly correlated with GFR.
Urinary Transferrin	76.5 kDa; more readily filtered than albumin	Increased levels have been associated with microalbuminuria
Urinary IgG, IgA, IgM	Variable molecular weights; indicators of increased glomerular permeability	Increased IgG levels have been associated with development of microalbuminuria; higher IgM levels associated with cardiovascular morbidity.
Urinary α1 microglobulin	27 kDa;	Indicates proximal tubular dysfunction. It correlates with albuminuria and glycemic control.
Urinary Retinol-binding protein	21 kDa; transports retinol from liver to tissue	Increased levels have been associated with DN progression.
Urinary Monocyte chemo-attractant protein-1	13 kDa; chemokine which activates macrophage and contributes to tubulointerstitial disease	Increased levels have been associated with advanced tubulointerstitial lesions
Serum and urinary Neutrophil gelatinase-associated lipocalin	25 kDa; increased after renal injury	NGAL increases significantly with albuminuria progression and cardio-renal morbidity.
Serum and urinary Kidney injury molecule 1(KIM-1)	Type 1 epithelial transmembrane protein, sensitive marker of proximal tubular injury	Increased levels in all DM patients independently of albuminuria; KIM-1 has been associated with progression of DN.

Liver-type fatty acid-binding protein	14 kDa, expressed in proximal tubule cells. Increased in ischemia, hypertension, hyperglycemia	Increased levels are associated with micro- and macroalbuminuria in T1D.
Urinary Matrix metalloproteinase-9, Urinary fibronectin, α-smooth muscle actin	Urinary mRNA markers of epithelial-mesenchymal transition.	Increased levels have been correlated with severity of DN.
Urinary podocyte markers (nephrin and synaptopodin)	Podocytes found in DN and other renal diseases	Increased levels have been detected in patients with normoalbuminuria and have been correlated with progression of DN.
Serum YKL-40 (Chitinase-3-Like Protein 1)	40-kDa secreted glycoprotein	Inflammatory marker and indicator of endothelial dysfunction, seems to increase in patients with diabetes and albuminuria.
Serum GDF-15 (Plasma growth differentiation factor-15)	Protein that belongs to the transforming growth factor beta superfamily	Increased GDF-15 values are associated with rapid decline in GFR and are considered as a predictor of all-cause cardiovascular mortality in patients with DN.

1.9.1 Neutrophil gelatinase-associated lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL), first purified and identified in 1993 by Kjeldsen et al. seems to be a promising biomarker [73,111,112]. NGAL is a 178 amino acid 25 kDa protein that belongs to the lipocalin protein family. It is mainly produced in renal tubules in response to structural kidney injury [115], but also, to a lesser degree, in the lung, trachea, stomach and colon, while it is also excreted in the urine [116]. NGAL, is freely filtered by the glomerulus, it is largely reabsorbed in the proximal tubules by

efficient megalin-dependent endocytosis [116]. NGAL as a renal biomarker was first described in 2003, following experimental renal ischemia in a mouse model[117].

Urinary NGAL (uNGAL) has been considered to be able to discriminate intrinsic acute kidney injury (AKI) from prerenal AKI and predict an unfavorable clinical course. In a recent study, there were validated cutoffs derived from patients' cohorts, where it was confirmed that a uNGAL <47 mg/l, made intrinsic AKI decidedly unlikely, whereas a value of uNGAL >104 mg/l was indicative of intrinsic AKI [118]. It is also observed that serum NGAL levels play a significant prognostic role in cardiovascular morbidity. It is recently reported that NGAL, can bind matrix metalloproteinase-9 (MMP-9) and inhibit its degradation, thereby sustaining MMP-9 proteolytic activity. It has been suggested that increased urinary excretion of NGAL and MMP-9 supports a role for NGAL/MMP-9 dysregulation in renal function and a susceptibility for the development of renal complications in diabetes mellitus 1 and 2. Experimental animal studies found that NGAL/MMP-9 activities preceded microalbuminuria in diabetic nephropathy [73]. Moreover a genetic study showed that genetic variations within the MMP-3/MMP-12 locus influence susceptibility to DN in type 1 diabetes [74].

Plasma NGAL measurements may be influenced by a number of coexisting variables including chronic kidney disease, chronic hypertension, systemic infections, inflammatory conditions, anemia, hypoxia and malignancies. Plasma and urinary NGAL is supported not to be influenced by age after the 5th year of age, while it is found that infants have higher s-and u-NGAL than children who are found to have values similar to adults [116]. In contrast to conventional markers, such as serum creatinine, blood urea nitrogen, or serum Cystatin C (CysC), NGAL is not considered as a marker of renal function, but rather reflects structural damage of renal cells. Consequently, it is rapidly detectable in response to injury and its increased levels were supposed to be independent of a functional deficit. In previous studies, NGAL was effective in the early diagnosis of AKI in several clinical settings [119–121] and was also validated for its significant prognostic role in cardiovascular morbidity [122]. The association between the early tubular interstitial damage in normoalbuminuric diabetic patients and NGAL was further supported by recently published studies[73,111,112].

1.9.2 Cystatin C

Cystatin C is a small-molecular-weight protein, and in particular, an endogenous cysteine proteinase inhibitor which is highly correlated with glomerular filtration rate[123]. This correlation is independent of inflammatory conditions, muscle mass, gender, body composition and age (after the age of 12 months old) [124]. Superiority of CysC over other markers of renal function decline marked by eGFR<60 mL/min, lie in its ability to remain unbound to protein and to be freely filtered across the glomeruli. In healthy subjects, CysC is almost freely filtered by the glomeruli and almost entirely reabsorbed in the proximal tubule like other low molecular weight proteins with no or only partial tubular secretion. Inter-individual variation in CysC accounts for 25% of its biological variability compared to 93% for creatinine. Increased CysC values with or without renal impairment are associated with increased cardiovascular morbidity risk and atherosclerosis progression in obese children [125,126]. Cystatin C is commonly quantified using either an automated particle-enhanced turbidimetric immunoassay or a particle-enhanced nephelometric immunoassay to measure the formation of antigen-antibody complexes. The nephelometric method is more sensitive and performs optimally in dilute solution, making it preferable for small sample volumes encountered in the pediatric population. It should also be noted that there are discrepancies in the determination of cystatin C in the same blood samples between the turbidimetric and the nephelometric immunoassay method, suggesting different reactivity to the antibodies against the cystatin C molecule, different standards, or different substrates. The reference range for young healthy persons ranges from 0.53 to 0.95 mg/l. Schwartz et al has recently shown that the reciprocal of cystatin C measured by the nephelometric method showed substantially stronger correlations with iGFR [127].

1.9.3 Uric Acid

Uric acid (UA) is the main byproduct of purine metabolism. Serum uric acid, has received renewed attention because of its potential causal role in renal function impairment, possibly due to its pro-oxidant, complement system activation, renin-

angiotensin-aldosterone system (RAAS) up-regulation, and nitric oxide inhibitory mechanisms rather than its role as a marker of renal function itself [65,128].

Moreover, several studies have shown an independent association of subtle changes in uric acid with the cross-sectional level of GFR within the normal range [129]. Recently, studies suggested that in patients with type 1 diabetes, high normal serum uric acid levels appear to be associated with impaired glomerular filtration rate (GFR) and that elevated serum uric acid levels are a strong predictor of the development of albuminuria in patients with type 1 diabetes [130–132]. Previous studies suggested uric acid as an early marker of DN that does not correlate with microalbuminuria[131]. Elevated UA has not only been demonstrated to be associated with renal dysfunction [133] but also with hypertension development and cardiovascular disease irrespective of renal involvement [134].

1.9.4 YKL-40

YKL-40, Chitinase-3-Like Protein 1, is a 40-kDa secreted glycoprotein which is an inflammatory marker and indicator of endothelial dysfunction and seems to increase in T1D patients with albuminuria. It is found to be overexpressed in certain solid tumors, such as breast cancer, colon, lung, kidney and ovary. In patients with malignancies, the serum concentrations of YKL-40 can often predict tumor stage, response to treatment, and prognosis. Increased levels of serum YKL-40, have also been observed in certain conditions characterized by inflammation and tissue remodeling, such as arthritis, severe bacterial infection, inflammatory bowel disease, and liver cirrhosis. The increased YKL-40 values in inflammatory processes and vascular dysfunction, suggest that YKL-40 may play a role in endothelial dysfunction [135]. Elevated levels of YKL-40 have been associated with the metabolic syndrome and cardiovascular morbidity. It has recently been proposed as an indicator of atherosclerotic disease progression [135,136]. In patients with type 1 diabetes, a positive correlation between elevated levels of plasma YKL-40 and albuminuria has been reported [137,138].

1.9.5 GDF-15

GDF-15 (Plasma growth differentiation factor-15), is a protein belonging to the transforming growth factor beta superfamily [139], which has been linked to inflammation and apoptosis and seems to be expressed in the heart in response to ischemia and in atherosclerotic plaques. A recent study showed that high levels of GDF-15 are a predictor of cardiovascular mortality and morbidity in patients with diabetic nephropathy [114,140]. GDF-15 also plays an important role in tumor-genesis and metastatic tumors. It has been observed that in many types of cancer, such as colorectal cancer, breast and prostate cancer, the expression of GDF-15 is significantly increased [141,142]. In addition, high levels of this marker are associated with rapid decline in glomerular filtration rate and renal dysfunction. Higher levels of GDF-15 have been suggested as predictors of all-cause cardiovascular morbidity and mortality in patients with diabetic nephropathy [114,140].

1.10 Glomerular filtration rate

Glomerular filtration rate (GFR) is defined as the volume of fluid filtered by the renal glomerular capillaries into the Bowman's capsule per unit of time. Glomerular filtration rate (GFR) is best evaluated by the clearance of iohexol (iGFR) [143]. The best and most accurate formula regarding estimated GFR for children has been debated the last years. Indeed, the 2009 modified Schwartz bedside GFR formula ‘’eGFR = 0.413 * height (cm)/creatinine (mg/dl) or eGFR 36.5 * height (cm)/creatinine (mol/L)’’ has been widely adopted for children because it has been correlated to iGFR [144,145].

Schwartz et al. have recently suggested another eGFR formula ‘’eGFR=39.8 $(ht(m)/Scr)^{0.456}(1.8/cystatinC)^{0.418}(30/BUN)^{0.079}1.076^{male}(ht(m)/1.4)^{0.179}$’’ that estimates the glomerular filtration rate in youth, by assessing several parameters (reciprocal of CysC measured by the immunonephelometric method, BUN, serum creatinine, gender and height) which showed substantially stronger correlations with iGFR (0.87 for nephelometric vs.0.74 for turbidimetric analyzed Cystatin C) [127]. This formula is

proposed as the best tool to accurately and precisely estimate GFR in childhood populations at study visits when iohexol is not indicated.

The Lund strategy suggested by A. Grubb and his team, appears to give interesting and reliable results in adults and children, though there is no real consensus [146,147]. This strategy consists of a multi-step evaluation of GFR by calculating eGFR with one creatinine-derived formula and one cystatin C-derived formula; in case of agreement between the two equations, the mean value is considered to be the reliable GFR; in case of disagreement, clinical explanation must be considered (e.g. decreased muscle mass or treatment with corticosteroids) and the formula known to be influenced is changed.

SPECIFIC PART

2 Early markers of diabetic nephropathy in children and young adults with type 1 diabetes

2.1 Aim of the study

The aim of this study was to determine the possible predicting roles of serum NGAL, serum YKL-40, serum GDF-15 serum CysC, serum uric acid and urinary NGAL, in unmasking an early glomerular and/or tubular injury and the possible association with renal function decline in asymptomatic young T1D patients with or without microalbuminuria.

2.2 Study design

This is an observational cross-sectional prospective long-term follow-up study. The sample size was calculated from a previously estimated standard deviation of the analyzed biochemical markers. With α=0.05 and a power of 80%, the patient group should consist of 54 patients; with an estimated dropout rate of 20%, the necessary sample size was calculated to be 70 patients. In total, at baseline, 70 patients were included in the study, and completed the baseline clinical examination and laboratory assessment. During the 12-15 months' follow up period, 14 patients dropped out either because they met exclusion criteria or discontinued the follow up.

During the follow-up period, a patient-group that completed both baseline and reevaluation at 12-15 months was evaluated. The patient group consisted of 56 T1D patients, with mean age 13.1 years (SD: 3.20) and mean diabetes duration 4.59 years (SD: 3.49) at enrollment, who were prospectively followed for at least 2 years at the

Diabetes Centre of the First Department of Pediatrics of the University of Athens, Aghia Sophia Children's Hospital, Greece.

The diagnosis of T1D was based in all participants of the patients' group on the presence of the high titer of at least one and mostly two of the known autoantibodies related to type 1 diabetes mellitus. Specifically, the percentage (%) of positivity for each autoantibody tested was: for GADA (72%), IA2 (71%) and IAA (45%). Ten of the patients presented with microalbuminuria at inclusion in the study, while five of them had persistent microalbuminuria at reevaluation and five patients were restored to normoalbuminuria. Three patients presented with newly diagnosed microalbuminuria at reevaluation. In total, eight patients were found to have microalbuminuria at reassessment.

Forty-nine healthy children with mean age 12.8 (SD: 6,6) who were referred to the Division of Endocrinology for growth evaluation but were found to be within the normal reference charts and willing to participate in the study, served as controls (Table 11). Informed consent was obtained from the parents of all participants prior to their inclusion in the study.

The study was approved by the Ethics Committee of the Aghia Sophia Children's Hospital and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Inclusion criteria for all participants were euthyroidism for at least 6 months prior to study enrollment as well as during follow-up. The exclusion criteria were the presence of active urinary tract infection, glucocorticoid medication, pregnancy, renal disease and any chronic disease other than T1D.

Table 11: Demographic characteristics of the patients and healthy controls that participated in the study

Demographic characteristics of T1D patients at baseline and controls							
	T1D			Controls			P-value
n	56			49			
Gender	Boys 57.1% (n= 32) Girls 42.9% (n= 24)			Boys 42.8% (n=21) Girls 57.2% (n=28)			0.31
	Mean	95% CI	SD	Mean	95% CI	SD	
Age	13.1	12.2- 13.9	3.2	12.8	10.9- 14.7	6.6	0.21
Age of diagnosis	8.5	7.6- 9.5	3.4	na (non applicable)			
BMI z-score	0.3	0.04 - 0.6	0.8	0.3	0.02 - 0.6	0.8	0.68
HbA1c	8.3 % (68 mmol/mol)	7.8 - 8.8% (63- 73	1.7	4.7 % (28 mmol/mol)	4.4 - 4.9 % (25-30	0.4	<0.001
T1D duration	4.5	3.6 - 5.5	3.4	na			
Microalbuminuria	18.5% presented microalbuminuria (10/56)			0 % presented microalbuminuria (0/49)			

Along with the study, we looked into the epidemiological data through a retrospective study of data acquisition on clinical manifestations during the long-term follow-up from the archives of our Diabetes Centre. We studied 567 individuals (51% males and 49% females) with type 1 diabetes diagnosed at the Division of Endocrinology, Metabolism and Diabetes, First Department of Pediatrics, University of Athens, ‘’Aghia Sophia’’ Children’s Hospital from 1990 to 2013. The diagnosis of T1D was based on the presence of the high titer of at least one and mostly two of the known autoantibodies related to type 1 diabetes mellitus. Specifically, the percentage (%) of positivity for each autoantibody tested was: for GADA (72%), IA2 (71%), IAA

(45%) and Islet Cell Cytoplasmic Autoantibodies (ICA) (35%). Fifty-seven percent of these patients presented with ketoacidosis at diagnosis. The urinary albumin excretion was assessed in 196 of the 567 patients.

2.2.1 Specimen collection, transportation and storage

After 12-hour fasting, a morning blood sample was obtained for blood urea nitrogen, creatinine (sCr), uric acid (UA), total cholesterol (tot chol), low density cholesterol (LDL), high density cholesterol (HDL), triglycerides and Glycated hemoglobin (HbA1c) determination. Blood was collected in special vials to be used for measurement of the specific markers NGAL, YKL-40, GDF-15 and Cystatin C. Part of the blood was centrifuged for separation of serum and stored at -80°C, until final assessments. Morning urine sample was also collected in special vials to be used for measurement of NGAL. Urinary albumin excretion was determined from a 24-hour urine collection.

2.2.1.1 Glycosylated hemoglobin (HbA1c) assessment

The assessment of HbA1c was carried out using the HPLC method, via Biorad's DIAMAT and VARIANT TM2 (DiaMed GmbH, Switzerland).

2.2.1.2 Urea, creatinine and uric acid measurement

Blood chemistry, including blood urea nitrogen (BUN), creatinine and uric acid was performed using Siemens ADVIA 1800 Clinical Chemistry Analyser (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

2.2.1.3 Microalbuminuria analysis

Urinary albumin excretion was determined from a 24-hour urine collection (microalbuminuria was defined with values between 30–300 mg/24 hours, measured on at least two of three measurements over a two- to three-month period). Urinary albumin was determined by nephelometry [(nephelometer Turbox) Orion, Espoo,

Finland], with commercially available reagents, and was expressed in micrograms per minute.

2.2.1.4 Serum NGAL analysis

Serum NGAL levels were measured using a commercially available ELISA (Bioporto, Gentofte, Denmark). The intra- and inter-assay coefficients of variation (CVs) were 5.6% and 6.4%, respectively. In this assay, the lowest detection limit of NGAL is 0.1 ng/ml and assay range is 0.2-20 ng/ml.

2.2.1.5 Urinary NGAL analysis

Urinary NGAL levels were measured using a commercially available ELISA (Bioporto, Gentofte, Denmark). The intra- and inter-assay coefficients of variation (CVs) were 5.6% and 6.4%, respectively. In this assay, the lowest detection limit of NGAL is 0.1 ng/ml and assay range is 0.2-20 ng/ml.

2.2.1.6 Serum GDF15 analysis

The serum GDF-15 concentrations were measured using a commercial enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). In this assay, the lowest detection limit of GFD-15 is 2.0 pg/ml. The intra-assay coefficient of variation range from 1.8-2.8%, while the inter-assay coefficient of variation range from 5.6-6.0% according to the manufacturer.

2.2.1.7 Serum YKL40 analysis

Serum YKL-40 levels were measured by a commercial two-site sandwich-type enzyme-linked immunosorbent assay (Quidel Corporation, San Diego, CA). In this assay, the lowest detection limit of YKL-40 is 5.4 ng/ml. The intra-assay coefficient of variation range from 5.6-6.6%, while the inter-assay coefficient of variation range from 6-7% according to the manufacturer.

2.2.1.8 Serum cystatin C analysis

CysC concentration was measured by an immunonephelometric technique using the BN Prospec nephelometer (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany). The nephelometric method is more sensitive and performs optimally in dilute solution, making it preferable for small sample volumes encountered in the pediatric population. With a range of 0.23–7.25 mg/L, this assay is currently the most precise automated assay across the clinical concentration range. The inter-assay coefficient of variation (CV) for the assay was 5.05% and 4.87% at mean concentrations of 0.97 and 1.90 mg/L, respectively.

2.3 Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). Correlation analysis is used to determine whether the values of two variables are associated using Pearson parametric correlation (Pearson's correlation coefficient r with P-value). Student t-test was performed as appropriate (paired samples t-test were used to test the null hypothesis that the average of the differences between a series of paired observations is zero when performed on the same subjects or independent samples t-test when performed between controls' and patients' group). Multiple regression analysis was a method used to examine the relationship between one dependent variable and one or more independent variables. The significance was defined at p-value <0.05, rho and 95% confidence interval (CI) for the correlation coefficient.

2.4 Results

2.4.1 Estimated Glomerular Filtration Rate (eGFR)

Paired samples t-test of repeated measurements revealed that mean S-eGFR value in T1D patients was increased at re-evaluation (mean S-eGFR at baseline= 90.72 with

SD= 19.8, mean S-eGFR at reevaluation= 97.5 with SD= 17.5, n=56, p=0.003) (figure 1). The same analysis showed a significantly increased CysC-eGFR at reevaluation (mean value at baseline= 96, 81 with SD= 11.23 mean CysC-eGFR at reevaluation= 101.44 with SD= 12.13, n=56, p=0.002) (figure 2) and an increase of L-eGFR at reevaluation, but this result was not statistically significant (mean value at baseline=120.7, mean value at reevaluation= 122.7, n=56). Regarding S-eGFR, 13 patients were found to have a decreased value at reevaluation, while for CysC-eGFR and L-eGFR, 14 and 23 patients respectively had a decreased value at reevaluation.

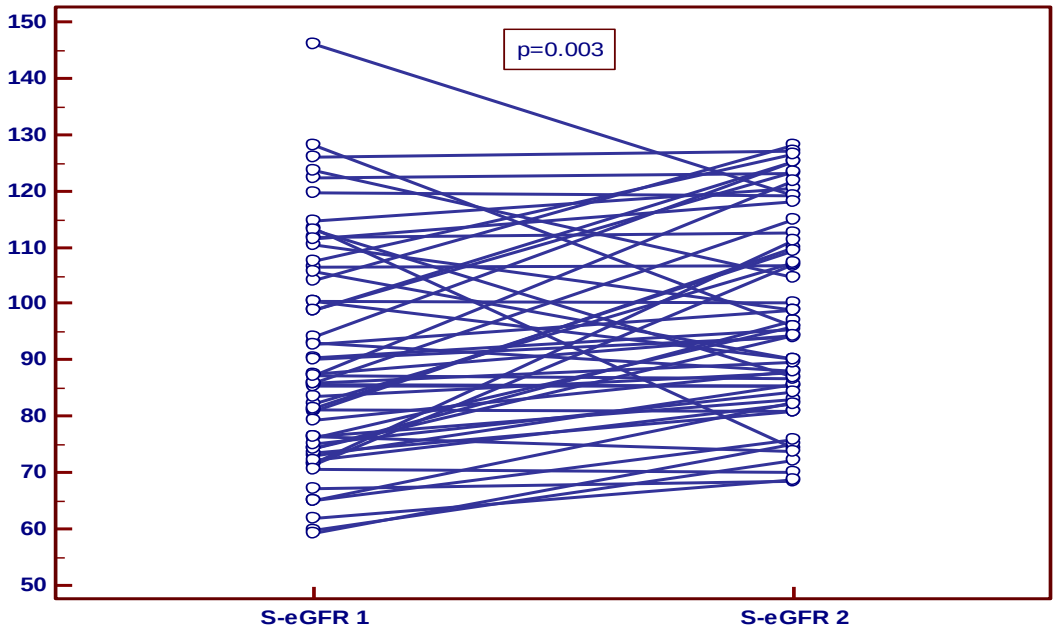


Figure 1: Paired samples t-test of repeated measurements revealed that mean S-eGFR (bedside formula Schwartz) value) in T1D patients was increased at re-evaluation (p=0.003) (1= at baseline, 2= after 12-15 months)

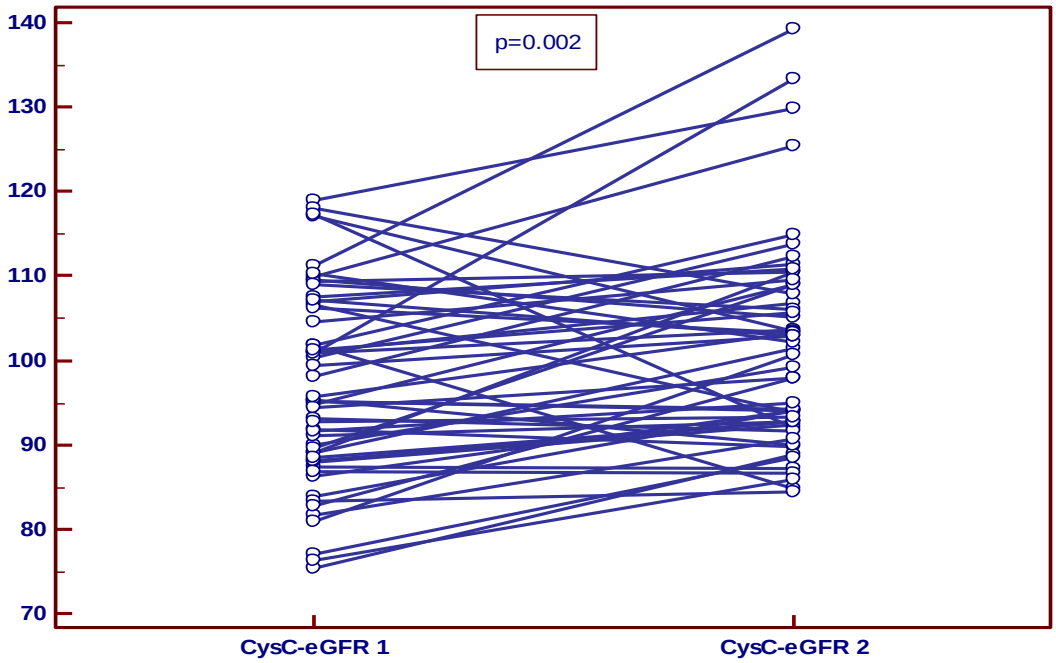


Figure 2: Paired samples t-test of repeated measurements revealed that mean CysC-eGFR (Schwartz et al. recently suggested pediatric eGFR formula for immunonephelometric measured CysC) in T1D patients was increased at re-evaluation (p=0.002) (1= at baseline, 2= after 12-15 months)

2.4.2 Urinary albumin excretion

The epidemiological study showed that 60 patients (30, 6%) from a total number of 196 who were examined for urinary albumin excretion, were found to perform microalbuminuria (MA). Survival analysis with Kaplan-Meier survival curve showed a mean age of microalbuminuria 15, 7 years with no statistically significant difference between the two genders (Figure 3).

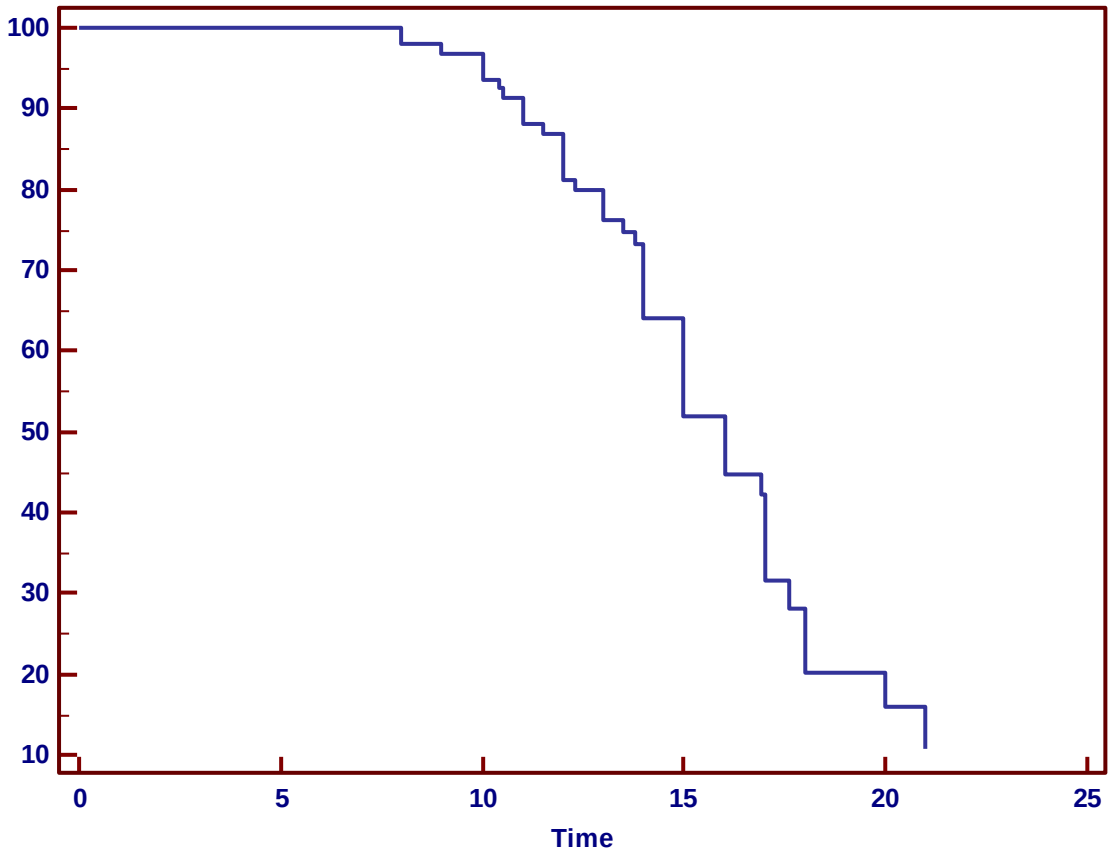


Figure 3: Survival analysis with Kaplan-Meier survival curve showed a mean age of microalbuminuria 15, 7 years

Another analysis with Kaplan-Meier survival curve showed a mean age of microalbuminuria 13,6 years for the patients who presented DKA at onset of the T1D, while the mean age for MA for those who had no DKA in their history was 17,4 years (p=0.024)(Figure 4). Paired samples t-test of repeated measurements revealed that mean value of urinary albumin in T1D patients was not significantly different between the two times of evaluation.

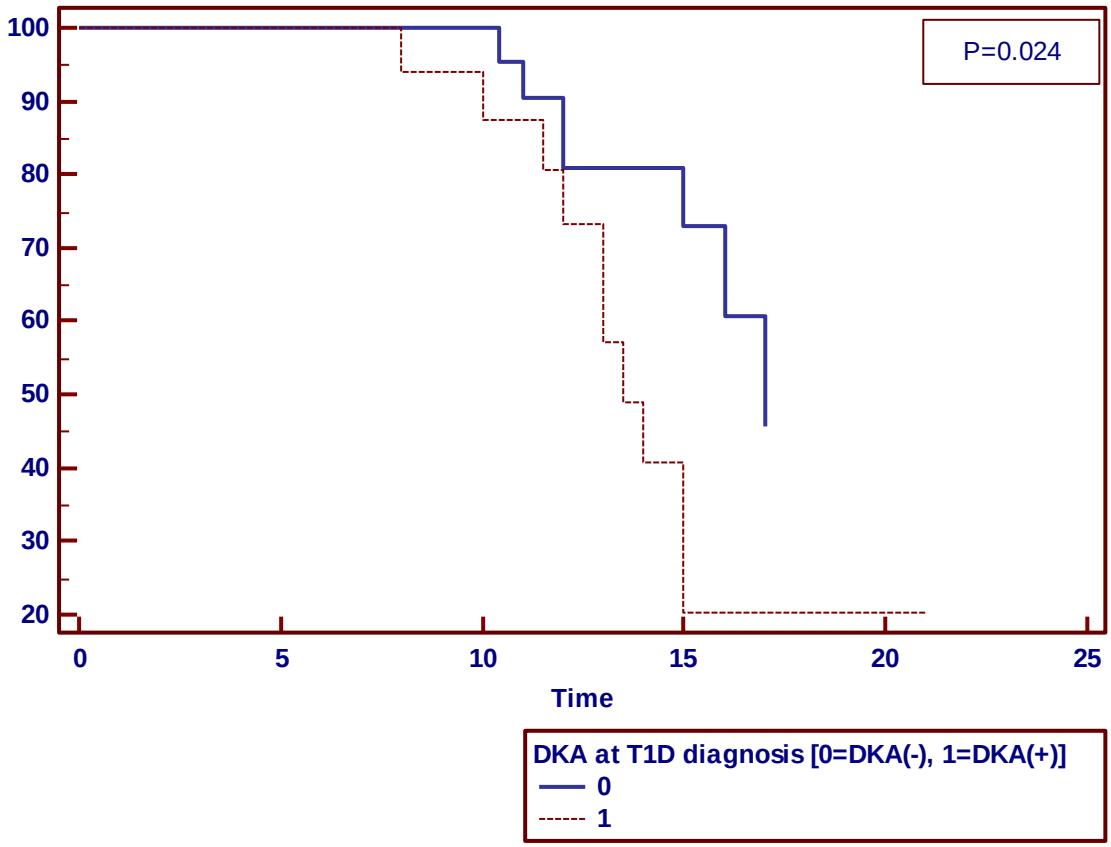


Figure 4: Kaplan-Meier survival curve showed a mean age of microalbuminuria 13,6 years for the patients who presented DKA at T1D onset, while the mean age for MA for those who had no DKA at onset was 17,4 years(p=0.024)

The results from the main study regarding microalbuminuria were the following:

MA had a positive correlation with the duration of T1D (r=0.36, p=0.02, n=56) and the advanced pubertal stages in males (r=0.46, p=0.01, n=56).

Microalbuminuria had a negative correlation with creatinine (r=-0.27, p=0.046, n=54 and r=-0.30, p=0.027, n=56 respectively) and BUN (r=-0.38, p=0.004, n=56 and r=-0.30, p=0.026, n=56 respectively) at both time-points of the evaluation. MA had a positive correlation with eGFR at both time-points of evaluation regarding CysCeGFR (r=0.32, p=0.017, n=56 and r=0.32, p=0.021, n=56) and S-eGFR (r=0.36, p=0.007, n=56 and r=0.43, p=0.0014, n=56) (figure 5).

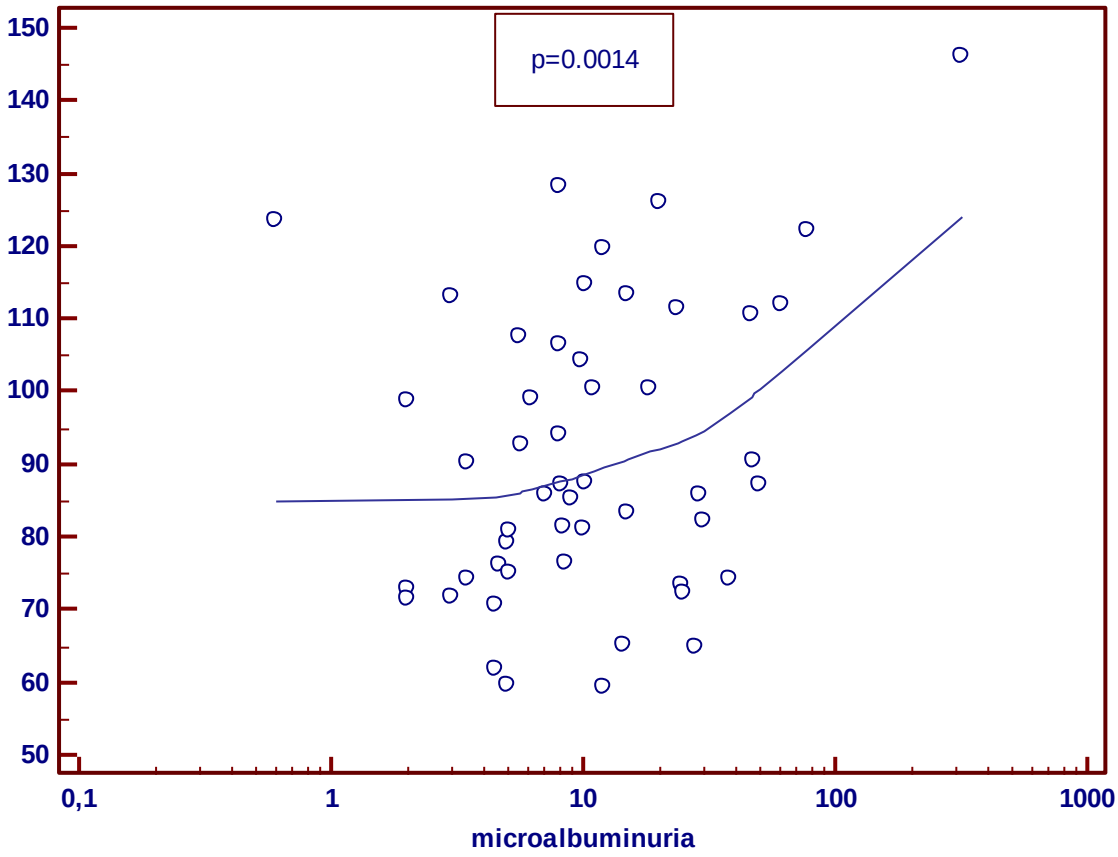


Figure 5: Microalbuminuria had a positive correlation with S-eGFR at both time-points of evaluation (p=0.0014)

No statistically significant correlation was found between MA and L-eGFR.

MA had a positive correlation with LDL at both time-points of evaluation (r=0.35, p=0.010, n=56 and r=0.43, p=0.004, n=56).

MA at baseline, had a positive correlation with DAP z-score at re-assessment (r=0.29, p=0.021, n=56) (Figure 6).

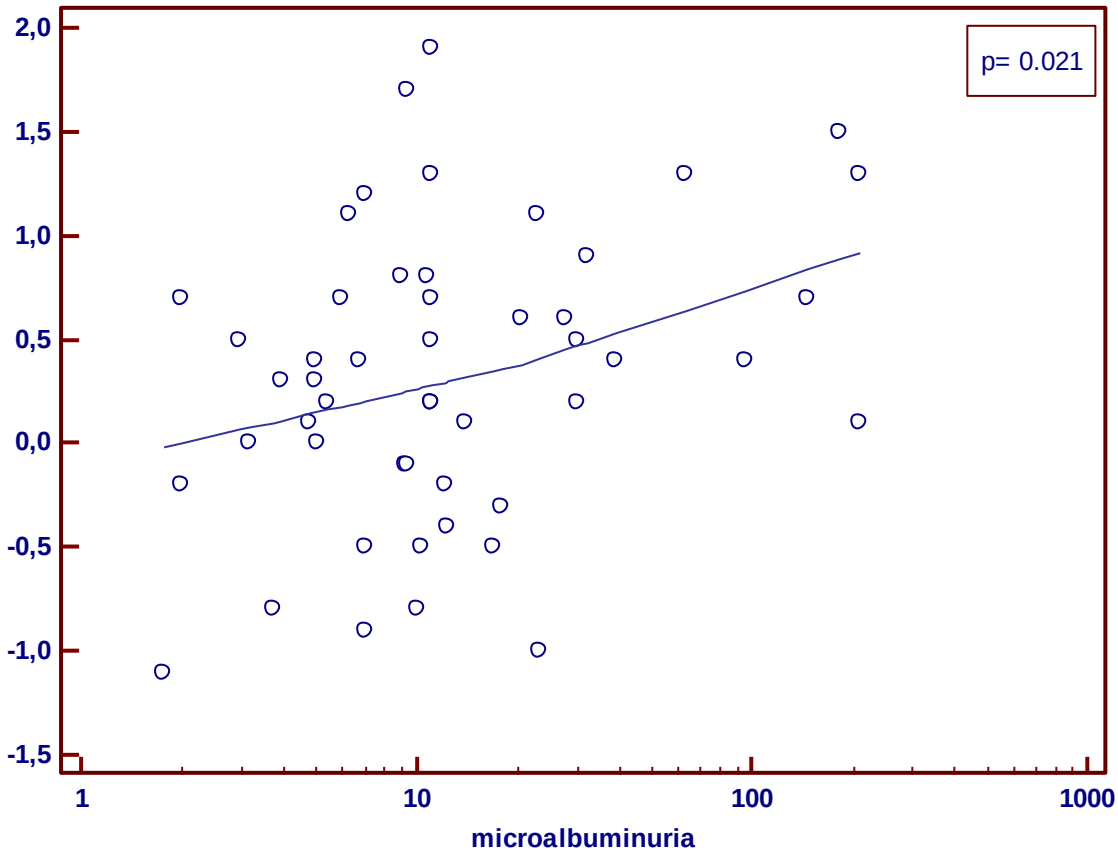


Figure 6: Microalbuminuria had a positive correlation with DAP z-score at re-evaluation

BMI z-score at baseline had a positive correlation with MA of reevaluation (r=0.36, p=0.027, n=56).

2.4.3 Serum Uric acid

Mean value of uric acid (UA) at baseline was not significantly different between T1D patients and healthy controls (mean: 3. 4 mg/dl for T1D patients, mean for controls: 3.1 mg/dl) according to Student t-test statistical analysis for independent samples. Paired samples t-test of repeated measurements revealed that mean UA value in T1D patients was not significantly different at re-evaluation (mean: 3. 5 mg/dl).

2.4.3.1 Serum uric acid and eGFR

UA at baseline had a negative correlation with both CysC-eGFR and S-eGFR at reevaluation (r=-0.40, p=0.005, n=56 and r=-0.40, p=0.004, n=56 respectively) (figure 7).

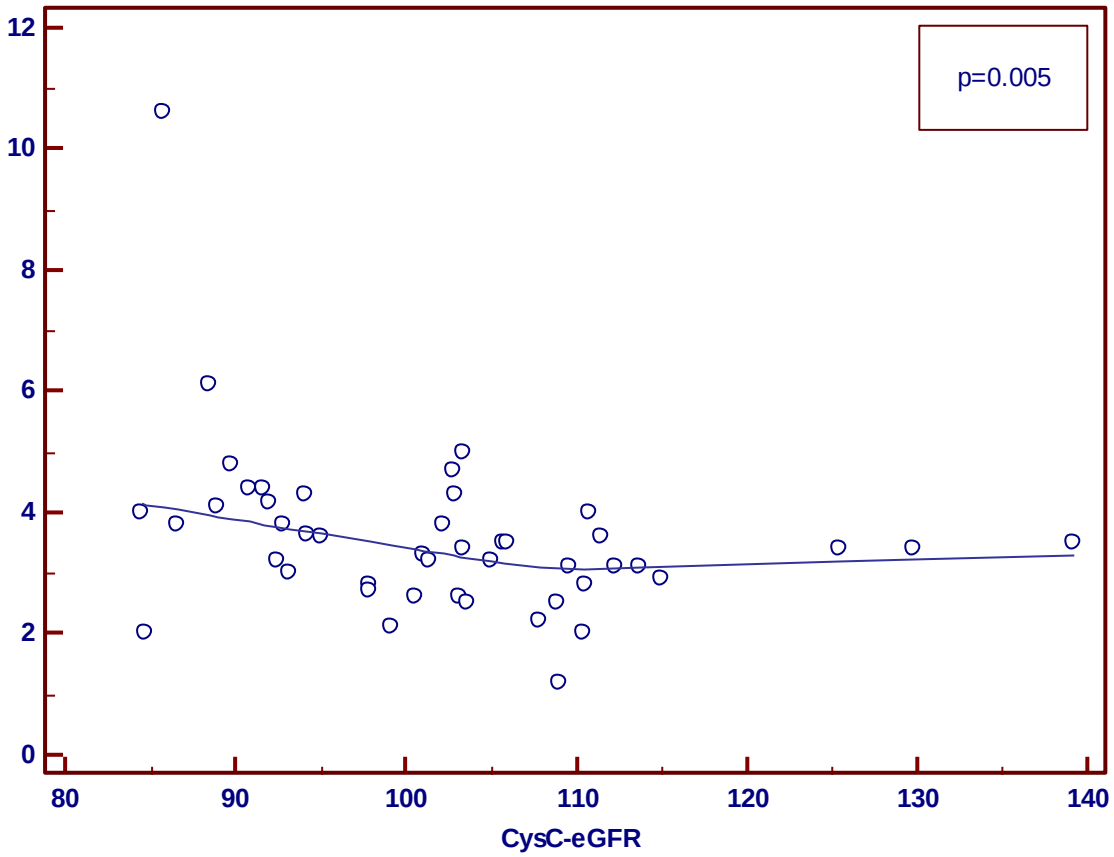


Figure 7: Uric acid at baseline had a negative correlation with CysC-eGFR at re-evaluation (p=0.005)

2.4.3.2 Serum uric acid and other biomarkers (Creatinine, Cystatin C, microalbuminuria, GDF-15, YKL-40, lipid profile, serum NGAL, urinary NGAL)

Uric acid had a positive correlation with serum creatinine at both time-points of assessment (r=0.39, p=0.005, n=56 and r=0.37, p=0.026, n=56 respectively). Moreover UA assessed at baseline was found to have a stronger positive correlation with creatinine at reevaluation (r=0.50, p=0.0004, n=56). At reevaluation, UA

correlated positively with Cystatin C (r=0.40, p=0.016, n=56). UA did not have any significant correlation with microalbuminuria, GDF-15, YKL-40, lipid profile, serum NGAL and urinary NGAL.

2.4.3.3 Serum uric acid and Arterial Pressure

No statistically significant correlations were found between UA and SAP- or DAP z-score.

2.4.3.4 Serum uric acid and diabetes control

No statistically significant correlations were found between UA and HbA1c.

2.4.3.5 Serum uric acid, puberty and somatometric parameters

UA had positive correlations with advanced pubertal stages regarding the pubertal stages of pubic hair and the increased testicular volume at both time-points of evaluation [r=0.41, p=0.010, n=56 and r=0.37, p=0.029, n=56 (pubertal stages for pubic hair) and r=0.49, p=0.020, n=25 and r=0.65, p=0.0016, n=25 (testicular volume)]. A positive correlation was also revealed between UA and height at both time-points of assessment (r=0.33, p=0.027, n=56 and r=0.40, p=0.013, n=56 respectively).

2.4.3.6 Serum uric acid and diabetes duration

Uric acid had a positive correlation with age at both time-points of assessment(r=0.313, p=0.031, n=56 and r=0.313, p=0.027, n=56) but it had no statistically significant correlation with the duration of the disease.

2.4.4 Serum NGAL

Mean value of sNGAL at baseline was not significantly different between T1D patients and controls (mean: 59.5 ng/ml for T1D patients, mean for controls: 62.6 ng/ml, p=0.393), but sNGAL mean value of T1D patients at reevaluation (mean: 67.6 ng/ml) was statistically significantly higher than the mean value of the controls' group (p<0.001) according to Student t-test statistical analysis for independent samples. Paired samples t-test of repeated measurements revealed that mean NGAL value in T1D patients was increased at re-evaluation (p=0.032) (Figure 8).

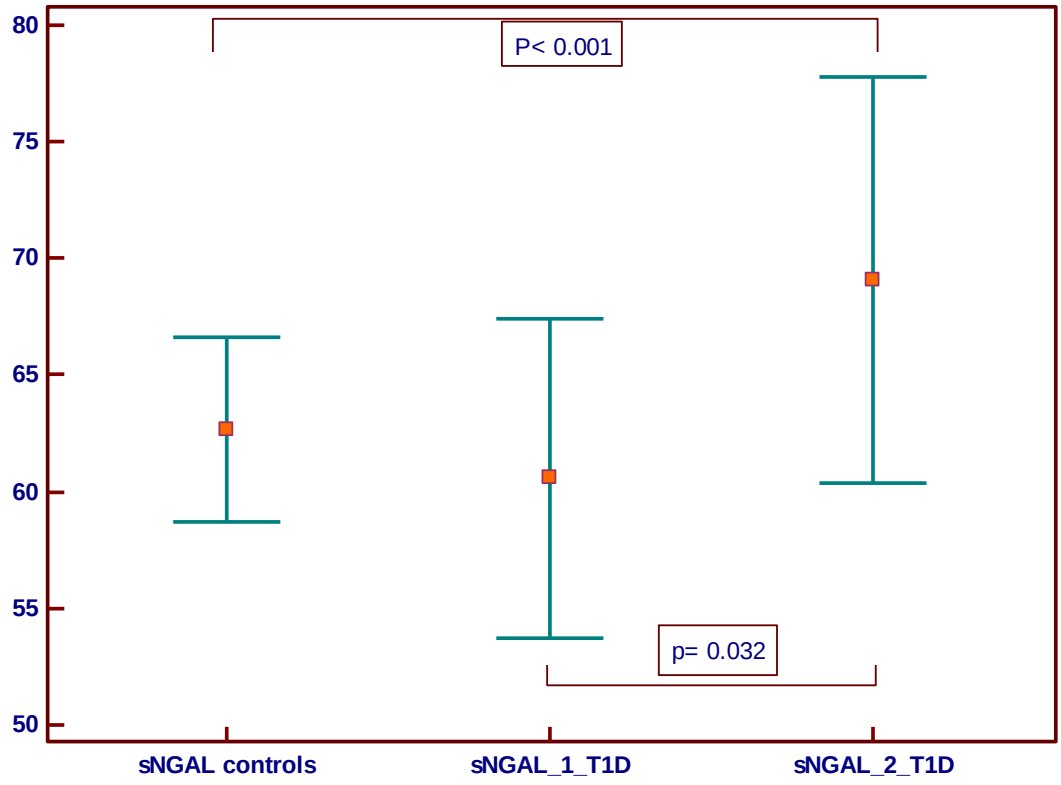


Figure 8: Mean value of sNGAL at baseline was not significantly different between T1D patients and controls (p=0.393), but sNGAL mean value of T1D patients at reevaluation was statistically significantly higher than the mean value of the controls' group (p<0.001). Mean sNGAL value in T1D patients was increased at re-evaluation (p=0.032) (1= at baseline, 2= after 12-15 months)

2.4.4.1 Serum NGAL and eGFR

At baseline, regarding the T1D patients' group, NGAL had no significant correlation either with S-eGFR or with CysC-eGFR, but it was negatively correlated with L-eGFR (r=-0.35, p=0.007, n=56). Moreover, at re-evaluation, NGAL had a negative correlation with S-eGFR (r=-0.26, p=0.049, n=56), CysC-eGFR(r=-0.31, p=0.019, n=56) and L-eGFR (r=-0.33, p=0.002, n=56) (figure 9).

In addition, the sNGAL values at baseline, were found to have a statistically significant negative correlation with the L-eGFR at reevaluation(r=-0.32, p=0.014, n=56) (figure 10).

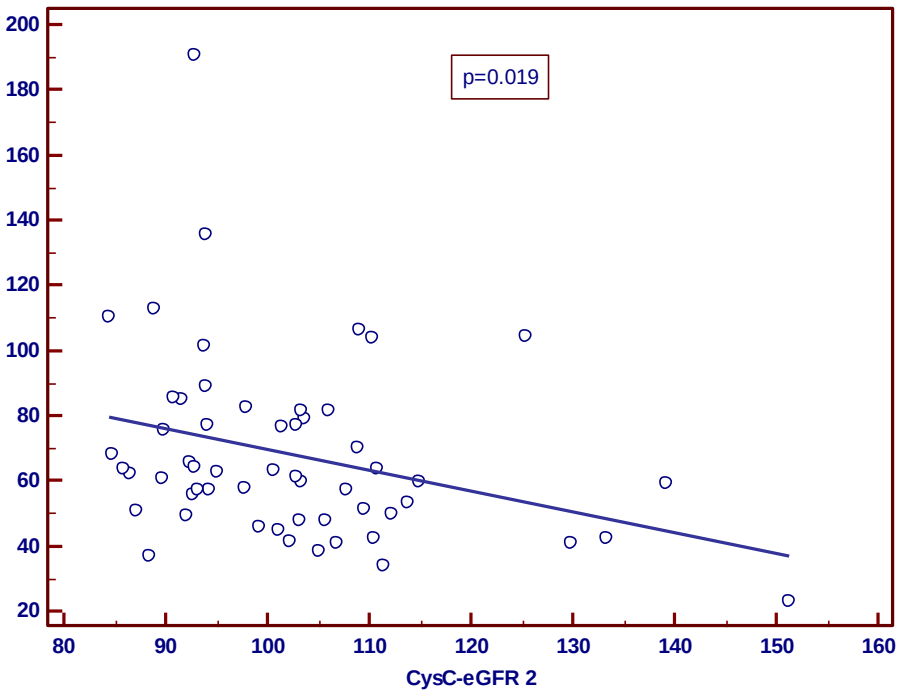


Figure 9: At 12-15 months' evaluation NGAL had a negative correlation with CysC-eGFR (p=0.019)

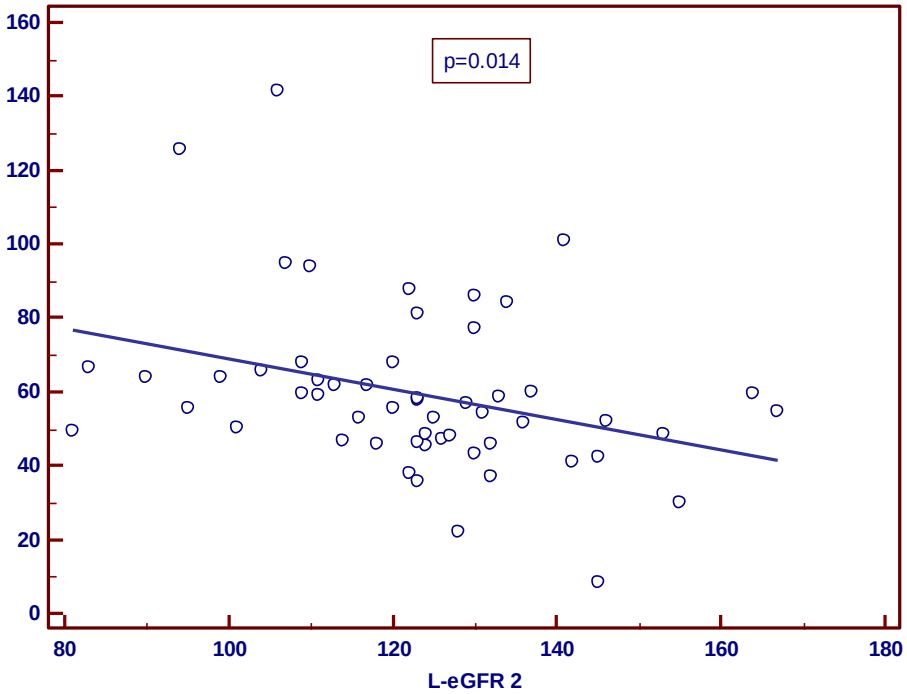


Figure 10: sNGAL values at baseline (1) had negative correlation with L-eGFR at re-evaluation (2) (p=0.014)

2.4.4.2 Serum NGAL and other biomarkers (Creatinine, Uric Acid, Cystatin C, microalbuminuria, GDF-15, YKL-40, lipid profile, urinary NGAL)

At baseline, regarding T1D patients' group, sNGAL had no significant correlation with CysC but at reevaluation, a positive correlation between these markers was revealed ($r=0.41$, $p<0.001$, $n=56$) (Figure 11).

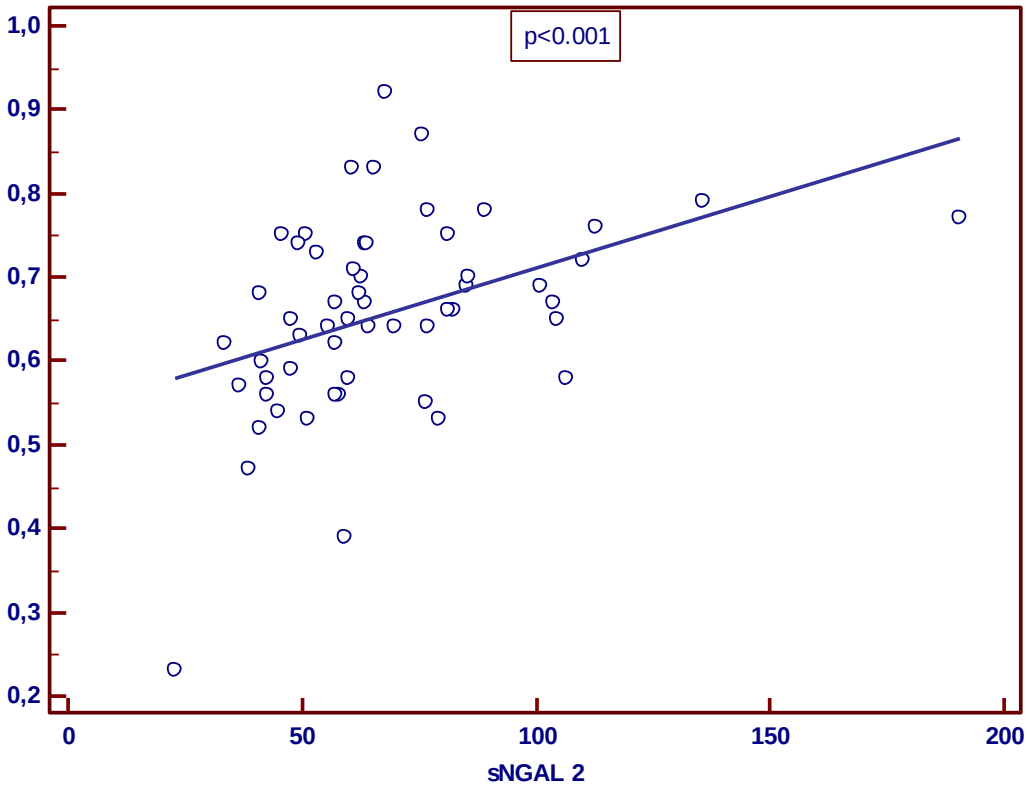


Figure 11: At 12-15 months' evaluation (2), sNGAL had a positive correlation with CysC (p<0.001)

At both time-points, sNGAL had a positive correlation with sCr (r=0.28, p=0.040, n=56 and r=0.32, p=0.010 n=56 respectively) (figure 12).

No statistically significant correlation was found between sNGAL and microalbuminuria, however regression analysis revealed that sNGAL values higher than 70 μg/L found to have a statistically significant positive correlation with microalbuminuria (r=0.29, p= 0.03, F-ratio: 4, 78).

SNGAL was found to correlate positively with YKL-40 at both time-points of evaluation (r= 0.35, p=0.007, n=56 and r= 0.31, p=0.021, n=56) (figure 13).

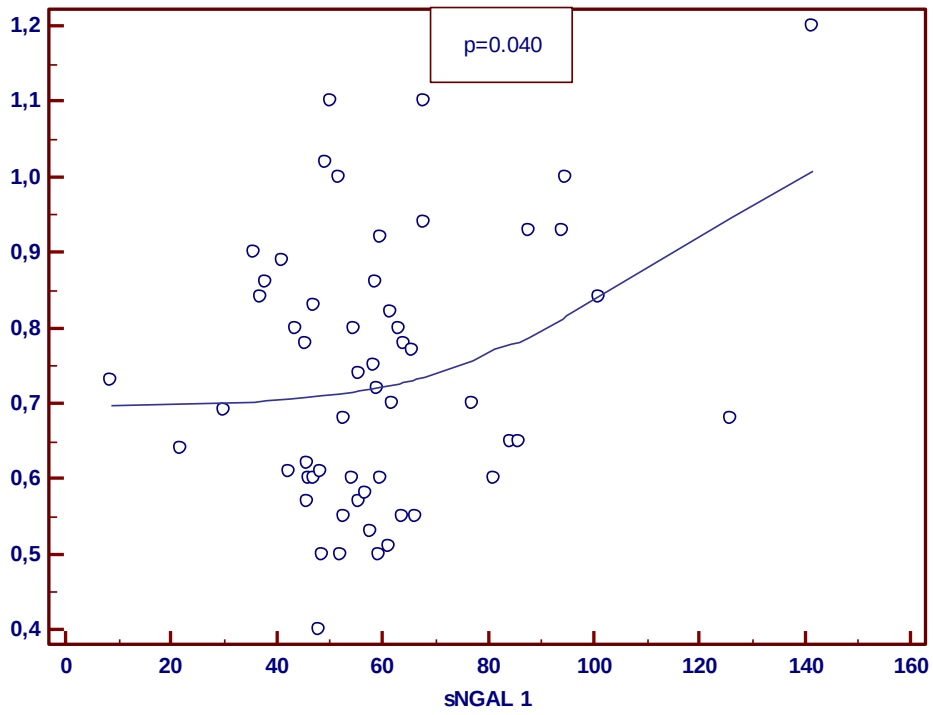


Figure 12: At baseline (1), sNGAL had a positive correlation with sCr (p=0.040)

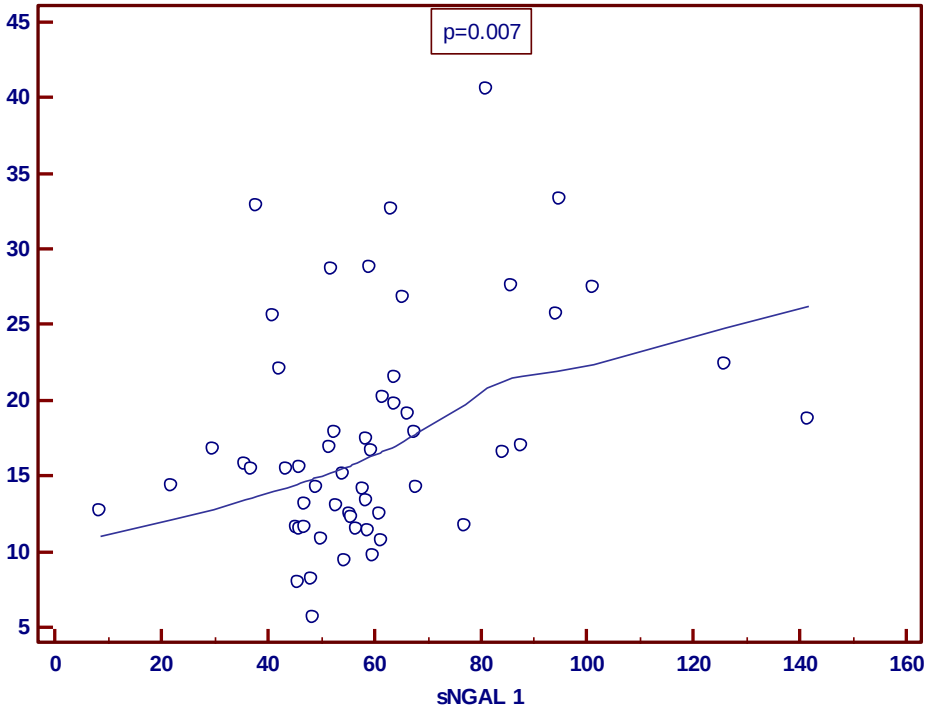


Figure 13: At baseline (1), sNGAL had a positive correlation with YKL-40 (p=0.007)

2.4.4.3 Serum NGAL and Arterial Pressure

sNGAL was positively correlated with SAP z-score (r=0.26, p=0.011, n=112) (figure 14). No significant correlation between sNGAL and DAP z-score was noted.

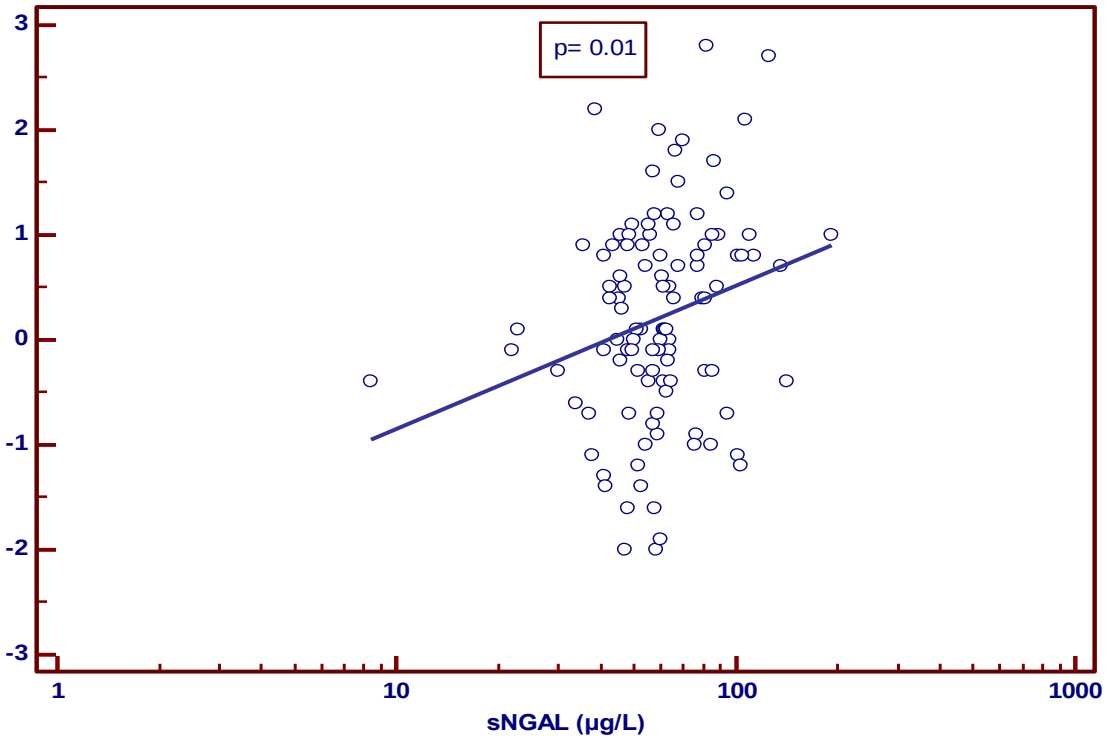


Figure 14: NGAL was positively correlated with SAP z-score (p=0.011)

2.4.4.4 Serum NGAL and diabetes control

HbA1c did not significantly correlate with NGAL either at baseline or at reevaluation.

2.4.4.5 Serum NGAL, puberty and somatometric parameters

A multiple regression analysis was performed between sNGAL at both time-points of assessment and stages of puberty but no statistically significant associations were revealed.

No correlation between sNGAL and either with BMI z-score or height z-score was revealed.

2.4.4.6 Serum NGAL and diabetes duration

The sNGAL values were found to correlate positively with the duration of T1D (p=0.04) (figure 15).

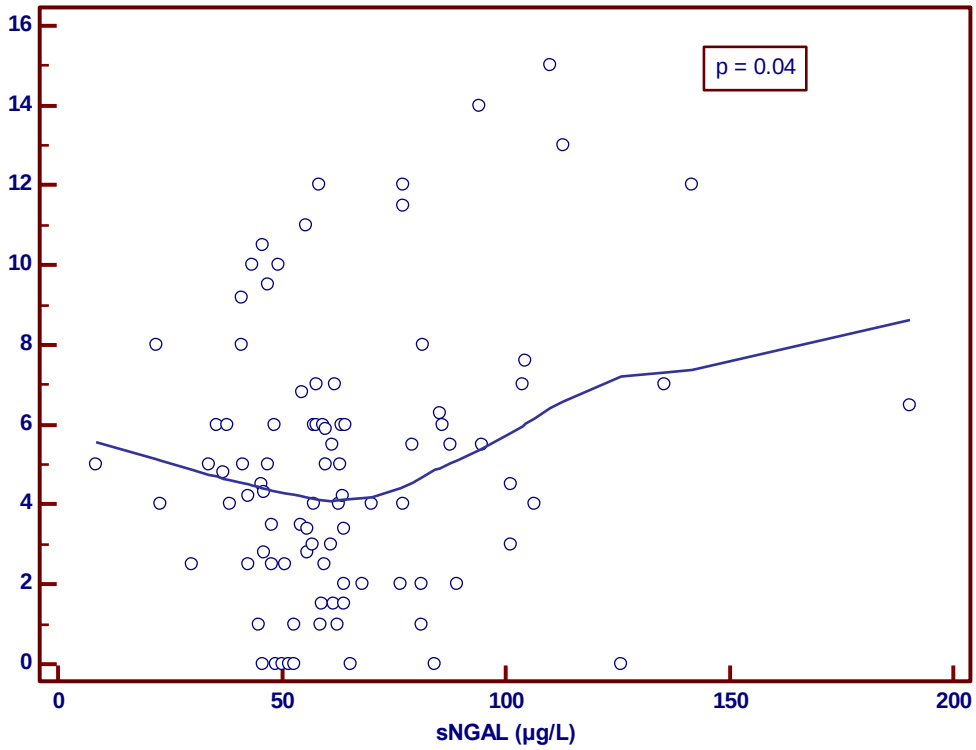


Figure 15: The sNGAL values were found to correlate positively with the duration of T1D (p=0.04)

2.4.5 Urinary NGAL

Mean value of uNGAL neither at baseline nor at reevaluation was significantly different between T1D patients (mean: 22.7 ng/ml and 33.6 ng/ml respectively) and controls (mean: 21,1 ng/ml) according to Student t-test statistical analysis for independent samples. Paired samples t-test of repeated measurements revealed that mean uNGAL value in T1D patients was not statistically significantly increased at re-evaluation (p=0.090).

2.4.5.1 Urinary NGAL and eGFR

No statistically significant correlation was found between uNGAL and eGFR estimated with the three different formulas mentioned above.

2.4.5.2 Urinary NGAL and other biomarkers (Creatinine, Uric Acid, Cystatin C, microalbuminuria, GDF-15, YKL-40, lipid profile)

Microalbuminuria had a positive correlation with uNGAL at reevaluation (r=0.33, p=0.012, n=56) (figure 16).

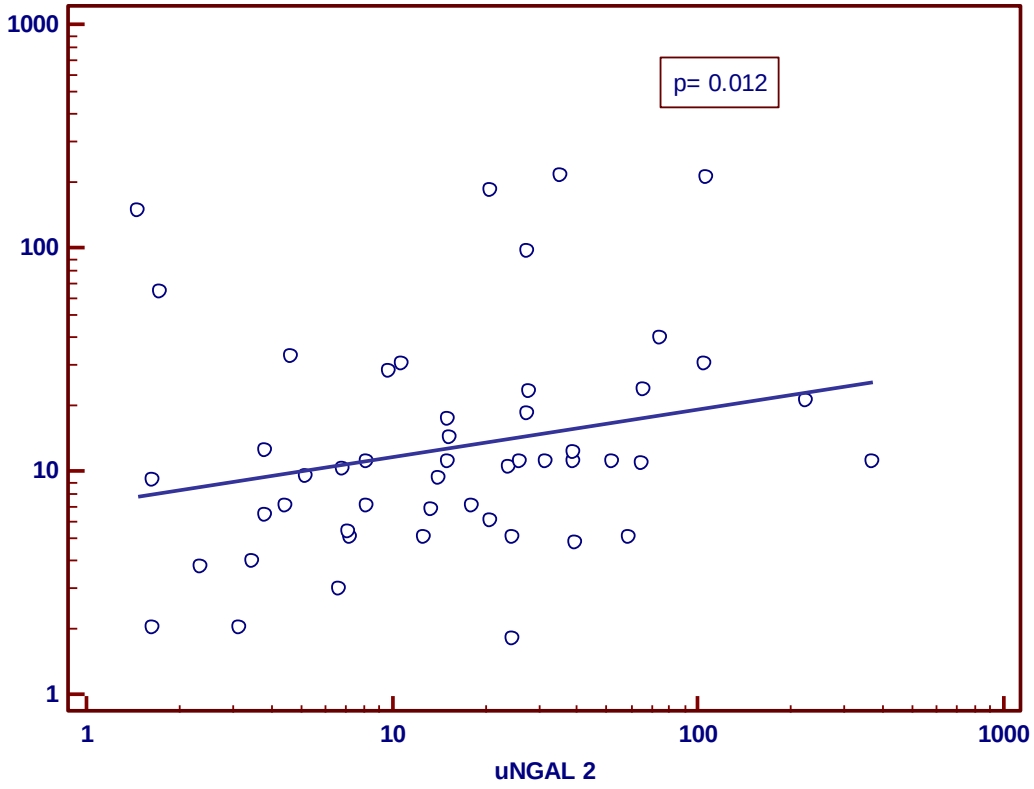


Figure 16: At 12-15 months' evaluation (2), microalbuminuria had a positive correlation with uNGAL (p=0.012)

UNGAL was found to correlate positively with LDL in both times of evaluation (r=0,36, p=0,014, n=56 and r=0,32, p=0,037 n=56 respectively) (figure 17) and Cholesterol values (r=0.24, p=0.021) at re-evaluation.

No statistically significant correlation was found between uNGAL and urea, creatinine, GDF-15, YKL-40, HDL, triglycerides, sNGAL or Cystatin C.

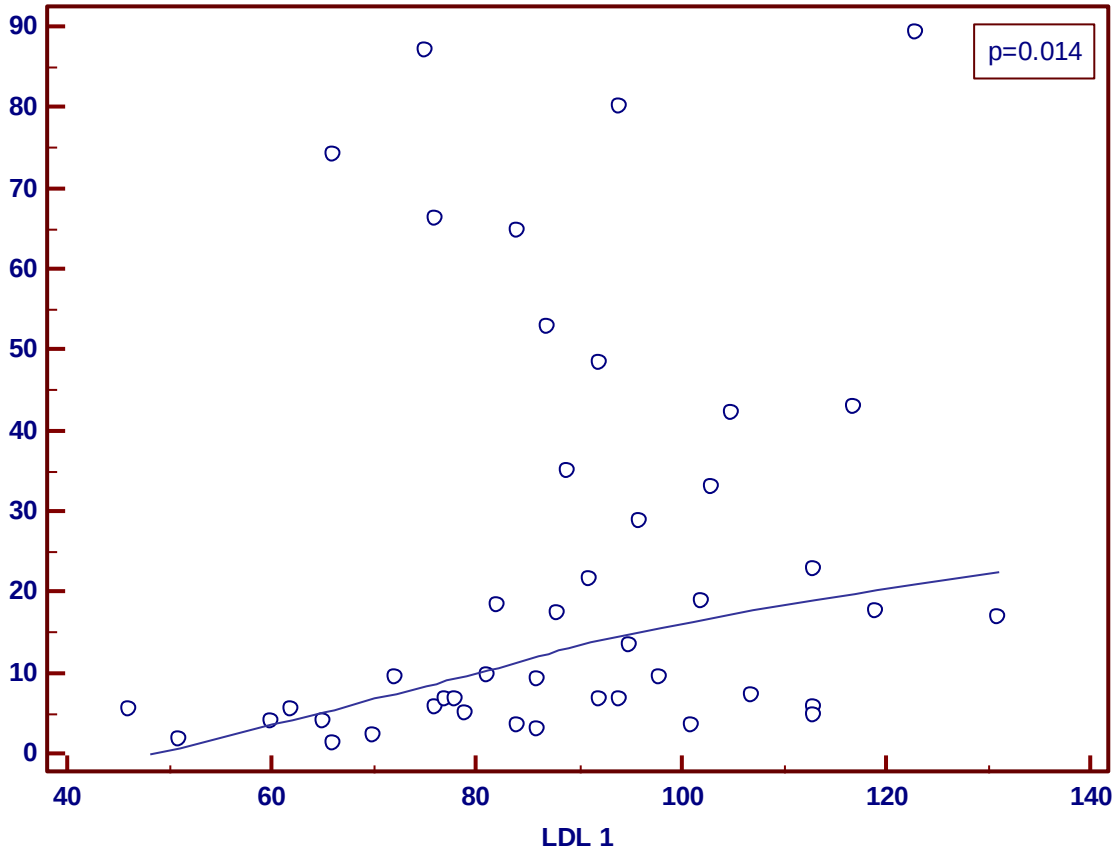


Figure 17: At baseline (1), uNGAL had a positive correlation with LDL (p=0.014)

2.4.5.3 Urinary NGAL and Arterial Pressure

No statistically significant correlation was found between uNGAL and systolic or diastolic arterial pressure z-scores

2.4.5.4 Urinary NGAL and diabetes control

No statistically significant correlation was found between uNGAL and the Glycated hemoglobin.

2.4.5.5 Urinary NGAL, puberty and somatometric parameters

The Urinary NGAL values were found to be significantly higher in females (r=0.35, p=0.004) but no association was revealed between uNGAL and the advanced Tanner stages of puberty in females. On the other hand, uNGAL correlated positively with testicular volume (r=0.33, p=0.021, n=49) (figure 18).

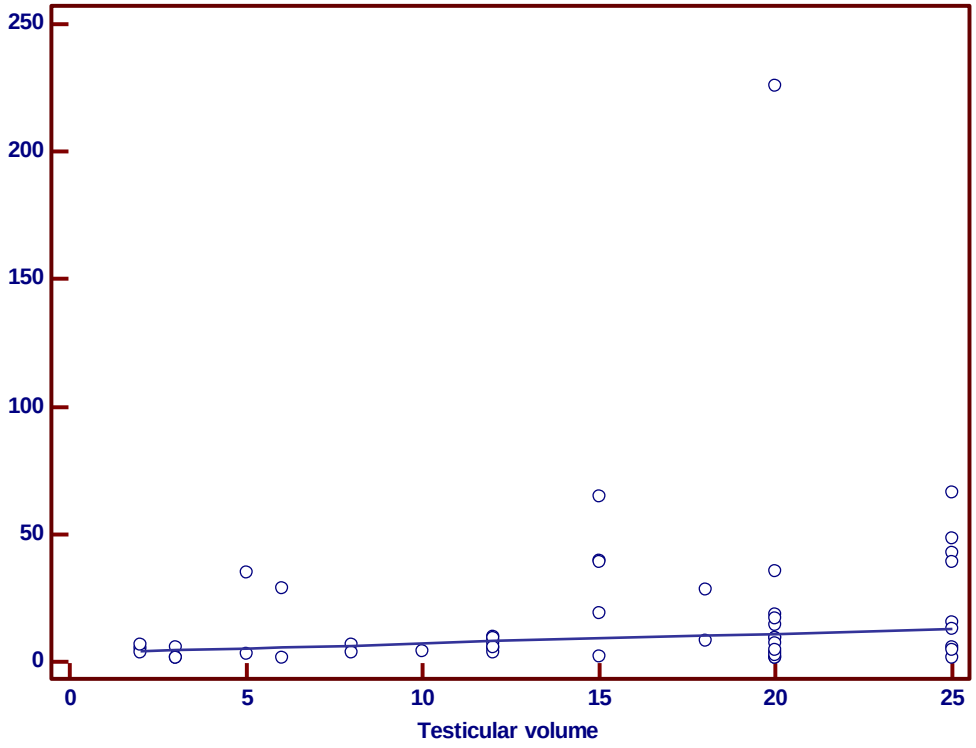


Figure 18: uNGAL correlated positively with the increased testicular volume (p=0.021)

No statistically significant correlation was found between uNGAL and body mass index z-score (BMI z-score) in both genders.

2.4.5.6 Urinary NGAL and diabetes duration

No statistically significant correlation was found between uNGAL and diabetes duration.

2.4.6 Cystatin C

Paired sample t-test of repeated measurements revealed that CysC mean value in T1D patients did not significantly differ between the two time points of assessment (p=0.61). No significant difference was observed between healthy controls' and T1D patients' CysC measurements (p=0.21).

2.4.6.1 Cystatin C and eGFR

Cystatin C value at baseline correlated negatively with S-eGFR of re-evaluation (r= -0.29, p= 0.028, n=56) (figure 19). Moreover, at re-evaluation, CysC had a negative correlation with S-eGFR (r= -0.29, p= 0.025, n=56).

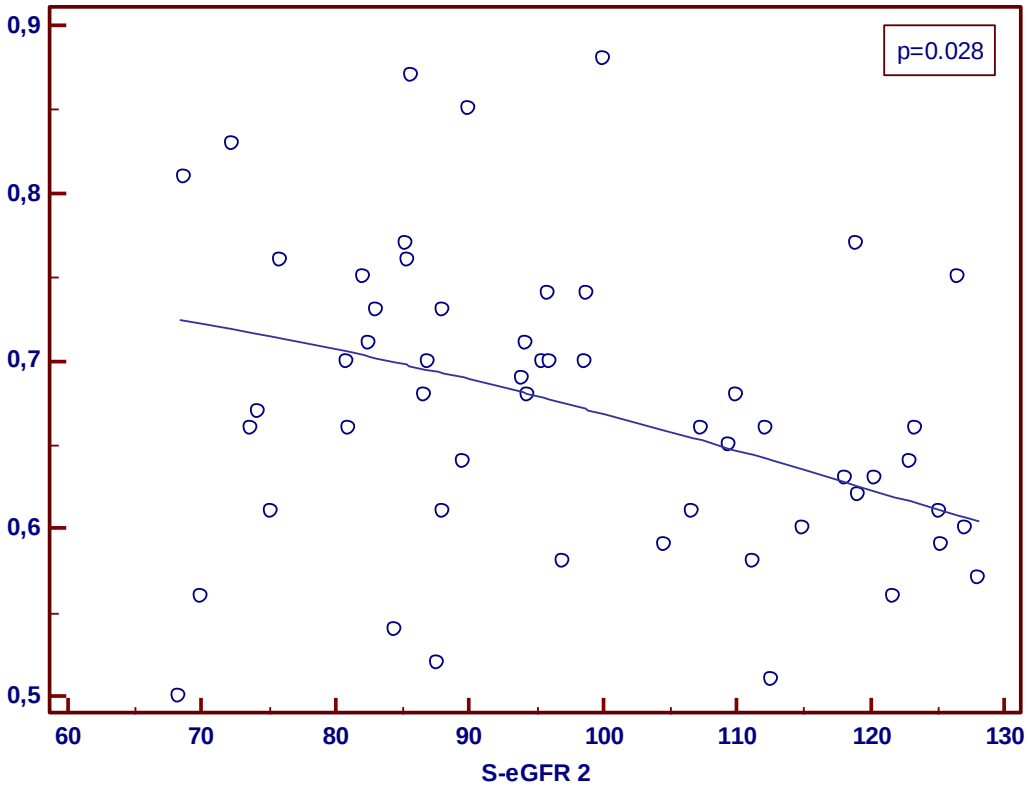


Figure 19: Cystatin C value measured at baseline (1) correlated negatively with S-eGFR assessed at re-evaluation (2) (p= 0.025)

2.4.6.2 Cystatin C and other biomarkers (Creatinine, Uric Acid, Cystatin C, microalbuminuria, GDF-15, YKL-40, lipid profile)

Baseline Cystatin C, correlated positively with creatinine value at reevaluation (r=0.37, p=0.006, n=56) (figure 20). Cystatin C at reevaluation, had a positive correlation with creatinine at the same point of time (r=0.35, p=0.009, n=56).

Cystatin C at both time-points of evaluation, had a positive correlation with uric acid (r=0.28, p=0.049, n=56 and r=0.41, p=0.014, n=56 respectively).

No significant correlation between Cystatin C and microalbuminuria, HDL, LDL, triglycerides, BUN, GDF-15 or YKL-40 was found.

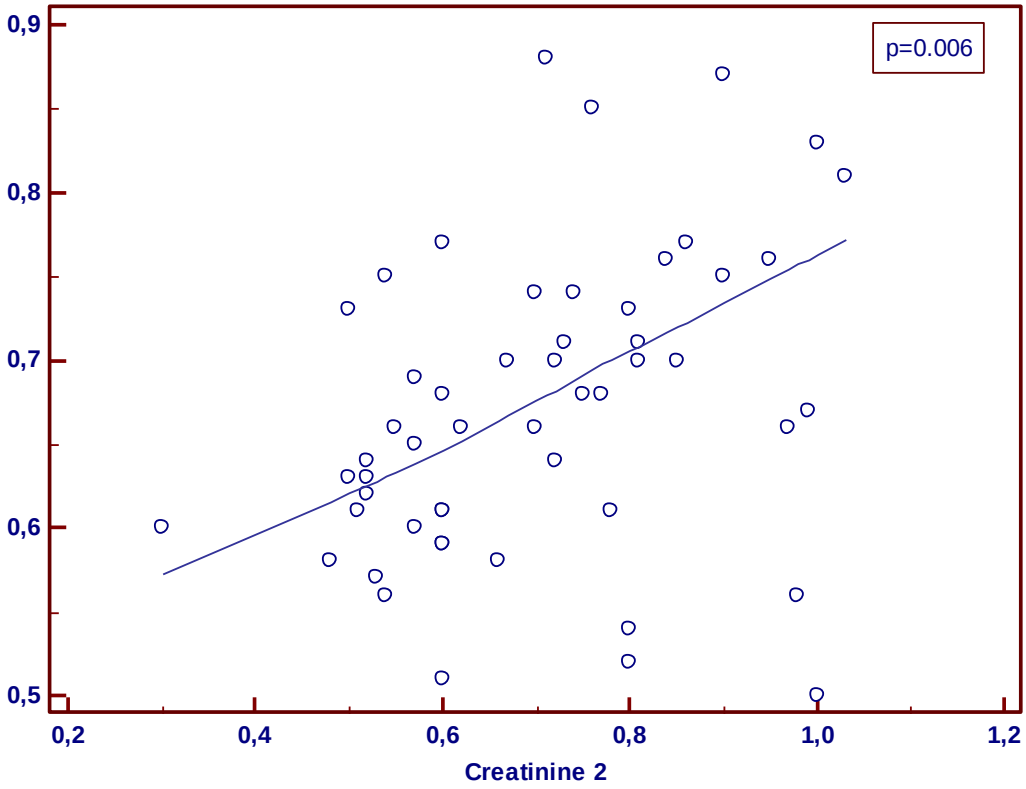


Figure 20: Cystatin C measured at baseline correlated positively with Creatinine at re-evaluation (2) (p=0.006)

2.4.6.3 Cystatin C and Arterial Pressure

No statistically significant correlation was found between Cystatin C and SAP z-score or DAP z-score (p=0.46).

2.4.6.4 Cystatin C and diabetic control

Cystatin C had no statistically significant correlation with HbA1c.

2.4.6.5 Cystatin C, puberty and somatometric parameters

A multiple regression analysis was performed regarding Cystatin C for BMI z-score and pubertal stages but no statistically significant correlations were revealed.

2.4.6.6 Cystatin C and diabetes duration

No statistically significant correlation was found between CystatinC and either age (p=0.79) or T1D duration (p=0.44).

2.4.7 YKL-40

Paired sample t-test of repeated measurements revealed that YKL-40 mean value in T1D patients did not significantly differ between the two time points of assessment (p=0.07). No significant difference was observed between controls' and T1D patients' YKL-40 values (p=0.21).

2.4.7.1 YKL-40 and eGFR

No statistically significant correlation was found between YKL-40 and the eGFR estimated with the three different equation formulas.

2.4.7.2 YKL-40 and other biomarkers (Creatinine, Uric Acid, Cystatin C, microalbuminuria, GDF-15, lipid profile)

We found no statistically significant correlations between YKL-40 and Uric Acid, Cystatin C, microalbuminuria, LDL or HDL.

YKL-40 correlated positively with total Cholesterol values at both time-points of assessment (r=0.29, p=0.042, n=56 and r=0.44, p=0.001, n=56) (figure 21) and triglycerides (r=0.47, p=0.0006, n=56) at re-evaluation.

YKL-40 correlated positively with GDF-15 at both time-points of evaluation (r=0.55, p<0.0001, n=56 and r=0.36, p=0.006, n=54 respectively).

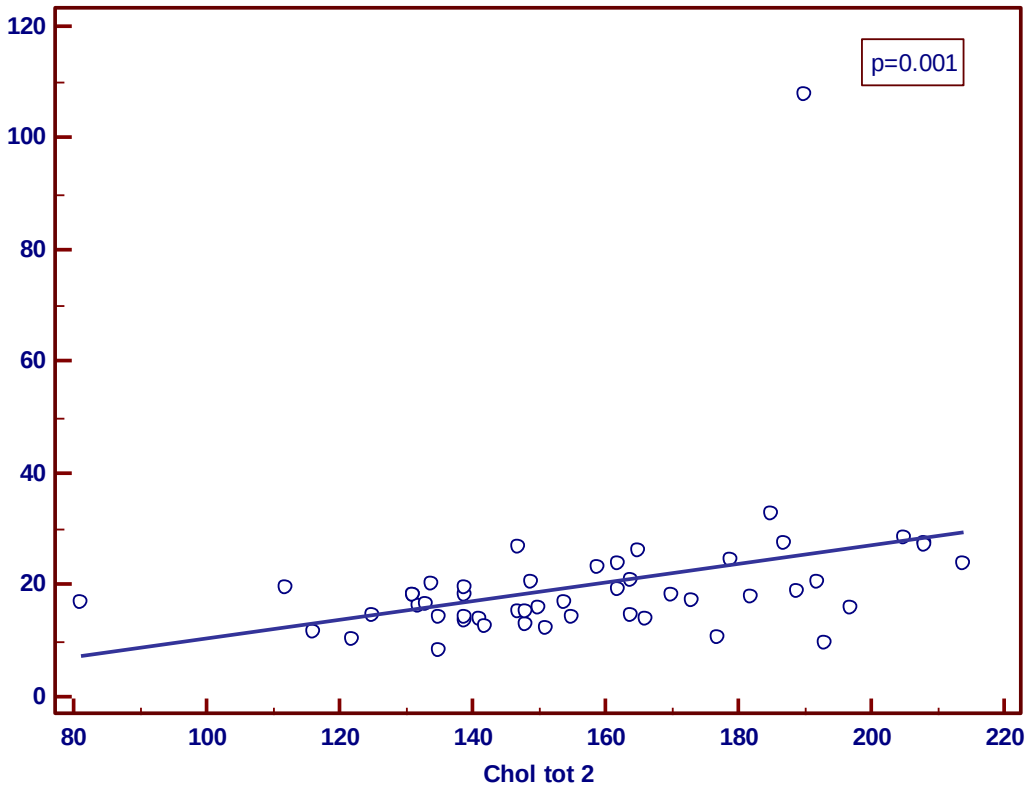


Figure 21: At 12-15 months' evaluation, YKL-40 correlated positively with total Cholesterol values (p=0.001)

2.4.7.3 YKL-40 and Arterial Pressure

YKL-40 correlated positively with Systolic arterial pressure (SAP) (r=0.23, p=0.019, n=100). Regression analysis revealed a positive correlation between SAP z-score and YKL-40 in female gender (p=0.041, n=51) (figure 22).

2.4.7.4 YKL-40 and diabetes control

YKL-40 had a positive correlation with HbA1c (r=0.3, p=0.028, n=112) (figure 23).

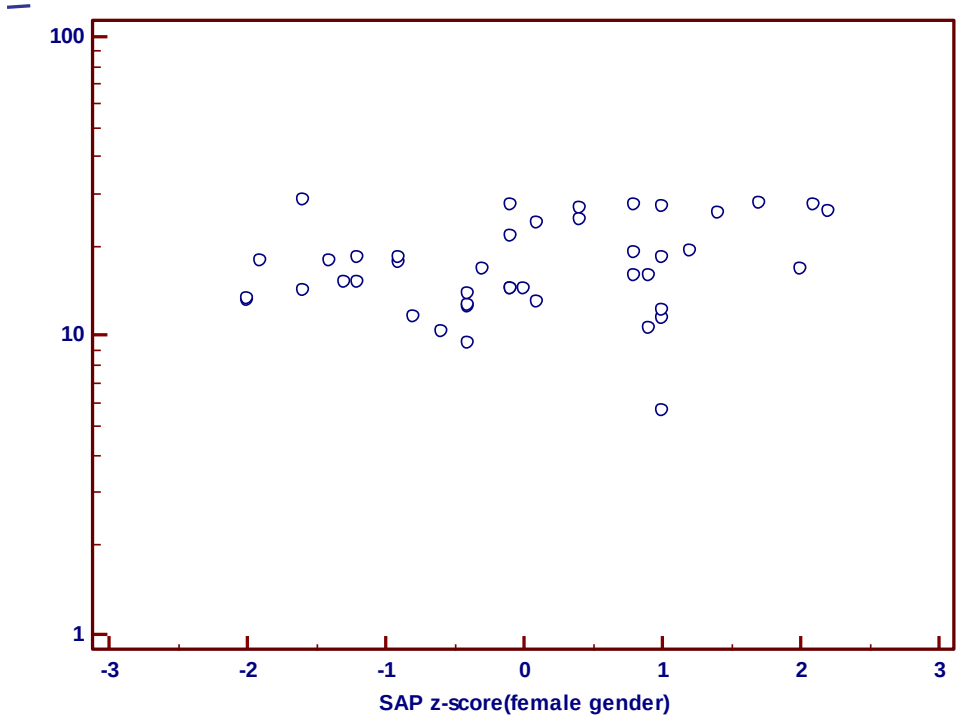


Figure 22: Regression analysis revealed a positive correlation between SAP z-score and YKL-40 in females (p=0.041)

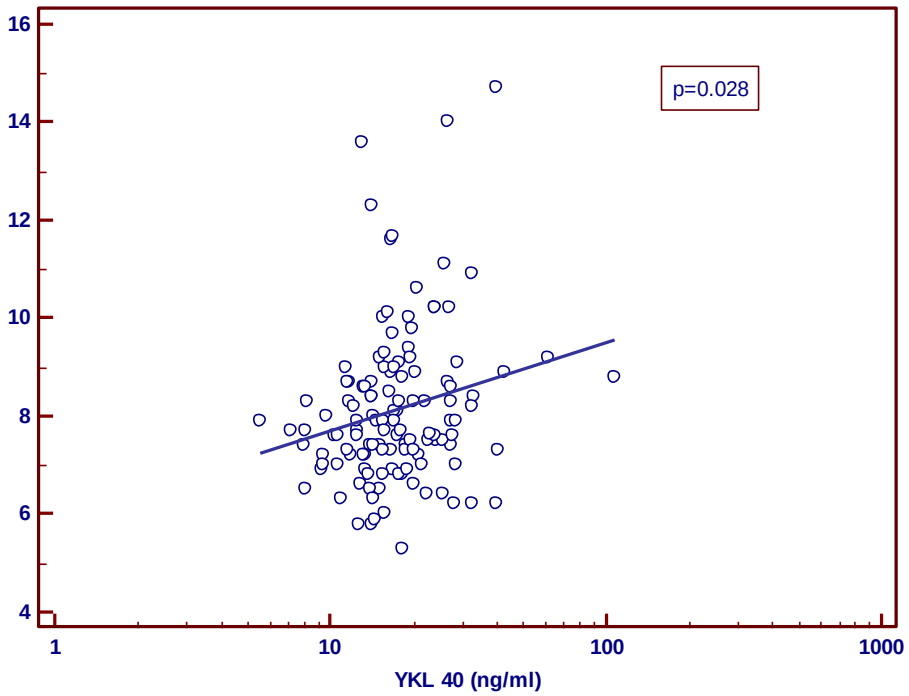


Figure 23: YKL-40 had a positive correlation with HbA1c (p=0.028)

2.4.7.5 YKL-40 puberty and somatometric parameters

YKL-40 had a positive correlation with the advanced Breast pubertal stage (r=0.28, p=0.041, n=51). No correlation either with BMI z-score or height z-score was revealed.

2.4.7.6 YKL-40 and diabetes duration

No statistically significant correlation was found between YKL-40 and diabetes duration. However, a positive correlation was found between YKL-40 and age (r=0.28, p=0.015, n=123).

2.4.8 GDF-15

Mean value of sGDF-15 at baseline was not significantly different between T1D patients and controls (mean: 289.5 pg/ml for T1D patients, mean for controls: 278.6 pg/ml, p=0.717), but s sGDF-15 mean value of T1D patients at re-evaluation (mean:

366.7 pg/ml) was statistically significantly higher than the mean value of the controls' group (p<0.0001) according to Student t-test statistical analysis for independent samples. Paired samples t-test of repeated measurements revealed that mean sGDF-15 value in T1D patients was increased at re-evaluation (p<0.001) (figure 24).

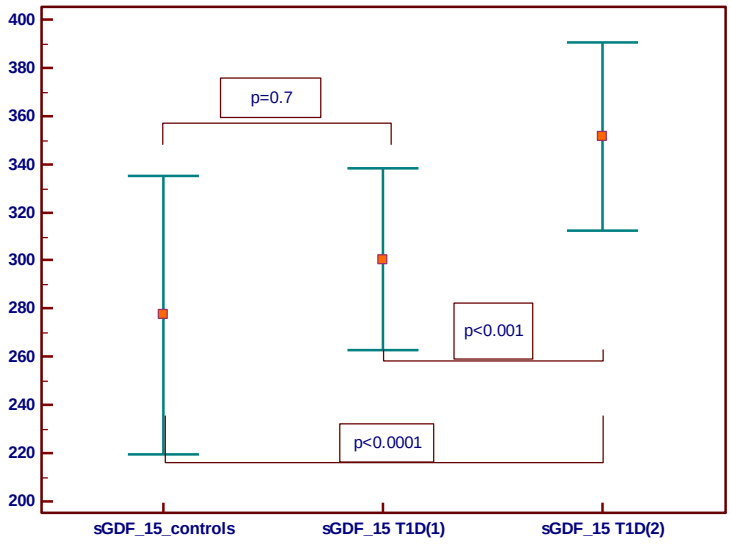


Figure 24: Mean value of sGDF-15 at baseline (1), was not significantly different between T1D patients and controls but at re-evaluation (2), it was statistically significantly higher than the mean value of the controls' group (p<0.0001), while mean sGDF-15 value in T1D patients was increased at re-evaluation (p<0.001)

2.4.8.1 GDF-15 and eGFR

GDF-15 had no statistically significant correlation with any of eGFR formulas at baseline, but it correlated negatively with CysC-eGFR(r=-0.27, p=0.039, n=56) at re-evaluation (figure 25).

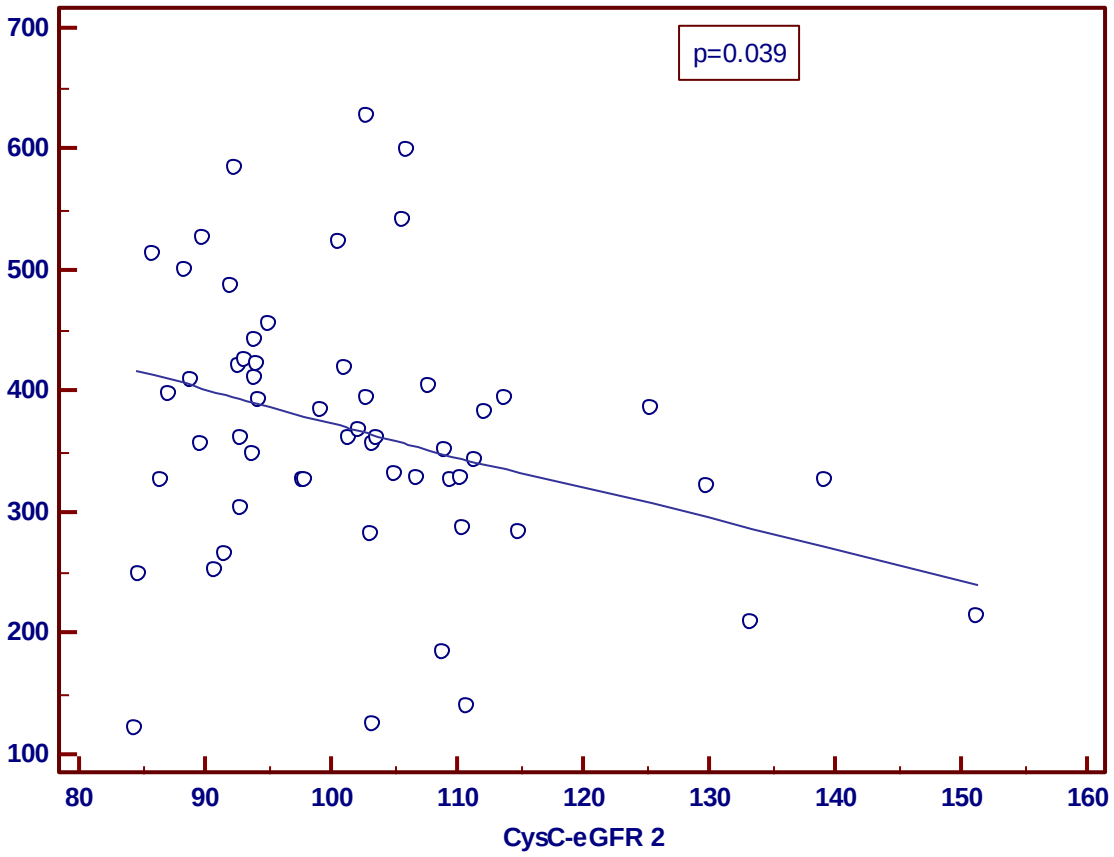


Figure 25: At 12-15 months' evaluation (2), GDF-15 correlated negatively with CysC-eGFR (p=0.039)

2.4.8.2 GDF-15 and other biomarkers (Creatinine, Uric Acid, Cystatin C, microalbuminuria, YKL-40, lipid profile)

At re-evaluation, GDF-15 correlated positively with total cholesterol values (r=0.29, p=0.033, n=54) and LDL (r=0.35, p=0.009, n=54).

2.4.8.3 GDF-15 and Arterial Pressure

No statistically significant correlation was found between GDF-15 and either SAP z-score or DAP z-score.

2.4.8.4 GDF-15 and diabetes control

GDF-15 had no statistically significant correlation with HbA1c.

2.4.8.5 GDF-15, puberty and somatometric parameters

A multiple regression analysis was performed between GDF-15 at both time-points of the evaluation and pubertal stages but no statistically significant correlation was revealed. No correlation either with BMI z-score or height z-score was revealed.

2.4.8.6 GDF-15 and diabetes duration

No statistically significant correlation was found between GDF-15 and age or T1D duration.

2.5 Discussion

Microalbuminuria has generally been considered as the earliest marker of the development of diabetic nephropathy and is often associated with established significant glomerular damage which has been traditionally believed to be the most frequent and main derangement in diabetic nephropathy. However, recent studies showed that MA may be temporary and does not necessarily reflect permanent renal impairment [90]. In addition, several lines of evidence suggest that early lesions in both glomerular and tubular structures may be present in normoalbuminuric subjects. Indeed, cohort studies including prepubertal children with average diabetes duration of 5-8 years revealed GBM thickening and mesangial expansion [83] while they also disclosed that longstanding normoalbuminuric T1D patients may have significant glomerulopathy lesions [23,98,111]. Moreover, recent studies have shown that pathophysiologic changes in DN include renal function decline associated with cellular and extracellular derangements in both the glomerular and tubulo-interstitial compartments [98]. In addition, several studies have provided evidence that hyperglycemia is not the only factor for diabetes-induced microvascular complication,

but other factors may play an important role with various pathophysiological mechanisms. The increased hyperglycemia-induced permeability of the glomeruli that is associated with hyperfiltration and microalbuminuria is not debated but the perception of microalbuminuria being the only marker for diagnosing and excluding the development of diabetic nephropathy has to be discussed and further investigated, in order to reduce the number of the young T1D normoalbuminuric patients who will progress to renal failure.

In our center, the percentage of T1D patients who had microalbuminuria, was 30, 6 % of the number of patients who were assessed. The mean age of microalbuminuria was significantly younger for the patients who have presented with ketoacidosis at the onset of the disease and this association may reflect a genetic or environmental predisposition regarding the severity of the course of T1D. It has been also supported that decreased c-peptide levels are associated with microvascular complications in T1D and T2D [148]. Apparently, the pancreatic cells' disability to secrete C-peptide in and insulin adequate levels early in the course of T1D, results not only in diabetic ketoacidosis but is also one of the triggering factors for the initiation of the pathophysiologic phenomena that lead to the microvascular complications in a younger age. The mean age of microalbuminuria was independent of the age of diagnosis or the duration of T1D. We found that the mean value of estimated eGFR for the patients' group, was increased at reevaluation, as expected, since the equation formulas take into account body height, which increases with advancing age in children and adolescents, but also because of the hyperfiltration in some patients. In our study, microalbuminuria was correlated negatively with BUN and creatinine and positively with the increased glomerular filtration rate, reflecting the hyperfiltration and the possible underlying early lesions of the glomeruli, findings which agree with those from previous studies [149]. We also found a positive correlation between the BMI z-score at enrollment and MA at reevaluation, suggesting that increased body mass index, a known factor of cardiovascular morbidity, associates with microalbuminuria. This finding agrees with a recently published study which suggested that obesity in youth with T1D associates with early alterations in renal function, compared to T1D patients with normal weight [150]. The positive correlations between microalbuminuria

and increased LDL values as well as DAP z-score at reevaluation, known markers of atherosclerosis and endothelial dysfunction, suggest that MA is multifactorial and a predictive marker of cardiovascular morbidity, findings which agree with previous studies. Moreover, a positive correlation between MA and the advanced pubertal stages, also known from previous studies, and the fact that in a number of patients MA was not persistent, is questioning whether the pubertal hormonal changes play any synergic role in the hyperfiltration with or without diabetes-induced glomerular injury.

Uric acid was found to be associated with creatinine and especially, the values assessed at baseline were found to have a stronger positive correlation with creatinine at reevaluation, suggesting a possibly predicting role of decreased renal function. Furthermore, uric acid had a positive correlation with Cystatin C, another renal injury marker. UA correlated negatively with eGFR. Moreover, uric acid at enrollment had a stronger negative correlation with CysC-eGFR of reassessment. These findings may reflect that uric acid is an indicator of renal function decline, even a predictor of future decline and appears to be more sensitive than creatinine. The association between age and advanced pubertal stages of pubic hair and testicular volume may reflect a hormonal, probably androgenic influence. In addition, a positive correlation between UA values and height, but not height z-score was found. Previous studies had shown an association between the height and the development of DN [23], with no obvious explanation. The association found between UA, a byproduct of purine metabolism, and somatometric parameters, may also be relevant to that finding.

As mentioned above, the mean value of estimated eGFR for the patients' group, was increased at reevaluation but a small number of patients had lower eGFR (the number varies depending on the different eGFR equation formula used) at reevaluation than at baseline and this finding was associated with a higher serum creatinine, cystatin C and sNGAL. Indeed sNGAL was found to have strong negative correlations with S-eGFR, CysC-eGFR and L-eGFR, indicating significant association with renal function decline.

SNGAL values higher than 70μg/L had a positive correlation with microalbuminuria, suggesting that increased NGAL values associate with this known marker of

glomerular injury. Nevertheless, urinary NGAL showed much different results, while it had no significant correlation with eGFR. It is known that NGAL is filtered in the glomeruli and it is reabsorbed by the proximal tubuli. UNGAL was found to have a positive correlation with microalbuminuria, a finding that may reflect a similar mechanism of indicating a glomerular lesion through increased permeability. It also had a positive correlation with LDL and total cholesterol implying an association with cardiovascular morbidity. Another finding similar to MA was the correlation with advanced pubertal stages. In this case, the question remains whether the pubertal hormonal changes can influence through hyperfiltration irrespective of structural lesion.

Our study underpins the value of NGAL as a biomarker of early renal damage in T1D, since sNGAL values increased during follow-up, it had a positive correlation with the duration of T1D, more specifically when eGFR was decreased and this may reflect a progress of the early renal structural damage occurring with the progress of the disease. SNGAL value demonstrated a significant negative correlation with the estimated GFR using three different methods, suggesting that a higher sNGAL value is associated with a glomerular function decline even if remaining within normal range. This finding is probably suggestive that sNGAL is associated with the occurrence of ERFD and early renal structure lesions. The number of the included patients who presented microalbuminuria was much lower than the number of patients who presented a decreased eGFR. Moreover, the fact that a percentage of normoalbuminuric patients, who were found to have renal function decline, presented higher sNGAL concentrations, while some patients with microalbuminuria had normal sNGAL concentrations, may imply that these two markers, i.e. microalbuminuria and sNGAL, may reflect different sites of renal damage during the process of DN establishment.

It is certainly necessary to identify markers of early tubular damage independently of albuminuria development in patients with early DN and progression, as it may play a significant role in the management of the normoalbuminuric renal insufficiency cases [64,151]. The significant positive correlation between NGAL, a known marker of structural

renal lesion, and established renal biochemical indexes such as sCr and CysC underpin its important role in the early diagnosis of renal dysfunction.

Systolic arterial pressure has been previously demonstrated to be a predictor of DN [63]. In our study, because it was specifically focused on a young population, we estimated the SAP z-score and found a positive correlation with NGAL. The positive correlation between sNGAL and systolic arterial pressure, even if the latter remains within normal range, can further reflect an association between sNGAL and the silent phase of DN. Since past studies had suggested that ambulatory blood pressure modest rises in T1D patients are associated with the silent phase up to 5 years before microalbuminuria appears [87], the role of sNGAL becomes more important in unravelling early renal injury. The association between the early tubular interstitial damage in normoalbuminuric people with T1D and NGAL is further supported by recently published studies [86,98,113]. The association between sNGAL and SAP z-score may also reflect an indirect role of NGAL as an endothelial dysfunction marker. Undoubtedly, further studies investigating endothelial dysfunction will further delineate the extent of microvascular damage in DN. According to multiple regression analysis, NGAL values were not influenced by age, age of T1D onset and puberty, suggesting independency from the several physiologic mechanisms occurring during puberty.

The diagnostic utility of Cystatin C is well documented in the acute setting [123,152]. Our study demonstrated that Cystatin C measured at baseline had a positive correlation with sCreatinine assessed at reevaluation, and this finding may imply a prognostic role in renal function decline. In addition, its negative correlation with eGFR, suggests that higher cystatin C values correlate with a decreased eGFR. These findings may support the predictive and more sensitive than other markers' role of Cystatin C in early Diabetic Nephropathy. Moreover, Cystatin C assessment is undoubtedly useful in the more accurate GFR determination as seen by the fact that using equation formulas of estimation of GFR with Cystatin C can detect more cases with renal dysfunction [146]. Its significant positive correlation with UA, which has been demonstrated to be associated with renal dysfunction [134], hypertension and cardiovascular disease, further supports the role of CysC as a prognostic cardiometabolic biomarker in diagnosing early diabetic microvascular complications[125]. Multiple regression analyses found that

CysC values were not influenced by age, T1D duration and puberty. However, CysC was found to have higher values in boys in the patients' group, and this fact may imply a gender dimorphism of this biomarker. This finding has not been previously reported, nor has it been replicated in our control group, and it therefore necessitates further validation through large-scale future studies.

YKL-40, had no statistically significant correlation with neither Creatinine, BUN, microalbuminuria nor eGFR. It had a positive correlation with total cholesterol values and SAP, and especially with SAP z-score in pre-hypertensive levels in young females with T1D, suggesting that these molecules may early unmask microvascular or endothelial dysfunction before overt cardiovascular events become evident.

Our study demonstrates also the value of sGDF-15 as a biomarker of early renal damage in T1D, since sGDF-15 had a negative correlation with CysC-eGFR, at reevaluation, reflecting its possible role in identifying an early renal structural injury, from either glomerular or tubular origin, with function decline. SGDF-15 values increased during follow-up, and this may reflect a progress of the early renal structural damage occurring with progress of the disease. It was also associated with total cholesterol and LDL suggesting that this marker may early unmask cardio-renal morbidity.

Uric acid, sNGAL, Cystatin C and GDF-15 had no significant correlation with Microalbuminuria and the fact that they correlated with decreased and not increased GFR like MA does, may reflect possibly different kind and sites of glomerulo-tubular lesions.

This study aimed in assessing the predictive value of other besides microalbuminuria early markers of renal injury, such as sNGAL, UA, GDF-15 and CysC, in young T1D patients by unraveling renal structural damage long before apparent renal dysfunction occurs.

Diabetic nephropathy has been associated with biomarker changes consistent with generalized endothelial dysfunction. Markers of endothelial dysfunction and arterial stiffness have also been associated with MA in diabetic populations [83,153]. Our study underpins the possible indirect role of sNGAL, UA, GDF-15 and CysC, in predicting

early cardiovascular morbidity. However prior to the clinical application, these biomarkers must undergo through rigorous validation in multiple cohorts [154].

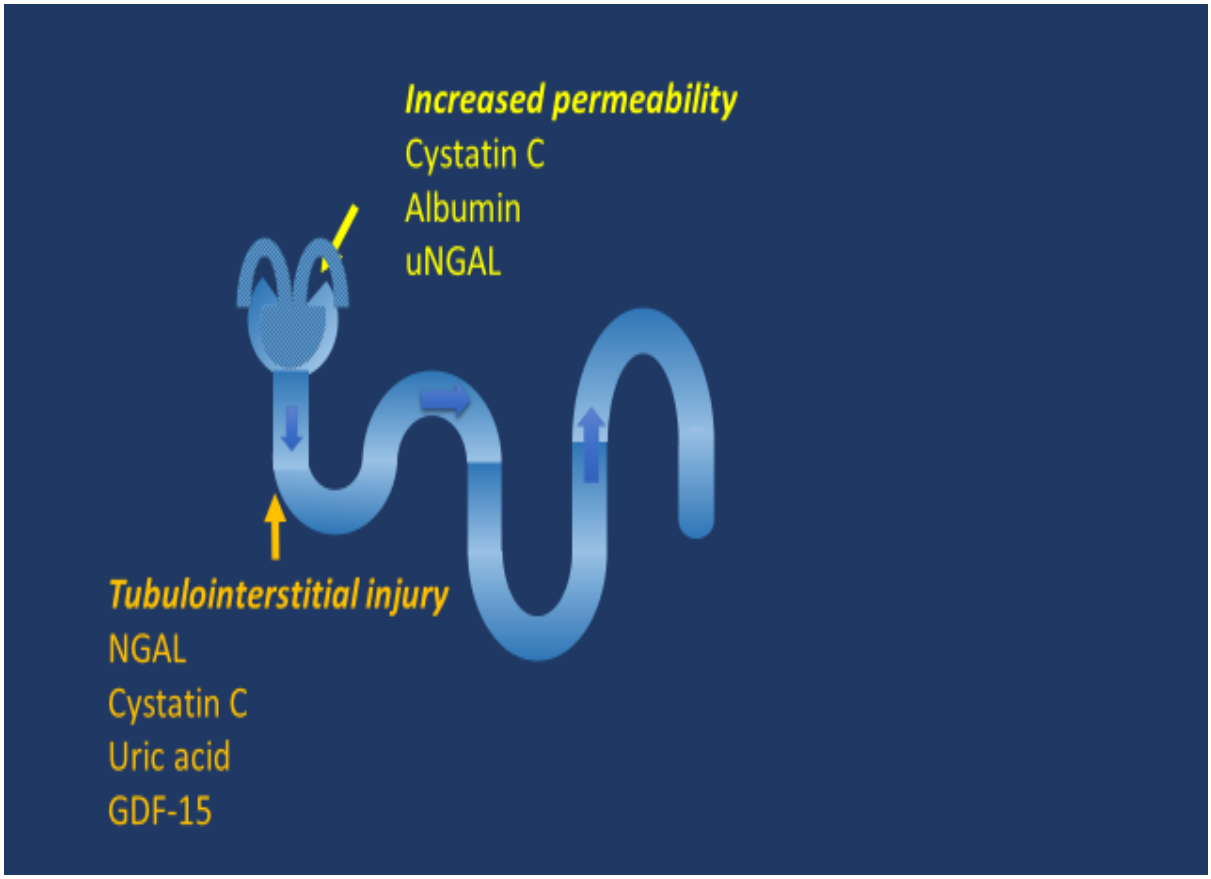
A limitation of our study was the fact that repeated measurements were obtained only in the children with T1D after 12-15 months and not in the control group as well. However, it is considered unethical to perform repeated venipunctures in healthy children, especially in assessing markers that have been shown not to be related to pubertal progression and age, while they do not increase without a backstage tissue injury [116]. Moreover, it is known that microalbuminuria does not occur in healthy children unless renal disease, hypertension, obesity or cardiovascular disease are present [155].

According to the National Kidney Foundation (NKF) and the Kidney Disease Outcomes Quality Initiative (KDOQI), a patient is considered to be in chronic kidney disease (CKD) state if he presents a GFR<60 mL/min per 1.73 m^2 for three months or more. Alternatively, any ongoing (at least three months) structural or functional abnormality of the kidney, regardless of GFR, that can be detected by pathological abnormalities or specific markers is considered CKD [68]. In addition, early renal function decline (ERFD), defined as a progressive loss of GFR over time even if it remains within normal range, is also supported to be associated with DN development. The significant negative correlations between NGAL, Cystatin C, uric acid, GDF-15 and GFR decline further support the prognostic role of these biomarkers in unmasking the structural renal damage before an overt renal impairment becomes evident.

To our knowledge, this is the first study to demonstrate the predictive role of NGAL, uric acid, GDF-15 and Cystatin C as early markers of DN in children and adolescents before severe overt nephropathy occurs.

Defining new predictors as supplementary tests to urinary albumin excretion for the early diagnosis of DN would accelerate the effective management and treatment approaches which are desperately needed in order to minimize the rates of severe cardiorenal morbidity and mortality in young T1D patients. Therefore, these data need to be confirmed by further large-scale longitudinal studies before being integrated in the DN risk assessment of young patients with T1D.

Table 12: Different biomarkers may unravel the early lesions of diabetic nephropathy depending on the kind and site of injury



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4 List of abbreviations

AGE	Advanced Glycation End products
AKI	Acute kidney injury
ALP	Alkaline phosphatase
BMI	Body Mass Index
BUN	blood urea nitrogen
Chol	Total Cholesterol
CKD	Chronic kidney disease
Cr	serum creatinine
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
CVD	Cardiovascular disease
Cys C	Cystatin C
CysC-eGFR	Schwartz et al. recently suggested pediatric eGFR formula for immunonephelometric measured CysC
DAP	Diastolic Arterial Blood Pressure
DKA	Diabetic ketoacidosis
DN	Diabetic Nephropathy
DR	Diabetic Retinopathy
ERFD	Early Renal Function Decline
GADA	Glutamic Acid Decarboxylase Antibody
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate

gGT	Gamma-glutamyltransferase
HbA1C	Glycated hemoglobin
HDL	High Density Cholesterol
HLA	Human Leukocyte Antigen
IA2A	tyrosine phosphatase IA2 Antibody
IAA	Insulin Autoantibody
ICA	Islet Cell Antibody
iGFR	GFR estimated by clearance of iohexol
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INS	Insulin gene
KDOQI	Kidney Disease Outcomes Quality Initiative
LDL	Low-density lipoprotein
L-eGFR	Lund strategy estimation of GFR
MA	Microalbuminuria
MHC	Major Histocompatibility Complex
Mt DNA	Mitochondrial DNA
MIC	Human related MHC class I chain-related genes
NGAL	Neutrophil gelatinase-associated lipocalin
NKC	Natural Killer Cells
NKF	National Kidney Foundation

OGTT	Oral Glucose Tolerance Test
PKC	Protein kinase C
PTPN22	Protein Tyrosine Phosphatase, Non-receptor 22
RAAS	Renin- Angiotensin- Aldosterone- System
SAP	Systolic Arterial Blood Pressure
S-eGFR	2009 modified Schwartz bedside GFR formula
SGOT	Aspartate aminotransferase
SGPT	Alanine aminotransferase
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TCR	T-Cell Receptors
TNF	Tumor Necrosis Factor
Trig	Triglyceride
UA	Serum Uric Acid
UK	United Kingdom
USA	United States of America
WHO	World Health Organization

5 Abstract

5.1 Abstract

Objective: Diabetic nephropathy constitutes a devastating complication in patients with type 1 diabetes mellitus (T1D) and its diagnosis is traditionally based on microalbuminuria. The aim of this prospective cross-sectional follow-up study was to explore the role of serum and urine neutrophil-gelatinase-associated lipocalin (NGAL) cystatin C (Cys C), YKL-40, Uric acid (UA) and GDF-15 in unravelling early diabetic nephropathy even in patients with normoalbuminuria.

Design: Fifty-six euthyroid patients with T1D, with mean age 13.1 (SD: 3.2) years, and 49 healthy controls with mean age 12.8 (SD: 6.6) were recruited. Besides standard blood chemistry and urinary albumin excretion, serum and Urinary NGAL (ELISA) cystatin C (nephelometry), YKL-40(ELISA) and GDF-15(ELISA) were measured at enrollment and after 12-15 months. GFR was calculated with the bedside Schwartz formula (S-GFR), Cystatin C Schwartz formula (CysC-eGFR) and the Lund strategy formula (L-eGFR).

Results: At baseline, mean sNGAL levels, uNGAL, sYKL-40, sGDF-15 and UA were not significantly different between children with diabetes and controls. At re-evaluation, mean NGAL value and mean eGFR value in patients with diabetes were increased ($p=0.032$ and $p=0.003$ respectively). sNGAL was positively correlated with cystatin C ($r=0.41$, $p<0.001$), systolic blood pressure z -score ($r=0.3$, $p=0.031$) and creatinine ($r=0.32$, $p=0.010$). sNGAL and uric acid correlated negatively with S-eGFR ($r=-0.26$, $p=0.049$ and $r=-0.26$, $p=0.049$ respectively) and CysC-eGFR ($r=-0.31$, $p=0.019$ and $r=-0.27$, $p=0.041$ respectively). sNGAL was negatively correlated with L-eGFR ($r=-0.33$, $p=0.002$) and GDF-15 had a negative correlation with CysC-eGFR ($r=-0.27$, $p=0.039$).

Cystatin C had a negative correlation with S-eGFR ($r=-0.29$, $p=0.025$), and a positive correlation with creatinine ($r=0.35$, $p=0.009$). No statistically significant correlation between NGAL, cystatin C, YKL - 40, GDF- 15 and microalbuminuria was found.

Conclusions: NGAL, GDF-15, uric acid and cystatin C, known markers of renal injury, correlate with renal function decline in T1D, suggesting that they may be used as supplementary tests to urine albumin excretion in order to unmask early renal dysfunction

Περίληψη

Στόχος: Η Διαβητική νεφροπάθεια αποτελεί μια σοβαρή επιπλοκή του Σακχαρώδη Διαβήτη τύπου 1 (ΣΔ1) και η διάγνωση της βασίζεται παραδοσιακά στην μικρολευκωματινουρία. Στόχος αυτής της μελέτης παρακολούθησης ήταν να διερευνήσει το ρόλο της λιποκαλίνης που σχετίζεται με τη ζελατινάση των ουδετερόφιλων (NGAL) τη Συστατίνη C, τη χιτινάση (YKL – 40), το ουρικό οξύ και τον αυξητικό παράγοντα διαφοροποίησης 15 (GDF–15) στη διάγνωση της πρώϊμης διαβητικής νεφροπάθειας, ακόμη και σε ασθενείς με νορμολευκωματινουρία.

Ασθενείς και μεθοδοι: Πενήντα έξι ευθυρεοειδικοί ασθενείς με ΣΔ1, με μέσο όρο ηλικίας 13,1(SD : 3.2) έτη, και 49 υγιείς μάρτυρες με μέσο όρο ηλικίας 12,8 (SD : 6.6) έλαβαν μέρος στη μελέτη. Εκτός του βασικού βιοχημικού ελέγχου του αίματος και της λευκωματίνης ούρων, μετρήθηκαν NGAL (ELISA), συστατίνη C (νεφελομετρία), YKL - 40(ELISA) και GDF- 15(ELISA) σε 2 χρόνους (0΄ και μετά 12-15 μήνες). Ο ρυθμός σπειραματικης διήθησης (GFR) υπολογίστηκε με τη μέθοδο bedside Schwartz (S-eGFR), με το προσφάτως προτεινόμενο μαθηματικό μοντέλο εκτίμησης GFR παιδιατρικών ασθενών με βάση τη συστατίνη C (νεφελομετρία) Schwartz (CysC-eGFR) και τη στρατηγική Lund (L-eGFR).

Αποτελέσματα: Στο χρόνο 0΄, τα μέσα επίπεδα sNGAL, Συστατίνης C, sYKL - 40 , sGDF - 15 και ουρικού οξέος δε διέφεραν σημαντικά μεταξύ των παιδιών με ΣΔ1 και των μαρτύρων. Στον επανέλεγχο, η μέση τιμή NGAL και η μέση τιμή eGFR σε ασθενείς με ΣΔ1 αυξήθηκαν σημαντικά (p=0.032 και p=0.003 αντίστοιχα). Η NGAL συσχετίστηκε θετικά με τη συστατίνη C (r= 0.41, p<0.001), το z-score της συστολικής αρτηριακής πίεσης (r=0,3, p=0.031) και την κρεατινίνη (r=0.32, p=0.010). Η NGAL και το ουρικό οξύ συσχετίστηκαν αρνητικά με το S-eGFR (r=-0.26 , p=0.049 και r=-0.26 , p=0.049 αντίστοιχα) και το CysC-eGFR(r=-0.31, p=0.019 και r=-0.27, p=0.041 αντίστοιχα). Η NGAL συσχετίστηκε αρνητικά με το L-eGFR (r=-0.33, p=0.002). Ο GDF-15 είχε αρνητική συσχέτιση με το CysC-eGFR (r=-0.27, p=0.039).

Η συστατίνη C είχε αρνητική συσχέτιση με το S–eGFR (r=-0.29, p=0.025), και θετική με την κρεατινίνη (r=0.35, p=0.009). Δεν παρατηρήθηκε στατιστικά σημαντική

συσχέτιση μεταξύ των NGAL, συστατίνης C, YKL–40, GDF-15 και της μικρολευκωματινουρίας.

Συμπεράσματα: Η NGAL, το ουρικό οξύ, η συστατίνη C και ο GDF – 15, γνωστοί δείκτες νεφρικής βλάβης, συσχετίζονται με έκπτωση της νεφρικής λειτουργίας στο ΣΔ1, και προτείνονται ως συμπληρωματικές δοκιμασίες μαζί με τη λευκωματίνη ούρων, στην έγκαιρη διάγνωση της διαβητικής νεφροπάθειας πριν η νεφρική δυσλειτουργία γίνει μη αναστρέψιμη.

6 Study protocol in Greek

ΠΡΩΤΟΚΟΛΛΟ ΕΡΕΥΝΑΣ ΔΙΔΑΚΤΟΡΙΚΗΣ ΔΙΑΤΡΙΒΗΣ

Πρώϊμη ανίχνευση διαταραχών νεφρικής λειτουργίας σε παιδιατρικούς ασθενείς και νεαρούς ενήλικες με σακχαρώδη διαβήτη τύπου 1.

Η διαβητική νεφροπάθεια, είναι η νόσος ή η βλάβη των νεφρών που εμφανίζεται ως επιπλοκή του σακχαρώδη διαβήτη τύπου 1 και 2. Πρόκειται για την κύρια αιτία νεφρικής ανεπάρκειας στις ΗΠΑ. Η ακριβής αιτία της διαβητικής νεφροπάθειας παραμένει άγνωστη, όμως συσχετίζεται άμεσα με τη μη ελεγχόμενη υπεργλυκαιμία. Ασθενείς που δεν έχουν λάβει εντατικό θεραπευτικό σχήμα με ινσουλίνη και έχουν πτωχό γλυκαιμικό έλεγχο, έχουν 30-40% πιθανότητες ανάπτυξης διαβητικής νεφροπάθειας εντός 20ετίας απο την έναρξη της νόσου. Όμως η διαβητική νεφροπάθεια δε θα συμβεί σε όλους τους ασθενείς με ΣΔ1. Παρόλο που συνδέεται με την υπεργλυκαιμία, ο κίνδυνος της ανάπτυξης διαβητικής νεφροπάθειας είναι γενετικά προσδιοριζόμενος. Σε ορισμένες μελέτες που έγιναν σε οικογένειες, φαίνεται η υψηλή συμπτωτικότητα της διαβητικής νεφροπάθειας και της σπειραματικής βλάβης, ανάμεσα σε αδέλφια με ΣΔ1.

Η διαβητική νεφροπάθεια συσχετίζεται με γονιδιακούς τόπους όπως την HLA περιοχή του χρωμοσώματος 6p. Ένα από τα γονίδια που συσχετίζονται με τη διαβητική νεφροπάθεια είναι το TRPC1(calcium-permeable nonselective transient receptor potential cation channel, subfamily C, member 1), το οποίο αποτελεί γονίδιο που σχετίζεται με την HNF4α(Nuclear receptor hepatic Nuclear factor 4^{α}) που είναι μια ρυθμιστική πρωτεΐνη με ουσιαστικό ρόλο σε μεταβολικές διαδικασίες, βρίσκεται στο ανθρώπινο χρωμόσωμα 3q22-24, περιοχή άμεσα σχετιζόμενη με τη διαβητική νεφροπάθεια. Η χαμηλή έκφραση του γονιδίου TRPC1 στους νεφρούς των ατόμων με διαβητική νεφροπάθεια συνηγορεί υπέρ της παραπάνω συσχέτισης. Άλλη μελέτη έδειξε συσχέτιση μεταξύ των πολυμορφισμών του γονιδίου της ερυθροποιητίνης και της αγγειοπάθειας αμφιβληστροειδούς και νεφρών σε άτομα με ΣΔ1. Η έλλειψη του

νεφροπροστατευτικού παράγοντα ENTPD1 (ο Ectonucleoside triphosphate diphosphohydrolase 1 ή CD39 είναι η κύρια αγγειακή εκτονουκλεοτιδάση, που αποτελεί αγγειακό προστατευτικό παράγοντα της διαβητικής νεφροπάθειας εφόσον τροποποιεί τη σπειραματική φλεγμονή αλλά και τη ρύθμιση της δημιουργίας θρόμβου), οι πολυμορφισμοί του γονιδίου ICAM-1(οι πολυμορφισμοί του γονιδίου ICAM-1 που βρίσκεται στο χρωμόσωμα 19p13 το οποίο συνδέεται με την εμφάνιση ΣΔ1, πιθανώς να σχετίζονται με την εμφάνιση της διαβητικής νεφροπάθειας), οι πολυμορφισμοί του γονιδίου UNC13B(αντικατάσταση στο ιντρόνιο 1 του γονιδίου UNC13B που φαίνεται να αυξάνει την απόπτωση στα σπειραματικά κύτταρα επί παρουσίας υπεργλυκαιμίας), οι μεταλλάξεις του γονιδίου ACE και το οικογενειακό ιστορικό μπορούν να παίξουν σημαντικό ρόλο στην ανάπτυξη της διαβητικής νεφροπάθειας.

Τελευταία φαίνεται πως στα αίτια της διαβητικής νεφροπάθειας περιλαμβάνονται και περιβαλλοντικοί παράγοντες, όπως το κάπνισμα, η φλεγμονή και το οξειδωτικό στρες
.

Ο κάθε νεφρός αποτελείται από εκατοντάδες χιλιάδες νεφρώνες. Ο κάθε νεφρώνας έχει το δικό του σπείραμα. Αυτές οι δομές βοηθούν στη διήθηση και τελικά την αποβολή άχρηστων ουσιών απο τον οργανισμό. Οι υψηλές τιμές γλυκόζης στο αίμα, μπορούν να προκαλέσουν βλάβη σε αυτές τις δομές με πάχυνση και ουλοποίηση. Με την πάροδο του χρόνου, όλο και περισσότερα αγγεία και νεφρικά σωληνάρια καταστρέφονται.

Η διαβητική νεφροπάθεια αρχικά εκδηλώνεται με πρωτεϊνουρία, ενώ με την εγκατάσταση της νόσου, επιδεινώνεται η νεφρική λειτουργία με αύξηση της ουρίας και της κρεατινίνη στο αίμα. Παρατηρείται συσσώρευση στο νεφρικό εξωκυττάριο στρώμα, ειδικά στο μεσάγγειο. Η πάχυνση της αγγειακής βασικής μεμβράνης και του μεσάγγειου των νεφρικών σωληναρίων, προάγει τη διάφορου βαθμού σπειραματοσκλήρυνση και τη νεφρική ανεπάρκεια. Η διάχυτη σπειραματοσκλήρυνση είναι πιο κοινή από την οζώδη διατριχοειδική σπειραματοσκλήρυνση (βλάβες Kimmelstiel-Wilson). Και οι δυο προκαλούν σημαντική λευκωματουρία. Η λευκωματουρία συχνά συνδυάζεται με υπέρταση, η οποία συσχετίζεται με τον αυξημένο κίνδυνο για θάνατο από καρδιαγγειακά συμβάματα σε διαβητικούς ασθενείς ακόμα και επί απουσίας νεφρικής ανεπάρκειας. Ο προσεκτικός έλεγχος των τιμών της

γλυκόζης, μπορεί να μειώσει την υπερδιήθηση και τη μικροαλβουμινουρία στα αρχικά στάδια της νόσου.

Κατά την πρόοδο της διαβητικής νεφροπάθειας, εμφανίζεται πρωτεϊνουρία διαφόρου βαθμού, που μπορεί να προκαλέσει νεφρωσικό σύνδρομο, με υποαλβουμιναιμία, οίδημα, αύξηση της LDL χοληστερόλης καθώς και προοδευτική αζωθαιμία. Η πρωτεινουρία, που σχετίζεται με τη διαβητική νεφροπάθεια, δε μειώνεται με την πρόοδο της νεφρικής δυσλειτουργίας. Με την πρόοδο της νεφρικής ανεπάρκειας, υπάρχει αύξηση του νεφρικού ουδού για την εμφάνιση γλυκοζουρίας. Επίσης, η υπέρταση και αθηροσκλήρυνση των εγκεφαλικών και στεφανιαίων αγγείων επιταχύνεται.

Στα πλαίσια της ανάγκης που προκύπτει για την πρώιμη ανίχνευση της διαβητικής νεφροπάθειας, διεξάγονται συνεχώς κλινικές μελέτες. Μία από τις σημαντικότερες μελέτες, είναι η International Diabetic Nephropathy Study (IDNS) που διεξήχθη στο Μόντρεαλ, τη Μιννεάπολη και το Παρίσι και στην οποία έλαβαν μέρος 243 παιδιά και νεαροί ενήλικες ηλικίας 10-40 ετών, νορμοτασικοί, με φυσιολογικό έως και υψηλό ρυθμό σπειραματικής διήθησης GFR και ρυθμό απέκκρισης αλβουμίνης <100 μg/min. Έγινε 2 φορές βιοψία νεφρού με μεσοδιάστημα 5 ετών κατά το οποίο γινόταν τακτική κλινική παρακολούθηση. Οι κύριες μορφομετρικές ανωμαλίες ήταν η αύξηση του πάχους της βασικής σπειραματικής μεμβράνης και του τμηματικού όγκου του μεσάγγειου καθώς και του μεσάγγειου στρώματος. Η συχνότητα των παραπάνω ανωμαλιών ήταν ανάλογη με τη διάρκεια του διαβήτη και παρατηρήθηκε σε διάστημα 2-8 έτη από την έναρξη της νόσου.

Παρόμοιες μελέτες με μικρότερο αριθμό ασθενών διεξήχθησαν και σε άλλα κέντρα της Ευρώπης και της Αμερικής με παρόμοια αποτελέσματα όσον αφορά τις νεφρικές αλλοιώσεις λίγα χρόνια μετά την έναρξη του ΣΔ, σε ασθενείς που παραμένουν νορμοαλβουμινουρικοί.

Είναι σημαντική λοιπόν η ανεύρεση νέων αναίμακτων μεθόδων πρώϊμης ανίχνευσης νεφρικής βλάβης με σκοπό την έγκαιρη αντιμετώπιση και πιθανώς την αναστρεψιμότητα αυτής. Θα γίνει έτσι, πιο εφικτή η προσέγγιση υποσχόμενων θεραπευτικών μεθόδων όπως φαίνεται από μελέτες. Για παράδειγμα, έρευνα δείχνει

πως δύναται να προληφθεί η κλινική διαβητική νεφροπάθεια με χορήγηση θειαμίνης και Benfotiamine. Μέχρι σήμερα έχουν μελετηθεί κάποιοι εργαστηριακοί δείκτες όπως το πρωτέωμα των ούρων και το smad1 των ούρων αλλά και κλινικοί όπως η εμφάνιση περιοδοντίτιδας. Όλοι αυτοί οι δείκτες φαίνεται να συνηγορούν με αρχόμενη νεφρική δυσλειτουργία.

Η έρευνα μας θα επικεντρωθεί στη μελέτη νεότερων δεικτών. Οι δείκτες αυτοί, είναι οι εξής: η Neutrophil gelatinase-B associated lipocalin (NGAL)/Lipocalin-2, ο YKL-40, ο GDF 15. Από αυτούς, η NGAL θα ανιχνευθεί τόσο στον ορό του αίματος, όσο και στα ούρα.

Οι παραπάνω δείκτες πλην του YKL40, έχουν μελετηθεί μεταξύ άλλων, μέχρι σήμερα στην οξεία νεφρική ανεπάρκεια και πιο συγκεκριμένα στην πρώϊμη ανίχνευση της οξείας νεφρικής βλάβης(AKI/ acute kidney injury). Η συστατίνη C, αποτελεί πλέον έναν πολύ καλό δείκτη της νεφρικής λειτουργίας που από μελέτες δείχνει να υπερτερεί της κρεατινίνης. Επίσης είναι ένας από τους παράγοντες κινδύνου για καρδιαγγειακή νόσο με αυξημένη θνησιμότητα σε διαβητικούς ασθενείς, ακόμα και όταν δεν είναι έκδηλη η νεφρική βλάβη.

Οι λιποκαλίνες επίσης έχουν συσχετιστεί σαφώς με το μεταβολικό σύνδρομο των ενηλίκων, ενώ δεν είναι ακόμα ξεκάθαρη η συσχέτιση τους με το μεταβολικό σύνδρομο των παιδιών.

Ο YKL-40, ο οποίος είναι ένας φλεγμονώδης δείκτης και δείκτης ενδοθηλιακής δυσλειτουργίας, φαίνεται να αυξάνεται σε διαβητικούς ασθενείς με αλβουμινουρία.

Ο δείκτης GDF-15 (Plasma growth differentiation factor-15), ο οποίος έχει συνδεθεί με τη φλεγμονή και την απόπτωση, φαίνεται πως εκφράζεται στην καρδιά ως απάντηση σε ισχαιμία και σε αθηρωματικές πλάκες. Πρόσφατη μελέτη έδειξε πως υψηλά επίπεδα του GDF-15 αποτελούν προγνωστικό δείκτη καρδιαγγειακής θνητότητας και νοσηρότητας σε ασθενείς με διαβητική νεφροπάθεια. Επίσης, τα υψηλά επίπεδα του παράγοντα αυτού συνδέονται με ταχεία πτώση του ρυθμού σπειραματικής διήθησης και επιδείνωση της νεφρικής λειτουργίας.

Σκοπός της μελέτης

Να μελετηθούν προδρομικά οι πρώιμοι δείκτες νεφρικής δυσλειτουργίας σε παιδιά και εφήβους με ΣΔ1.

Ασθενείς και μέθοδοι

1) Οι ασθενείς που θα μελετηθούν, είναι παιδιά που πάσχουν από σακχαρώδη διαβήτη τύπου 1 και παρακολουθούνται από το Διαβητολογικό Κέντρο της Μονάδας Ενδοκρινολογίας, Μεταβολισμού και Διαβήτη της Α Παιδιατρικής Κλινικής του Πανεπιστημίου Αθηνών στο Νοσοκομείο Παίδων «Αγ. Σοφία» και νεαροί ενήλικες πάσχοντες από σακχαρώδη διαβήτη τύπου 1 που παρακολουθούνται στη Β' Προπαιδευτική Παθολογική Κλινική, Μονάδα Έρευνας και Διαβητολογικό Κέντρο Παν/μίου Αθηνών του Νοσοκομείου «Αττικό».

2) Οι δείκτες θα μελετηθούν με προοπτική μελέτη και με μέθοδο cross sectional σε 60 έως 70 ασθενείς που πάσχουν από σακχαρώδη διαβήτη τύπου 1. Οι ασθενείς θα είναι παιδιά, έφηβοι και νεαροί ενήλικες ηλικίας έως 30 ετών. Επίσης, θα υπάρχει ομάδα 20 έως 40 εθελοντών υγιών ατόμων αντίστοιχων ηλικιακών ομάδων, στους οποίους θα γίνει μία φορά η μέτρηση των δεικτών. Η εργαστηριακή παρακολούθηση θα γίνεται ανά 6-12 μήνες παράλληλα με:

    - Δείκτη γλυκαιμικής ρύθμισης HbA1c
    - Ουρία, κρεατινίνη, ουρικό οξύ και ρυθμό σπειραματικής διήθησης
    - Συλλογή ούρων 24ώρου με μέτρηση μικροαλβουμίνης
    - Κλινική εξέταση και μέτρηση αρτηριακής πίεσης

Σημασία της μελέτης

Η μελέτη επικεντρώνεται στην πρώϊμη ανίχνευση της νεφρικής βλάβης που προκαλείται από τη μικροαγγειοπάθεια, η οποία είναι μια από τις συχνότερες επιπλοκές του σακχαρώδη διαβήτη σε παιδιά και ενήλικες. Στόχος της ενωρίς διάγνωσης είναι η έγκαιρη θεραπεία και η βελτίωση της ποιότητας της ζωής των διαβητικών παιδιών και ενηλίκων

ΣΥΜΦΩΝΗΤΙΚΟ ΕΘΕΛΟΝΤΙΚΗΣ ΣΥΜΜΕΤΟΧΗΣ

Α΄ ΠΑΙΔΙΑΤΡΙΚΗ ΚΛΙΝΙΚΗ ΠΑΝΕΠΙΣΤΗΜΙΟΥ ΑΘΗΝΩΝ

ΚΑΘΗΓΗΤΗΣ- ΔΙΕΥΘΥΝΤΗΣ: Γ. ΧΡΟΥΣΟΣ

ΜΟΝΑΔΑ ΕΝΔΟΚΡΙΝΟΛΟΓΙΑΣ ΜΕΤΑΒΟΛΙΣΜΟΥ ΚΑΙ ΔΙΑΒΗΤΗ

<u>ΣΥΜΦΩΝΗΤΙΚΟ ΕΘΕΛΟΝΤΙΚΗΣ ΣΥΜΜΕΤΟΧΗΣ</u>

Ο/Η ……, κηδεμόνας του …… ……., δηλώνω τη συγκατάθεσή μου για τη συμμετοχή του παιδιού μου στη μελέτη για την Πρώϊμη ανίχνευση διαταραχών νεφρικής λειτουργίας σε παιδιατρικούς και νεαρούς ασθενείς με σακχαρώδη διαβήτη τύπου 1 σε παιδιά και νεαρούς ενήλικες. Το παιδί μου παρακολουθείται για το σακχαρώδη διαβήτη τύπου 1 από το οποίο πάσχει, στο εξωτερικό ιατρείο της μονάδας Ενδοκρινολογίας-Διαβήτη-Μεταβολισμού στην Α΄ Παιδιατρική Κλινική Πανεπιστημίου Αθηνών στο Νοσοκομείο Παίδων «Αγ. Σοφία».

Το παιδί μου στα πλαίσια της κλινικής εκτίμησης θα εξεταστεί από το ιατρικό προσωπικό και θα δοθούν οι απαραίτητες εργαστηριακές εξετάσεις.

Το παιδί μου στα πλαίσια της κλινικής μελέτης θα υποβληθεί σε εργαστηριακό έλεγχο αλλά και κλινική εξέταση ανά 3 μήνες.

Επίσης, κατανοώ ότι το παιδί μου δεν θα επιβαρυνθεί με επιπλέον αιμοληψία, δεν υπάρχει κανένας κίνδυνος για την υγεία του, και δεν θα επιβαρύνει οικονομικά η ανωτέρω μελέτη ούτε το παιδί μου ούτε το Νοσοκομείο Παίδων «Αγ. Σοφία». Έχω ενημερωθεί ότι η συμμετοχή του παιδιού μου στη μελέτη είναι απόλυτα οικειοθελής και διατηρώ το δικαίωμα να τη διακόψει χωρίς καμία επίπτωση στην χορήγηση ιατρικής φροντίδας που του προσφέρεται.

Ο συναινών κηδεμόνας

Ο ενημερώσας ιατρός

7 Approval of the study from the Ethics' committee

ΓΕΝΙΚΟ ΝΟΣΟΚΟΜΕΙΟ ΠΑΙΔΩΝ « Η ΑΓΙΑ ΣΟΦΙΑ »

ΕΠΙΣΤΗΜΟΝΙΚΟ ΣΥΜΒΟΥΛΙΟ
ΠΡΟΣ κ. ΝΕΚΤΑΡΙΑ ΠΑΠΑΔΟΠΟΥΛΟΥ

Αθήνα, 30 Ιουνίου 2010

ΑΠΟΣΠΑΣΜΑ ΠΡΑΚΤΙΚΟΥ ΣΥΝΕΔΡΙΑΣΕΩΣ 10-05-10

ΠΑΡΟΝΤΕΣ :

Κ.ΠΡΟΥΝΤΖΟΥ-ΚΑΣΣΙΟΥ ΠΡΟΕΔΡΟΣ ΕΠΙΣΤΗΜΟΝΙΚΟΥ ΣΥΜΒΟΥΛΙΟΥ, Δ/ντρια Α΄Παιδιατρικού τμήματος
Π. ΜΑΜΜΗ ΑΝΤΙΠΡΟΕΔΡΟΣ ΕΠΙΣΤΗΜΟΝΙΚΟΥ ΣΥΜΒΟΥΛΙΟΥ, Δ/ντρια Αναισθησιολογικού τμήματος
Γ. ΚΑΛΑΒΡΟΥΖΙΩΤΗΣ ΜΕΛΟΣ, Δ/ντής Α΄Καρδιοχειρουργικού τμήματος
Π. ΚΑΡΡΑ ΜΕΛΟΣ, Επιμελήτρια Β΄Ακτινοδιαγνωστικού τμήματος
Γ. ΑΥΓΕΡΙΝΟΥ ΜΕΛΟΣ, Ειδικευόμενη Ιατρός Αιματολογίας
Ι. ΠΑΠΑΣΩΤΗΡΙΟΥ ΜΕΛΟΣ, Δ/ντής Βιοχημικού Εργαστηρίου
Α. ΓΙΑΓΤΖΙΔΟΥ ΜΕΛΟΣ, ΤΕ Νοσηλευτριών

ΘΕΜΑ : Έγκριση διεξαγωγής ερευνητικής μελέτης με τίτλο «Πρώιμη ανίχνευση διαταραχών νεφρικής λειτουργίας σε παιδιατρικούς και νεαρούς ασθενείς με σακχαρώδη διαβήτη τύπου 1».
Επιστημονικά Υπεύθυνος : κ. ΧΡ. ΚΑΝΑΚΑ Επίκουρος Καθηγήτρια Παιδιατρικής Ενδοκρινολογίας Α΄Παιδιατρικής Κλινικής Πανεπιστημίου Αθηνών.

ΣΧΕΤ. : Αρ.πρωτ. 897/18-01-10

Το Επιστημονικό Συμβούλιο κατά την τελευταία συνεδρίασή του έλαβε υπόψη του την ανωτέρω αίτηση της Επίκουρου Καθηγήτριας κ. Χρ. Κανακά, που αφορά στην έγκριση διεξαγωγής ερευνητικής μελέτης με τίτλο «Πρώιμη ανίχνευση διαταραχών νεφρικής λειτουργίας σε παιδιατρικούς και νεαρούς ασθενείς με σακχαρώδη διαβήτη τύπου 1».
Ύστερα από μελέτη και αναλυτική συζήτηση, διαπιστώθηκε ότι η ανωτέρω ερευνητική μελέτη, η οποία εκπονείται στα πλαίσια διδακτορικής διατριβής της υποψήφιας διδάκτορος κας Νεκταρίας Παπαδοπούλου, πληροί όλες τις προϋποθέσεις για τη διεξαγωγή της. Όπως αναφέρει η επιβλέπουσα της μελέτης Επίκουρος Καθηγήτρια κ. Χρ. Κανακά , το Νοσοκομείο και η οικογένεια δε θα επιβαρυνθούν οικονομικά από τη μελέτη και ότι δε θα υπάρχει κίνδυνος για την υγεία των παιδιών.
Κατόπιν τούτων, ομόφωνα το Επιστημονικό Συμβούλιο εισηγείται την έγκριση διεξαγωγής της ανωτέρω μελέτης .

Η ΠΡΟΕΔΡΟΣ
ΤΟΥ ΕΠΙΣΤΗΜΟΝΙΚΟΥ ΣΥΜΒΟΥΛΙΟΥ

ΠΡΟΥΝΤΖΟΥ – ΚΑΣΣΙΟΥ ΚΥΡΙΑΚΗ

‘’Translation in English’’

SCIENTIFIC COUNCIL

TO MRS. NEKTARIA PAPADOPOULOU

Athens, 30 June 2010

MEETING ABSTRACTS 10-05-10

Present at the meeting:

K. Prountzou-Kassiou President of the Scientific Council, Director Of The 1st Pediatric Department

P. Mammi, Deputy Director of the Anesthesia Department

P. Kalavrouziotis, Director of the Cardiosurgery Department

P. Karra Member, Md 1st Radiodiagnostic Department

G. Avgerinou, Member Resident Doctor, Hematology Department

I. Papasotiriou, Member Director of the Biochemistry Department

A. Giatzidou, Member

SUBJECT: Approval of holding of the research study entitled ‘’ early markers of diabetic nephropathy in children and young adults with type 1 Diabetes Mellitus’’

Scientific coordinator: Mrs. Christina Kanaka, Associate Professor For Pediatric Endocrinology, Juvenile Diabetes And Metabolism, Medical School, Division Of Endocrinology, Metabolism And Diabetes, First Department Of Pediatrics, University Of Athens, ″Aghia Sophia″ Children’s Hospital University Of Athens

Ref. No: 897/ 18-01-10

The scientific council at its last meeting, took note of the above application by Associate Professor Christina Kanaka, that in approving holding of the research study entitled ‘’ Early markers of diabetic nephropathy in children and young adults with type 1 Diabetes Mellitus’’. After research and detailed discussion, we concluded that the above study, which was prepared within the PhD thesis for the resident doctor-PhD Student Nektaria Papadopoulou, meets all the requirements to conduct. As mentioned by the Scientific Coordinator, Associate Professor Christina Kanaka, the hospital and the family will not bear the financial burden of the study and that there will be no health risk for the children.

That said, the scientific council unanimously recommended the approval to conduct this study.

THE PRESIDENT OF THE SCIENTIFIC COUNCIL

Signature

PROUNTZOU-KASSIOU KIRIAKI

8 Publication in English

Papadopoulou-Marketou N, Skevaki C, Kosteria I, Peppa M, Chrousos GP, Papassotiriou I, Kanaka-Gantenbein C: ‘’NGAL and Cystatin C: two possible early markers of diabetic nephropathy in young patients with type 1 diabetes mellitus: one year follow-up’’ Hormones 2014 Nov 5; DOI: 10/14310 horm.2002.1520

Research paper

NGAL and cystatin C: two possible early markers of diabetic nephropathy in young patients with type 1 diabetes mellitus: one year follow up

Nektraria Papadopoulou-Marketou,[1,2] Chrysanthi Skevaki,[3] Ioanna Kosteria,[1] Melpomeni Peppa,[4] George P. Chrousos,[1,5] Ioannis Papassotiriou,[3] Christina Kanaka-Gantenbein[1]

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ABSTRACT

OBJECTIVE: Diabetic nephropathy constitutes a major long-term complication in patients with type 1 diabetes mellitus (T1D) and its diagnosis is based on microalbuminuria. The aim of this observational follow-up study was to explore the role of neutrophil-gelatinase-associated lipocalin (NGAL) and cystatin C in unravelling early diabetic nephropathy even in patients with normoalbuminuria. DESIGN: Fifty-six euthyroid patients with T1D, with mean age 13.1 (SD: 3.2) years, and 49 healthy controls with mean age 12.8 (SD: 6.6) were recruited. Besides standard blood chemistry and urinary albumin excretion, serum NGAL (ELISA) and cystatin C (nephelometry) were measured at enrollment and after 12-15 months. GFR was calculated with the bedside Schwartz formula (eGFR) and the Lund strategy formula (L-eGFR). RESULTS: At baseline, mean NGAL levels were not significantly different between children with diabetes and controls. At re-evaluation, mean NGAL value and mean eGFR value in patients with diabetes were increased ($p=0.032$ and $p=0.003$ respectively). At both baseline and reevaluation, NGAL was positively correlated with cystatin C($r=0.41$, $p<0.001$), systolic arterial pressure z-score($r=0.3$, $p=0.031$) and creatinine($r=0.32$, $p=0.010$). NGAL correlated negatively with eGFR($r=-0.26$, $p=0.049$) and L-eGFR($r=-0.33$, $p=0.010$). Cystatin C had a negative correlation to eGFR($r=-0.29$, $p=0.025$) and a positive one with creatinine($r=0.35$, $p=0.009$) at reevaluation. No statistically significant correlation was found between cystatin C and microalbuminuria ($p=0.736$). CONCLUSIONS: NGAL and cystatin C, known markers of renal injury, correlate with renal function decline in T1D, suggesting that they may be used as supplementary tests to urine albumin excretion in order to unmask early renal dysfunction.

Key words: Cystatin C, diabetes-induced nephropathy, NGAL, type 1 diabetes, youth

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Received: 00-00-2013, Accepted: 00-00-2014

Informed consent was obtained from the parents of all participants prior to their inclusion in the study.

The study was approved by the Ethics Committee of the Aghia Sophia Children's Hospital and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Inclusion criteria for all participants were euthyroidism for at least 6 months prior to study enrollment as well as during follow-up. The exclusion criteria were the presence of active urinary tract infection, glucocorticoid medication, pregnancy, renal disease and any chronic disease other than T1D.

After 12-hour fasting, a morning blood sample was obtained for blood urea nitrogen, creatinine (sCr), uric acid (UA) and Glycated hemoglobin (HbA1c) determination. Blood was collected in special vials to be used for measurement of the specific markers NGAL and CysC. Part of the blood was centrifuged for separation of serum and stored at -80°C, until final assessments. Urinary albumin excretion was determined from a 24-hour urine collection (microalbuminuria was defined with values between 30-300 mg/24 hours, measured on at least two of three measurements over a two- to three-month period) and was measured by nephelometry.

CysC concentration was measured by an immuno-nephelometric technique using the BN Prospec nephelometer (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany). The nephelometric method is more sensitive and performs optimally in dilute solution, making it preferable for small sample volumes encountered in the pediatric population. With a range of 0.23-7.25 mg/L, this assay is currently the most precise automated assay across the clinical concentration range. The inter-assay coefficient of variation (CV) for the assay was 5.05% and 4.87% at mean concentrations of 0.97 and 1.90 mg/L, respectively.

The GFR was calculated according to the eGFR[35] and L-eGFR[36] equation methods mentioned above.

Serum NGAL levels were measured using a commercially available ELISA (Bioporto, Gentofte, Denmark). The intra- and inter-assay coefficients of variation (CVs) were 5.6% and 6.4%, respectively.

The values of systolic and diastolic arterial blood pressure (SAP and DAP respectively), body mass index (BMI) and the height are expressed in z-scores.

Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). Correlation analysis is used to determine whether the values of two variables are associated using Pearson parametric correlation (Pearson's correlation coefficient *r* with P-value). Student t-test was performed as appropriate (paired samples t-test were used to test the null hypothesis that the average of the differences between a series of paired observations is zero when performed on the same subjects or independent samples t-test when performed between controls' and patients' group). Multiple regression analysis was a method used to examine the relationship between one dependent variable and one or more independent variables. The significance was defined at p-value <0.05, rho and 95% confidence interval (CI) for the correlation coefficient.

RESULTS

Paired samples t-test of repeated measurements revealed that mean eGFR value in T1D patients was increased at re-evaluation (mean eGFR at baseline = 90.72 with SD = 19.8, mean eGFR at reevaluation = 97.5 with SD = 17.5, n=56, p=0.003). The same analysis showed an increase of the L-eGFR at reevaluation, but this result was not statistically significant (mean value at baseline=120.7, mean value at reevaluation= 122.7, n=56). Regarding eGFR, 13 patients were found to have a decreased value at reevaluation, while for L-eGFR, 23 patients had a decreased value at reevaluation. Mean value of NGAL at baseline was not significantly different between T1D patients and controls (mean: 59.5 for T1D patients, mean for controls: 62.6, p=0.393), but NGAL mean value of T1D patients at reevaluation (mean: 67.6) was statistically significantly higher than the mean value of the controls' group ($p<0.001$) according to Student t-test statistical analysis for independent samples. Paired samples t-test of repeated measurements revealed that mean NGAL value in T1D patients was increased at re-evaluation (p=0.032) (Figure 1). At baseline,

formula has been widely adopted because it has been strongly correlated to iGFR.[34,35] Moreover, formulas that assess both creatinine and cystatin C, like the Lund strategy (L-eGFR),[36] are considered to provide more accurate and reliable estimation of the GFR.

The aim of this study was to determine the possible predicting roles of serum NGAL and serum CysC, as supplementary tests to the urinary albumin excretion, in unmasking an early renal structural injury and renal function decline in asymptomatic, normoalbuminuric young T1D patients.

RESEARCH DESIGN AND METHODS

This is an observational cross-sectional long-term follow-up study. During a 12-15 months' follow-up period, a patient-group that completed both baseline and reevaluation at 12-15 months was evaluated. The patient group consisted of 56 T1D patients, with mean age 13.1 years (SD: 3.20) and mean diabetes duration 4.59 years (SD: 3.49) at enrollment, who were prospectively followed for at least 2 years at the Diabetes Centre of the First Department of Pediatrics of the University of Athens, Aghia Sophia Children's Hospital, Greece. The follow-up is scheduled to continue and reevaluation of the biomarkers will take place at a third point of time, three years after the baseline assessment.

The diagnosis of T1D was based in all participants of the patients' group on the presence of the high titer of at least one and mostly two of the known autoantibodies related to type 1 diabetes mellitus. Specifically, the percentage (%) of positivity for each autoantibody tested was: for Glutamic Acid Decarboxylase Autoantibodies (GADA) (72%), Insulinoma-Associated-2 Autoantibodies (IA2) (71%), Insulin Autoantibodies (IAA) (45%) and Islet Cell Cytoplasmic Autoantibodies (ICA) (35%). Ten of the patients presented with microalbuminuria at inclusion in the study, while five of them had persistent microalbuminuria at reevaluation and five patients were restored to normoalbuminuria. Three patients presented with newly diagnosed microalbuminuria at reevaluation. In total, eight patients were found to have microalbuminuria at reassessment.

Forty-nine healthy children with mean age 12.8 (SD: 6,6) who were referred to the Division of Endocrinology for growth evaluation but were found to be within the normal reference charts and willing to participate in the study, served as controls (Table 1).

Table 1. Demographic characteristics of T1D patients at baseline and conrols

	T1D			Controls			P-value
n	56			49			
Gender	Boys 57.1% (n= 32) Girls 42.9% (n= 24)			Boys 42.8% (n=21) Girls 57.2% (n=28)			0.31
	Mean	95% CI	SD	Mean	95% CI	SD	
Age	13.1	12.2- 13.9	3.2	12.8	10.9- 14.7	6.6	0.21
Age of diagnosis	8.5	7.6- 9.5	3.4	na			
BMI z-score	0.33	0.04 - 0.62	0.8	0.32	0.02 - 0.61	0.8	0.68
HbA1c	8.36 % (68 mmol/mol)	7.88 - 8.85% (63- 73 mmol/mol)	1.7	4.70 % (28 mmol/mol)	4.4- 4.9 % (25-30 mmol/ mol)	0.4	<0.001
T1D duration	4.5	3.6 - 5.5	3.4	na			
Microalbuminuria (daily secretion of urinary albumin ≥30mg but ≤300mg)	18.5% presented microalbuminuria (10/56)			0 % presented microalbuminuria (0/49)			

na: not applicable.

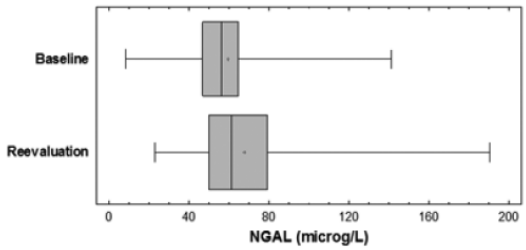

Figure 1. Comparison of NGAL plasma lenels at baseline and at re-evaluation after one year (p=0.032). Boxes represent the interquartile range; lines inside boxes represent the median value; cross represents mean marker; whiskers represent the lowest and highest obserbvation, respectively.

regarding the T1D patients' group, NGAL had no significant correlation either with eGFR (p=0.067) or with CysC (p=0.179), but it was negatively correlated with L-eGFR (r=-0.35, p=0.007, n=56). At re-evaluation, NGAL was positively correlated to CysC (r=0.41, p=0.0014, n=56) (Figure 2), SAP z-score (r=0.29, p=0.031, n=56) (Figure 3) and sCr (r=0.32, p=0.010 n=56). Moreover, at re-evaluation, NGAL had a negative correlation with both eGFR (r=-0.26, p=0.049, n=56) and L-eGFR (r=-0.33, p=0.002, n=56) (Figure 4). No statistically significant correlation was found between NGAL and microalbuminuria; however, regression analysis revealed that NGAL values higher than 70 μg/L at both time points of assessment had a positive correlation with

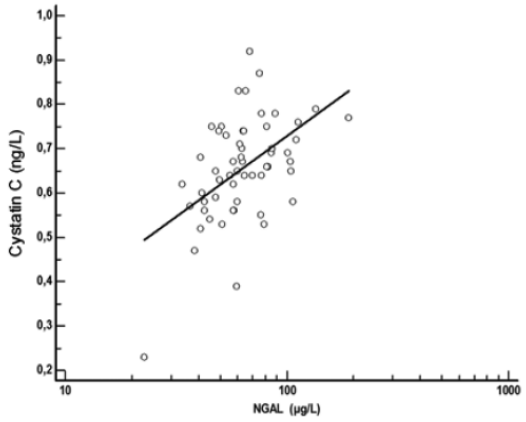

Figure 2. NGAL had a positive correlation to Cystatin C in patients with T1D at re-evaluation (r=0.41, p=0.001, n=56).

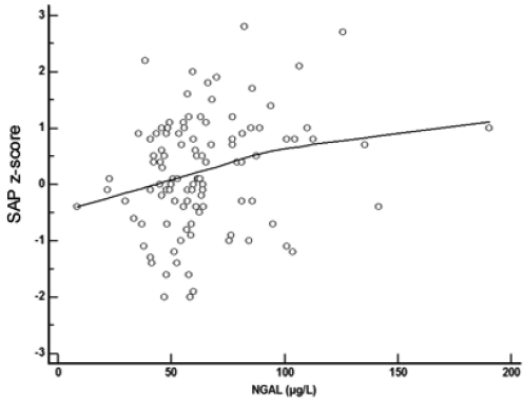

Figure 3. NGAL had a positive correlation to systolic arterial pressure (SAP) z-score patients with type 1 diabetes mellitus at re-evaluation (r=0.3, p=0.03, n=56).

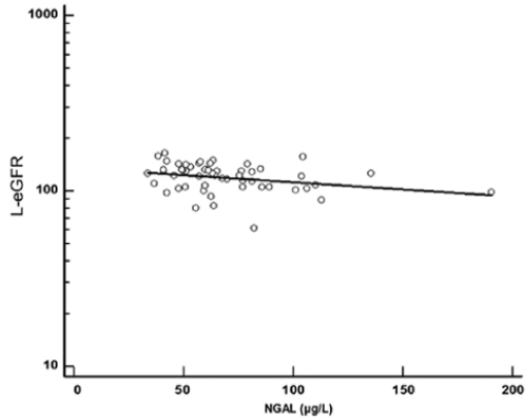

Figure 3. NGAL had a negative correlation to eGFR estimated with the Lund strategy formula in patients with type 1 diabetes mellitus at re-evaluation (r=0.28, p=0.04, n=56).

the presence of microalbuminuria (r=0.29, p= 0.038).

NGAL value, both at baseline and at re-evaluation, did not significantly correlate either with HbA1c or with BMI z-score, age or diastolic arterial pressure z-score. Furthermore, a multiple regression analysis was performed between NGAL at both time points of evaluation and age, age of T1D onset, BMI z-score, pubertal stages, sex and height z-score, but no statistically significant associations were revealed. However, higher NGAL values correlated positively with T1D duration (p=0.049).

Paired sample t-test of repeated measurements revealed that CysC mean value in T1D patients did not significantly differ between the two time points of assessment (p=0.61). No significant difference was observed between controls' and T1D CysC measurements (p=0.21). At reevaluation, CysC had a negative correlation to eGFR (r=-0.29, p= 0.025, n=56) and a positive one with sCr (r=0.35, p=0.009, n=56). At re-evaluation, UA in T1D was positively correlated with CysC (r=0.41, p=0.016, n=56). No statistically significant correlation was found between CysC and microalbuminuria (p=0.75), HbA1c (p=0.24), BMI z-score (p=0.38), age (p=0.79), SAP z-score (p=0.38), DAP z-score (p=0.46) and T1D duration (p=0.44). A multiple regression analysis was performed between CysC at both time points of evaluation and age, age of T1D onset, T1D duration, pubertal stages, BMI z-score, sex and height z-score and a statistically significant positive correlation between CysC and male sex in the patients' group (r=0.47, p<0.001) was revealed. No statistically significant correlation was found between the male sex and cystatin C in the controls' group. A multiple regression analysis was performed between sCreatinine at both time points of evaluation and age as well as T1D duration and a statistically significant positive correlation between age of assessment and sCr was found (r=0.50, p=0.0014), but no significant association with T1D duration was demonstrated (p=0.372). A multiple regression analysis was performed between eGFR, L-eGFR and age as well as T1D duration, but no statistically significant association was found.

No participant from the control group presented microalbuminuria.

During follow-up all participants had eGFR greater than 60 ml/min.

DISCUSSION

Microalbuminuria has generally been considered as the earliest marker of diabetic nephropathy development and is often associated with established significant glomerular damage. However, recent studies showed that MA does not necessarily reflect permanent renal impairment. In addition, several lines of evidence suggest that early structural damage in both glomerular and tubular structures may be present in normoalbuminuric subjects. Indeed, cohort studies including prepubertal children with average diabetes duration of 5-8 years revealed GBM thickening and mesangial expansion,[15] while it also disclosed that longstanding normoalbuminuric T1D patients may have significant glomerulopathy lesions[16,18,19] The pathophysiologic changes in DN therefore include renal function decline associated with cellular and extracellular derangements in both the glomerular and tubulo-interstitial compartments.[18]

The first results of this long-term observational study aimed in assessing the predictive value of early markers of renal injury, such as NGAL and CysC, in young T1D patients by unraveling renal structural damage long before renal dysfunction occurs.

We found that the mean value of estimated GFR for the patients' group was increased at reevaluation, as expected, since the equation formulas take into account body height, which increases with advancing age in children and adolescents. The cases where eGFR was lower at reevaluation than at baseline were related to a higher serum creatinine or cystatin C. NGAL correlated negatively with eGFR and L-eGFR, indicating an association with renal function decline. NGAL was positively correlated with CysC, SAP z-score, Cr and T1D duration. NGAL values higher than 70μg/L were associated with the presence of microalbuminuria. Our study underpins the value of NGAL as a biomarker of early renal damage in T1D, since NGAL values increased during follow-up more specifically when eGFR was decreased and this may reflect a progress of the early renal structural damage occurring with advancing T1D duration. NGAL value demonstrated a significant negative correlation with the estimated GFR using two different methods, suggesting that a higher NGAL value is associated with a glomerular function decline even if remaining within normal values, this probably suggestive of the occurrence of early lesions. The number of the included patients who presented microalbuminuria was much lower than the number of patients who presented a decreased eGFR. Moreover, the fact that a percentage of normoalbuminuric patients, who were found to have renal function decline, presented higher NGAL concentrations, while some patients with microalbuminuria had normal NGAL concentrations, may imply that these two markers,

i.e. microalbuminuria and NGAL, may reflect different sites of renal damage during the process of DN establishment.

It is certainly necessary to identify markers of early tubular damage independently of albuminuria development in patients with early DN and progression, as it may play a significant role in the management of the normoalbuminuric renal insufficiency cases.[36,37] The significant positive correlation between NGAL, a known marker of structural renal lesion, and established renal biochemical indexes such as sCr and CysC underpin its important role in the early diagnosis of renal dysfunction.

SAP has been previously demonstrated to be a predictor of DN.[3] In our study, because it was specifically focused on a young population, we estimated the SAP z-score and found a positive correlation with NGAL. This fact, independently of DN, may reflect an indirect role of NGAL as an endothelial dysfunction marker. Undoubtedly, further studies investigating endothelial dysfunction will further delineate the extent of microvascular damage in DN. According to multiple regression analysis, NGAL values were not influenced by age, age of T1D onset and puberty, suggesting independency from the several physiologic mechanisms occurring during puberty.

A limitation of our study was the fact that repeated measurements were obtained only in the children with T1D after 12-15 months and not in the control group as well. However, it is considered unethical to perform repeated venipunctures in healthy children, especially in assessing markers that have been shown not to be related to pubertal progression and age, while they do not increase without a backstage tissue injury.[23] Moreover, it is known that microalbuminuria does not occur in healthy children unless renal disease, hypertension, obesity or cardiovascular disease are present.[38]

The diagnostic utility of CysC is well documented in the acute setting.[28,29] Our study demonstrated that CysC had a positive correlation with sCr and a negative correlation with eGFR, suggesting that higher cystatin C values correlate with a decreased eGFR. These findings may support the predictive role of CysC in early DN. Moreover, cystatin C assessment is undoubtedly useful in the more accurate GFR determination.[30] Its significant positive correlation with UA, which has been demonstrated to be associated with renal dysfunction,[10] hypertension and cardiovascular disease, further supports the role of CysC as a prognostic cardiometabolic biomarker in diagnosing early diabetic microvascular complications.[31] Multiple regression analyses found that CysC values were not influenced by age, duration of T1D and puberty. However, CysC was found to have higher values in boys in the patients' group, and this fact may imply a gender dimorphism of this biomarker. This finding has not been previously reported, nor has it been replicated in our control group, and it therefore necessitates further validation through large-scale future studies.

According to *the National Kidney Foundation (NKF) and the Kidney Disease Outcomes Quality Initiative (KDOQI),* a patient is considered to be in chronic kidney disease (CKD) state if he presents a GFR<60 mL/min per 1.73 m^2 for three months or more. Alternatively, any ongoing (at least three months) structural or functional abnormality of the kidney, regardless of GFR, that can be detected by pathological abnormalities or specific markers is considered CKD.[40] The significant correlation between NGAL, cystatin C and GFR decline further support the prognostic role of NGAL and cystatin C in unmasking the structural renal damage before an overt renal impairment becomes evident. The association between the early tubular interstitial damage in normoalbuminuric people with T1D and NGAL is further supported by recently published studies.[17,18,20] To our knowledge, this is the first study to demonstrate the predictive role of NGAL and CysC as early markers of DN in children, adolescents and young adults before severe overt nephropathy occurs. These findings remain to be further confirmed at the final evaluation of this long-term study as well as by further prospective studies.

Defining new predictors as supplementary tests to urinary albumin excretion for the early diagnosis of DN would accelerate the effective management and treatment approaches which are desperately needed in order to minimize the rates of severe cardiorenal morbidity and mortality in young T1D patients. Therefore, these data need to be confirmed by further large-scale longitudinal studies before be-

ing integrated in the DN risk assessment of young patients with T1D.

CONFLICT OF INTEREST

None for all authors.

FUNDING

No conflict of interest or funding sources exist for any author.

AUTHORS' CONTRIBUTIONS

N.P. researched data and wrote the manuscript. C.S. contributed to the biochemical measurement I.K. contributed to the research of data. M.P. contributed to the research of data. G.C. contributed to the discussion and reviewed/edited the manuscript. I.P. contributed to the biochemical measurement and contributed to the discussion. C.K.-G. contributed to the discussion and reviewed/edited the manuscript.

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9 Curriculum Vitae

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Education and research experience

- Current position: Consultant physician in Endocrinology and Diabetology, Department of Endocrinology, Metabolism and Diabetes, University hospital of Linkoping, Sweden
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- March 2002-August 2003: Rural service, Lefkimmi, Corfu, Greece.
- 2002: Medical degree, Medical school University of Ioannina

Publications, Presentations and Abstracts

- ✓ *Ioannis Papassotiriou, Nektaria Papadopoulou-Marketou, Chrysanthi Skevaki, George P. Chrousos, Christina Kanaka-Gantenbein*. ‘’Association of the newly discovered cardio-renal biomarkers cystatin c, NGAL, GDF-15 and YKL-40 with arterial pressure elevation in young patients with type 1 diabetes’’, American Society of Hypertension (ASH), 2014(poster presentation)

- ✓ *Nektaria Papadopoulou-Marketou, Chrysanthi Skevaki, Ioanna Kosteria, Melpomeni Peppa, George P. Chrousos, Ioannis Papassotiriou, Christina Kanaka-Gantenbein*. ‘’NGAL and Cystatin C: two possible Early Markers of Diabetic Nephropathy in Young Patients with Type 1 Diabetes Mellitus: One year follow up’’ Hormones 2014; DOI: 10.14310/horm.2002.15201

- ✓ *Nektaria Papadopoulou-Marketou, Chrysanthi Skevaki, Melpomeni Peppa, Ioanna Kosteria, Ioannis Papassotiriou, George Chrousos, Christina Kanaka-Gantenbein*. ‘’NGAL and Cystatin C as early markers of diabetic nephropathy in patients with type 1 diabetes mellitus’’ 39th Annual Conference of the International Society for Pediatric and Adolescent Diabetes (ISPAD), Gothenburg, 2013(oral presentation)

- ✓ *Nektaria Papadopoulou, Chrysanthi Skevaki, Ioanna Kosteria, Melpomeni Peppa, Ioannis Papassotiriou, George Chrousos, Christina Kanaka-Gantenbein* “NGAL: An early marker of diabetic nephropathy?” 51st Annual Meeting of the European Society for Pediatric Endocrinology(ESPE), Milan 2013(poster presentation)

- ✓ *Antonia Dastamani, Niki Philippas, Nektaria Papadopoulou, Paraskeyi Pervanidou, Christina Kanaka-Gantenbein and George Chrousos* ‘’Diet Combined with Desserts with a Low Glycemic Index/Glycemic Load has a Positive Effect on Metabolic Syndrome Parameters in Overweight/Obese Children’’. ENDO Boston, 2011 (poster presentation)

- ✓ *Flora Tzifi, Maria Kanariou, Marianna Tzanoudaki, Nektaria Papadopoulou, George Chrousos, Christina Kanaka-Gantenbein* Cd4+ T Cell Receptor Vβ Repertoire Analysis In Newly Diagnosed Children With Diabetes Type 1 In Comparison To Children With A Systemic Autoimmune Disease (Systemic Lupus Erythematosus) And Age-Matched Healthy Controls,

ISPAD(International Society for Pediatric and Adolescent Diabetes), Istanbul 2012 (poster presentation)

- ✓ *Nektaria Papadopoulou, Ioanna Kosteria, Demosthenes Malliopoulos, George Chrousos, Catherine Dakou-Voutetakis, Christina Kanaka-Gantenbein*. ‘’Clinical manifestations of diabetes type 1 in children and andolescents; Seasonal distribution of diagnosis in Greece. 20-years’ experience from a pediatric Diabetes Center’’. (Panhellenic Congress of Endocrinology, Thessaloniki, 2011) (poster presentation)

- ✓ *Nektaria Papadopoulou, Ioanna Kosteria, Demosthenes Malliopoulos, George Chrousos, Catherine Dakou-Voutetakis, Christina Kanaka-Gantenbein*. Comorbidity between type 1diabetes and other autoimmune diseases. 20-years’ experience of a pediatric diabetes center (Panhellenic Congress of Endocrinology, Thessaloniki, 2011) (oral presentation)

- ✓ *Nektaria Papadopoulou, Maria-Christina Zennaro, Christina Kanaka Gantenbein, George Chrousos, Evangelia Charmandari. ‘’*A de novo mutation in the gene of the Mineralocorticoid Receptor, that causes Pseudohypo-aldosteronism type 1’’ (Panhellenic Congress of Endocrinology, Thessaloniki, 2011) (poster presentation)

- ✓ *Antonia Dastamani, Niki Philippas, Nektaria Papadopoulou, Paraskeyi Pervanidou, Christina Kanaka-Gantenbein and George Chrousos ΄΄*Diet Combined with Desserts with a Low Glycemic Index Has a Positive Effect on Metabolic Syndrome Parameters in Overweight Children’’ ESPE (European Society Pediatric Endocrinology) , Prague,2010(poster presentation)

- ✓ *Antonia Dastamani, Niki Philippas, Nektaria Papadopoulou, Paraskeyi Pervanidou, Christina Kanaka-Gantenbein and George Chrousos ΄΄*The beneficial effect of Low Glycemic Index on Metabolic Syndrome Parameters in

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- ✓ *Aikaterini Masgala, Niki Petroglou, Stavros Anevlavis, Athanasios Tzavaras, Evaggelos Marinakis, Nektaria Papadopoulou, Eleftherios Anevlavis*. ''Diagnostic quality and value of sedimentation rate, CRP and leucocytes in bacterial infections''. Internal Medicine Conference, Thessaloniki, 2006(oral presentation)

Research Experience

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Teaching Experience

Instructor at problem based learning student-groups, Medical School University of Linkoping, Sweden

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Mother tongue: Greek

Other languages	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C2	C2	C2	C2
Swedish	C2	C2	C2	C2	C2
French	B1	B1	B1	B1	B1
Italian	B2	B2	B2	B2	B2