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The possible role of serum NGAL and MMP-9 in the assessment of kidney impairment and cardiovascular risk in children and adolescents with type 1 diabetes mellitus or obesity

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Abstract. – OBJECTIVE: Type 1 diabetes mellitus (DM1) and obesity represent two chronic pediatric diseases characterized by increased risk for renal impairment and cardiovascular disease. The potential usefulness of neutrophil gelatinase-associated lipocalin (NGAL) and matrix metalloproteinase-9 (MMP-9), two novel biomarkers, for predicting early kidney injury or increased cardiovascular risk in children and adolescents with DM1 or obesity, was investigated in this cross-sectional study.

PATIENTS AND METHODS: Serum samples were obtained from children and adolescents aged 12.7 ± 3.8 years old with DM1 (n = 38) or obesity (n = 34) and normal renal function, as well as from healthy controls (n = 24). NGAL and MMP-9 concentrations were measured using commercially available sandwich ELISA kits (NGAL: DY1757-05, MMP-9: DMP900; R&D systems, Minneapolis, MN, USA).

RESULTS: NGAL serum values were found significantly higher in patients with obesity but not in those with DM1. A positive correlation was found in patients with DM1 with diabetes duration, and in the total population with body mass index (BMI) z-score. Also, serum MMP-9 levels were significantly increased in patients with DM1 and in patients with obesity compared to controls.

CONCLUSIONS: Circulating NGAL and MMP-9 levels may prove useful as surrogate biomarkers to creatinine, glomerular filtration rate (GFR), and albumin excretion rate for early detection of kidney injury and cardiovascular complications in children and adolescents with DM1 or obesity.

Key Words:
NGAL, MMP-9, Children, Diabetes mellitus type 1, DM1, Obesity, Biomarkers.

Introduction

Type 1 diabetes mellitus (DM1) represents a complex chronic disease of autoimmune origin with an accelerating prevalence worldwide. It is characterized by the destruction of the insulin-producing pancreatic beta cells and progressive metabolic derangement that is directly associated with macrovascular and microvascular complications[1]. More than 30% of patients with DM1 develop diabetic kidney disease (DKD), which is the leading cause of end-stage kidney disease (ESKD), presenting as reduced renal function and/or albuminuria[2]. Other DM1 complications include retinopathy, neuropathy, and cardiovascular disease (CVD)[2].

In general, DKD is caused by multiple factors, including genetic predisposition and environmental components, such as obesity and lifestyle, while arterial hypertension and uncontrolled hyperglycemia further increase the morbidity and mortality risk associated with DKD[3]. Many implicated genes have been identified, broadening our understanding of the involved pathophysiological mechanisms. So far, the most frequently used tools for assessing the severity of renal

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impairment are microalbuminuria and glomerular filtration rate (GFR)[3]. However, both markers have restrictions in their use, including reduced sensitivity. Therefore, novel, sensitive diagnostic and predictive biomarkers of kidney damage have been proposed to estimate the risk for DKD with the intent of integrating them into clinical practice. Among numerous biomarkers that have been examined, neutrophil gelatinase-associated lipocalin (NGAL) has been proposed as a promising useful marker for early detection of chronic kidney disease (CKD)[3]. NGAL is an innate immune protein of 25 kDa that belongs to the lipocalin superfamily and is expressed by activated neutrophils but is also stored in other human tissues[4]. It can also be produced in kidney tubular cells as a response to ischemia and tubular injury[5]. In acute kidney injury, NGAL levels increase due to NGAL overexpression in distal nephrons and impaired tubular reabsorption[6].

Obesity is another common chronic disease with multifactorial causes, characterized by the accumulation of adipose tissue, energy homeostasis imbalance, and metabolic dysregulation. Pediatric obesity represents a public health concern as it has reached epidemic proportions worldwide and is associated with the development of cardiovascular complications and premature death in adults[7]. Impaired insulin sensitivity, type 2 diabetes (DM2), dyslipidemia, hypertension, atherosclerosis, and peripheral and coronary artery disease are leading causes of obesity-related morbidity and mortality[8,9].

In addition, it is well-established that inflammation plays a key role in obesity and its complications. Research has shown that obesity is linked to a low-grade chronic inflammatory process due to the secretion of adipokines that are involved in extracellular matrix (ECM) remodelling[10]. Similarly, obesity-related comorbidities, such as hypertension, are also characterized by an inflammatory process mediated by proteolysis of the ECM[10]. This ECM degradation is mediated by matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases responsible for precise proteolysis and synthesized by vascular, endothelial, and inflammatory cells that are involved in the degradation of proteins such as collagen, elastin, and fibronectin[10]. Among them, MMP-9 is considered a key player in tissue remodelling[11] and is overexpressed in adults with obesity[12]. MMP-9 degradation is inhibited by NGAL, resulting in sustained MMP-9 proteolytic activity. Disorders in MMP-9 activity, gene expression, and urine concentrations have been reported in patients with diabetes-associated nephropathy and in other proteinuric kidney diseases.

According to the literature, there appears to be a strong relationship between kidney disease and CVD. It has been found that in patients with ESKD, the risk of CVD is overwhelming[13] and that the vascular alterations involved are associated with the remodeling of extracellular matrix[14] due to disordered MMP activity[15]. Therefore, NGAL and MMP-9 serum concentrations could hypothetically depict this relationship.

The aim of the present study was to investigate the possible role of serum NGAL and MMP-9 in identifying children and adolescents with DM1 or obesity and kidney dysregulation or increased cardiovascular risk, taking into consideration age, sex, duration of diabetes, diabetes regulation, and severity of obesity.

Patients and Methods

Study Population

This is a single-center cross-sectional study of 38 children and adolescents with DM1 and 34 children and adolescents with obesity, aged two to 18 years old, who were seen in routine follow-up visits in the outpatient clinic of the division of pediatric endocrinology and diabetes of a tertiary hospital, the University Hospital of Patras in Greece. Twenty-four healthy controls were also included in the study. Exclusion criteria for participation included DM1 diagnosis less than six months prior to recruitment, presence of any other chronic diseases, and thyroid disorders. All patients were monitored by protocol for anthropometric indices (weight, height, BMI) and pubertal status using Tanner stages. In order to classify the different degrees of weight status, we used the body mass index (BMI) z-score adjusted for age and sex. A BMI z-score of > 2 was used to define obesity.

The study was approved by the research ethics committee of the University Hospital of Patras (No.: 353, date: 02/09/2015) and it was conducted in accordance with the Helsinki Declaration as revised in 2013. Written informed consent from the parents or legal representatives of the participating children and adolescents and informed assent from the participants were obtained.

Serum NGAL, MMP-9, and Standard Biochemical Measurements

Serum NGAL and MMP-9 concentrations were measured in children and adolescents with T1DM

and obesity and in the controls. They were associated with age, DM1 duration, glucose control, lipid profile, renal function, and degree of albuminuria. All obtained serum samples were appropriately stored at -80°C after separation from clotted blood by centrifugation for 10 min at 1,200 x g at 4°C until assayed. NGAL and MMP-9 concentrations were measured using commercially available sandwich ELISA kits (NGAL: DY1757-05, MMP-9: DMP900; R&D systems, Minneapolis, MN USA). The tests were performed according to the manufacturer's recommended protocols.

Total serum cholesterol (Chol), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), creatinine, and hemoglobin A1c (HbA1c) levels were measured using an automated analyzer [ADVIA® 2400 Chemistry System, Siemens (Erlangen, Germany)]. The estimated glomerular filtration rate (eGFR) was calculated according to the revised bedside Schwartz formula as indicated by the Kidney Disease Improving Global Outcomes (KDIGO) guidelines (https://www.kidney-international.org/article/S0085-2538(23)00766-4/fulltext).

Statistical Analysis

All analyses were performed with either the SPSS statistical package (version 16.0 SPSS Inc., Chicago, IL, USA) or GraphPad Prism (version 5.00 for Windows, GraphPad Software; San Diego, CA, USA). All statistical tests were two-sided, with significance set at $p < 0.05$.

Quantitative variables are presented as mean and standard deviation when normally distributed or median and interquartile range (IQR) when skewed. The Kolmogorov-Smirnov test was used for normality analysis. Categorical data are presented as frequencies and percentages (n, %). For examining the differences in the means of NGAL or MMP-9 serum concentrations between children with DM1 or obesity and controls, we used ANOVA with Bonferroni post-hoc analysis in normally distributed data or Kruskal-Wallis test with Dunn's post-hoc analysis in skewed data. Furthermore, patients with DM1 were divided into two groups according to their mean HbA1c levels calculated using HbA1c measurements of the past year of follow-up (mean HbA1c < 7%: group with optimal glucose control; HbA1c > 7%: group with suboptimal glucose control). Comparisons between measured markers and HbA1c or Tanner stages were performed using independent samples *t*-test or Mann-Whitney test in case of violation of normality. Moreover, correlation Spearman's rho was used to test the relationship between demographic, clinical or biochemical variables with serum NGAL and MMP-9 levels.

Results

Overall, 96 children and adolescents were enrolled in the study. Baseline demographic characteristics of both groups of patients and controls are shown in Table I. There were 38 patients with DM1, 34 patients with obesity, and 24 control subjects. The mean duration after DM1 diagnosis was 3.4 ± 2.6 years. There were 27 patients with more

Table I. Basic clinical and biochemical characteristics of controls and patients with DM1 and obesity.

	Controls (n = 24)	DM1 (n = 38)	Obesity (n = 34)	*p*-value
Age (years)	9.9 ± 3.4	12.7 ± 3.8	12.2 ± 2.8	0.08
Sex (males/females)	7/17	17/21	15/19	ns
BMI z-score	0.53 ± 0.8	0.52 ± 0.9	2.2 ± 0.5	< 0.001
Serum creatinine (mg/dl)	0.68 ± 0.13	0.71 ± 0.12	0.66 ± 0.13	ns
eGFR (ml/min/1.73 m^2)	95 ± 17	93 ± 23	97 ± 16	ns
TSH	1.89 ± 0.69	1.82 ± 0.71	2.41 ± 1.1	0.01
FT4	1.24 ± 0.19	1.24 ± 0.17	1.25 ± 0.21	ns
CHOL	158.6 ± 28.4	167.5 ± 31.5	160.6 ± 39.4	ns
LDL	93.2 ± 21.8	94.5 ± 24.9	93.9 ± 38.7	ns
HDL	62.7 ± 12.9	58.5 ± 10.9	50.4 ± 13.5	< 0.001
TG	65.1 ± 24.8	67.2 ± 26.7	90.6 ± 60.1	0.03

BMI: body mass index, eGFR: estimated glomerular filtration rate, TSH: thyroid stimulating hormone, FT4: free T4, CHOL: cholesterol, LDL: low-density lipoprotein, HDL: high-density lipoprotein, TG: triglycerides.

Table II. Neutrophil gelatinase-associated lipocalin and MMP-9 serum concentrations between controls and patients with either DM1 or obesity.

	Controls (n = 24)	DM1 (n = 35)	Obesity (n = 38)	*p*-value
NGAL (pg/ml)	36,396 ± 10,750	44,894 ± 18,489	75,079 ± 48,551	< 0.001
MMP-9 (ng/ml)	282.6 ± 145.1	437.1 ± 221	559 ± 345.9	< 0.001

DM1: type 1 diabetes mellitus, NGAL: neutrophil gelatinase-associated lipocalin, MMP-9: matrix metalloproteinase-9.

than one year of DM1 duration and eight patients with a duration of over five years. Well-controlled DM1 (as defined in methods) was observed in 17 children. Twenty-two patients were prepubertal, while the rest were pubertal. All the patients were euthyroid and had normal GFR and normoalbuminuria.

Neutrophil Gelatinase-Associated Lipocalin

Post-hoc analyses showed that serum NGAL concentrations were significantly increased in patients with obesity in comparison to both controls and patients with DM1. In the latter group, although NGAL concentrations were moderately increased, this did not reach statistical significance (Table II, Figure 1A). Furthermore, in patients with DM1, NGAL concentrations showed a positive correlation with diabetes duration (Spearman rho 0.364, $p = 0.044$). However, patients with different diabetes duration (> 1 year *vs.* < 1 year, or > 5 years *vs.* < 5 years) did not show significantly different serum NGAL concentrations, although they were marginally higher in the groups of larger DM1 duration (46,807 ± 19,995 *vs.* 30,972 ± 7,345 pg/ml, $p = 0.13$, and 53,400 ± 14,823 *vs.* 41,760 ± 20,327 pg/ml, $p = 0.15$, respectively). Glycaemic control expressed as mean HbA1c < 7% *vs.* > 7% did not affect NGAL levels. Finally, in patients with DM1, different pubertal statuses, as expressed by Tanner stages, did not significantly affect serum NGAL concentrations, although prepubertal children showed modestly higher levels (61,869 ± 26,618 *vs.* 50,145 ± 20,426 pg/ml, $p = 0.07$).

No significant correlations were found in patients with obesity. When all study participants were examined together, serum NGAL concentrations correlated positively to BMI z-score (Spearman rho 0.401, $p = 0.004$).

Matrix Metalloproteinase-9 (MMP-9)

Serum MMP-9 concentrations were overall found to be significantly increased in patients with DM1 and obesity in comparison to controls (Table II, Figure 1B). In post-hoc analyses, MMP-9 serum concentrations were found to be margin-

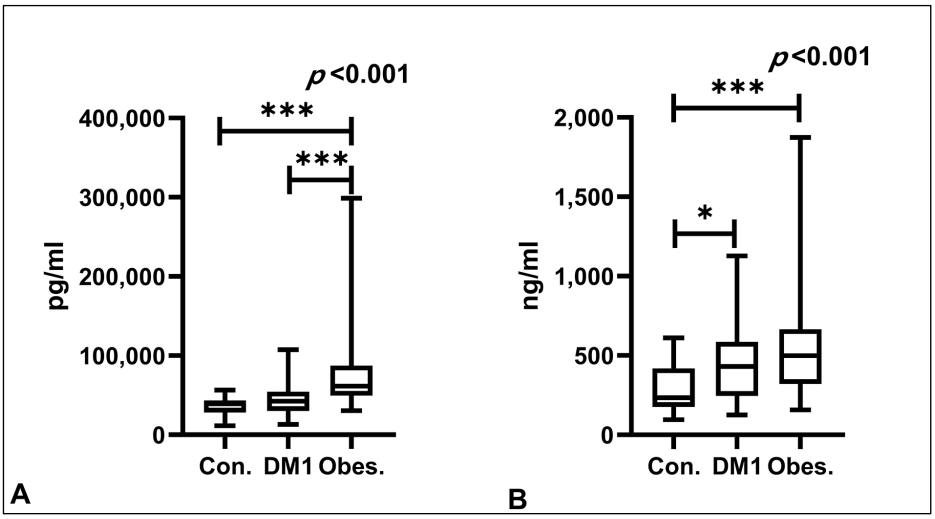

Figure 1. Mean values of markers NGAL and MMP-9 in serum of children with type 1 diabetes mellitus (DM1), obesity (obes), and controls (Con). **A**, NGAL serum concentrations. **B**, MMP-9 serum concentrations. *: $p < 0.05$, ***: $p < 0.001$.

ally increased in patients with obesity in comparison to those with DM1, but this difference was not significant. Patients diagnosed with DM1 for different time periods (> 1 year *vs.* < 1 year, or > 5 years *vs.* < 5 years) did not show significantly different serum MMP-9 levels although they were marginally higher in the groups of larger duration (462.8 ± 227.2 *vs.* 267.4 ± 63.5 ng/ml, $p = 0.1$, 533.8 ± 284.1 *vs.* 404.1 ± 193.3 ng/ml, $p = 0.16$, respectively). Suboptimal glucose control expressed as mean HbA1c > 7% did not affect MMP-9 concentrations. Finally, in patients with obesity, different pubertal statuses, as expressed by Tanner stages, did not affect serum MMP-9 concentrations.

When all study participants were examined together, serum MMP-9 levels did not correlate to any biochemical or other factors. In patients with either DM1 or obesity, serum MMP-9 concentrations also did not show any correlation with any of the examined indices.

Discussion

The findings of the present study demonstrate that NGAL concentrations are significantly higher in children and adolescents with obesity compared to healthy controls. Interestingly, the difference was not significant when children and adolescents with DM1 were compared to their healthy peers. This finding is of particular importance since elevated serum NGAL concentrations have been associated with acute kidney injury (AKI) and chronic kidney disease[16]. NGAL has been proposed as an effective diagnostic marker of AKI in different clinical settings[17,18], and as such, it has raised great expectations. Of interest, it is considered to perform better as a marker of AKI in children than in adults[17,19].

NGAL has also been proposed as a marker of CKD progression. An inverse relationship has been found between serum and urine NGAL and GFR decrease[20]. Kidney injury is commonly estimated by endogenous creatinine, an index of kidney function. However, increased blood creatinine concentrations primarily reflect GFR. Therefore, they are not considered ideal for the estimation of kidney injury if the GFR is not significantly reduced, as creatinine is influenced by diet, muscle mass, medications, sex, and ethnicity. In contrast to creatinine and other previously used markers, such as blood urea nitrogen and cystatin C, NGAL does not reflect renal function but renal cell damage. More specifically, it has been reported that increased NGAL concentrations are not only caused by decreased kidney clearance but also by increased production by damaged tubular epithelial cells. Therefore, whereas increased creatinine reflects the loss of functional nephrons, increased NGAL depicts the rate of progression of CKD[21]. In addition, creatinine is produced in the liver. Therefore, hepatic parenchymal dysfunction has a direct impact on its concentrations[22].

Other commonly used diagnostic markers for CKD include GFR, albuminuria, and proteinuria. However, their sensitivity is limited[23]. GFR is a measure of total nephron function. The use of inulin clearance for the measurement of GFR is considered the gold standard method but has been abandoned in clinical practice as it is laborious. The Schwartz formula is used instead as a measure of estimated GFR, which uses serum creatinine, height, and the coefficient k, which is proportional to muscle mass and depends on age and sex. The error of GFR has been shown to be ± 30-40% in children[24], and in younger than two years old children, GFR can be underestimated due to urinary tract immaturity.

Albuminuria is not always sensitive as a renal marker in children, as pediatric CKD is often caused by congenital anomalies which are associated with tubular loss of albumin as well[25]. There is also mounting evidence suggesting that glomerular or tubular damage is not always dependent on albuminuria, as it may be present in the absence of microalbuminuria[26,27].

Microalbuminuria, defined as a urinary albumin excretion rate of 30-300 mg/day or urinary albumin-to-creatinine ratio of 30-300 mg/g, is used for the screening of diabetes nephropathy (DN) in DM1[28]. It represents probably the most devastating complication of DM1 as it is associated with a high incidence of ESKD, cardiovascular disease, and premature death. Microalbuminuria is present in 12-16% of adolescents with DM1[29] and is directly linked to puberty and poor glycaemic control[30]. Significant glomerular damage is believed to have already been established when albuminuria is detected. Nonetheless, regression of microalbuminuria is frequently seen in patients with DM1, particularly in the early stages, and even if it persists, it is usually not accompanied by impaired kidney function. In addition, a significant portion of non-albuminuric patients present with renal impairment[31,32]; therefore, numerous studies[33,34] have challenged the diagnostic value of microalbuminuria in diabetic nephropathy and have proposed novel early biomarkers for early

detection of DN. Among them, NGAL is one of the most promising tubular markers since DN is caused by both glomerular and tubular interstitial injury[27,28]. NGAL is detected before the onset of proteinuria or alterations of the GFR, and it has also been associated with early tubular damage in nonalbuminuric patients with DM1.

A study by Papadopoulou-Marketou et al[35], demonstrated that serum and urine NGAL concentrations were not significantly different in children and adolescents with DM1 compared to controls on initial evaluation, which is in line with our findings[35]. However, they were significantly higher in the diabetes group on re-evaluation 12-15 months later, which may suggest that there is a critical time point when kidney injury that can be captured by NGAL develops. In agreement with the above hypothesis is the finding by both the study by Papadopoulou-Marketou et al[35] and our present study, that serum NGAL is positively correlated with diabetes duration. Notably, our population of children and adolescents with DM1 had a younger mean age (12.7 *vs.* 13.1 years) and smaller duration of diabetes (3.4 *vs.* 4.59 years), which could account for the higher, but not significantly higher NGAL concentrations compared to the controls. It could be speculated that if the participating population was older, larger or the duration of diabetes longer, this difference may have reached statistical significance. Further large-scale prospective studies are required so that safer conclusions can be drawn. Remarkably, no correlations were found by our group between NGAL and GFR, creatinine levels, age of DM1 patients, lipidemic profile, or any of the other studied parameters.

On the other hand, the significantly higher NGAL concentrations in the children and adolescents with obesity compared to the controls suggest that kidney injury is present from an early age in this population. This finding confirms recent literature reports indicating that pediatric obesity is associated with an increased risk of kidney injury, CKD, and ESKD[36,37]. Again, since the onset of obesity-related renal disease is insidious and asymptomatic, and since in the presence of functional reserve and normal kidney function, GFR is maintained within the normal range, markers that directly reflect kidney injury are needed. In a study by Polidori et al[38], urinary NGAL concentrations were found increased in prepubertal children with obesity[38]. In contrast, Gul et al[39], failed to demonstrate a relationship between urine NGAL and obesity, but it should be noted that the obesity group was small[39].

Our finding of significantly increased serum NGAL concentrations in the obese population but not in the age-matched population with DM1 may suggest that obesity represents a stronger risk factor for tubular renal injury and cardiovascular morbidity than DM1. This is one of the most important findings of the present study. This study is the first to examine both children and adolescents with DM1 and children and adolescents with obesity, which adds to the importance of the findings.

In addition, our data demonstrated significantly elevated MMP-9 concentrations in children and adolescents with DM1 and in those with obesity compared to the controls. MMP-9 concentrations have been implicated in diseases characterized by inflammation and oxidative stress[40,41]. As previously mentioned, MMPs modulate the extracellular matrix, and their production is enhanced by increased glucose concentrations. Indeed, increased serum MMP-9 levels and MMP-9 excretion have been observed in patients with DM1 compared to non-diabetic individuals[42,27]. It has also been shown that advanced glycation end-products produced secondary to chronic hyperglycemia increase MMP-9 expression[43], which is also positively correlated with HbA1c concentrations. In children with DKA, MMP-9 levels are even higher compared to children with DM1 but without DKA[44].

Moreover, MMP-9 concentrations have been found to be significantly higher in adult patients with T1DM and retinopathy compared to non-diabetic patients ($p < 0.001$) and patients with T1DM but without retinopathy ($p < 0.05$). MMP-9 concentrations have also been found elevated in adults with DM1 and subclinical neuropathy, nephropathy, or atherosclerosis[45]. It is well known that renal function decline in DM1 is associated with both glomerular and tubulointerstitial injury and that diabetic nephropathy is linked to generalized endotheliopathy and cardiovascular disease[46]. Disordered remodeling of ECM by MMPs is associated with vascular damage[47]. Therefore, MMP-9 has been proposed as a surrogate biomarker of retinopathy and other early diabetic complications in patients with T1DM[48,49].

Increased serum MMP-9 concentrations have been found in adults with obesity[50], metabolic syndrome[51], and type 2 diabetes[52]. A positive correlation has been found between MMP-9 expression and BMI, whereas insulin sensitivity has been negatively correlated with MMP-9[53]. MMP-9 activation has been associated with increased cardiovascular disease and mortality rates in adults[54].

Furthermore, increased MMP-9 concentrations have been reported in hypertensive compared to normotensive adults[55], although a causal relationship between MMP-9 and hypertension has not clearly been established. Interestingly, elevated MMP-9 concentrations have been associated with obesity and cardiac hypertrophy in treatment-resistant hypertensive patients, suggesting crosstalk among obesity, components of ECM, and cardiac remodelling[56], rendering this biomarker as a potential predictor of cardiovascular disease.

Differences have been found in MMP-9 haplotypes between children and adolescents with obesity and controls, indicating that genetic factors may account for the development of obesity-related cardiovascular complications[57]. It has also been demonstrated that children and adolescents with obesity present higher mean intima-media thickness values of the carotid arteries, which are linked to chronic inflammatory processes in cardiovascular disease mediated by MMP-9[58].

It has been hypothesized that MMP-9 elevation in DM1 reflects the underlying inflammation[42]. In the same context, the same may be true for obesity, as inflammation is a common characteristic for both entities.

The current study has certain limitations, including the small sample size. Another limitation is that the circulating concentrations of the studied biomarkers do not necessarily reflect the tissue concentration and matrix degradation[59]. Therefore, larger studies in tissues and on a molecular level may be useful to confirm our findings.

Notably, although dyslipidemia is considered one of the laboratory predictors of diabetes nephropathy, but also of endothelial damage and cardiovascular disease[60], no association was established between the NGAL or MMP-9 and the lipid profile of the two studied groups.

Conclusions

Novel non-invasive biomarkers are required for the estimation of renal impairment with evolutionary potential and unmasking early cardiovascular complications in children and adolescents with DM1 and obesity, two of the most common and complex chronic pediatric diseases. Our results are in line with previous findings favoring the possible predictive role of NGAL and MMP-9 as early markers of renal impairment and asymptomatic cardiovascular morbidity before creatinine levels are increased and independently of microalbuminuria. NGAL has recently emerged as a promising biomarker of acute and chronic kidney damage, able to detect tubular renal injury before creatinine levels are increased. As shown by our results, this may be of clinical relevance for children and adolescents with DM1, but particularly for children and adolescents with obesity. MMP-9 may also serve as an early marker of asymptomatic cardiovascular morbidity in the clinical context of both DM1 and obesity during childhood and adolescence. Early diagnosis may enable early intervention with potentially effective therapies and treatment approaches, which may prevent or minimize the rates of severe renal and cardiovascular morbidity in patients with T1DM or obesity.

Conflict of Interest

All authors declare no conflict of interest.

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Authors' Contributions

Study conception and design were performed by E. Kostopoulou, D. Goumenos, B.E. Spiliotis, and M. Papasotiriou. Material preparation and data collection were performed by D. Kalavrizioti and P. Davoulou. Statistical analyses were conducted by P. Plotas and E. Papachristou. The first draft of the manuscript was written by E. Kostopoulou, and M. Papasotiriou, while X. Sinopidis, G. Dimitriou, and B.E. Spiliotis critically reviewed the manuscript. All authors commented on the manuscript draft versions and approved the article version to be published.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

This study was performed in accordance with the principles of the Declaration of Helsinki. The Research Ethics Committee of the University Hospital of Patras granted approval (No. 353, dated 02-09-15).

Informed Consent
Informed consent was provided by the parents or legal representatives of all participants.

AI Disclosure
The authors did not use artificial intelligence or assisted technologies in the production of the study, including figures.

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