BMC Pediatrics

RESEARCH

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Association of the apoptotic markers Apo1/Fas and cCK-18 and the adhesion molecule ICAM-1 with Type 1 diabetes mellitus in children and adolescents

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Abstract

Background Type 1 diabetes mellitus (T1DM) is characterized by immune and metabolic dysregulation. Apo1/Fas is implicated in maintaining homeostasis of the immune system. Cytokeratin-18 (cCK-18) is a predictive marker of liver disorders in T2DM. Intercellular adhesion molecule-1 (ICAM-1) is considered to increase susceptibility to diabetes mellitus. All three markers are associated with endothelial function, apoptosis and diabetes-related complications. The possible role of Apo1/Fas, cCK-18 and ICAM-1 was investigated in children and adolescents with T1DM.

Method Forty-nine (49) children and adolescents with T1DM and 49 controls were included in the study. Somatometric measurements were obtained and the Body Mass Index (BMI) of the participants was calculated. Biochemical parameters were measured by standard laboratory methods and Apo1/Fas, cCK-18 and ICAM-1 were measured using appropriate ELISA kits. The statistical analysis was performed using the IBM SPSS Statistics 23 program.

Results Apo1/Fas ($p=0.001$), cCK-18 ($p<0.001$) and ICAM-1 ($p<0.001$) were higher in patients with T1DM compared to the controls. Apo1Fas was negatively correlated with glucose ($p=0.042$), uric acid ($p=0.026$), creatinine ($p=0.022$), total cholesterol ($p=0.023$) and LDL ($p=0.005$) in the controls. In children and adolescents with T1DM, Apo1/Fas was positively correlated with total cholesterol ($p=0.013$) and LDL ($p=0.003$). ICAM-1 was negatively correlated with creatinine ($p=0.019$) in the controls, whereas in patients with T1DM it was negatively correlated with HbA1c ($p=0.05$).

Conclusions Apo1/Fas, cCK-18 and ICAM-1 may be useful as serological markers for immune and metabolic dysregulation in children and adolescents with T1DM. Also, Apo1/Fas may have a protective role against metabolic complications in healthy children.

Keywords Apo1/Fas, cCK-18, ICAM-1, Type 1 diabetes mellitus, Children

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Background

Type 1 diabetes mellitus (T1DM) is characterized by severe loss of insulin-producing pancreatic β-cells through an autoimmune process that involves production of autoantibodies and T cell responses to autoantigens [1–4].

Programmed cell death, also known as apoptosis, is essential for normal tissue maintenance and repair. It is characterized by the expression of different proteins such as a glycosylated surface protein, Fas, alternatively known as CD95 or Apo1/Fas [5]. The Fas pathway is implicated in maintaining homeostasis of the immune system, cell-mediated cytotoxicity and suppression of the immune response [6]. FasL, a tumour necrosis factor-related type II transmembrane protein that binds to Fas on target cells, initiates an apoptosis signalling cascade [7]. This cascade results in proteolysis of cellular proteins and cleavage of internucleosomal DNA [8]. Fas is known to be expressed by β-cells in mice and humans. It has been shown that exposure to FasL or inflammatory cytokines increases Fas expression and beta-cell apoptosis [9]. Chronic hyperglycaemia in children leads to oxidative stress and inflammation [10], which can upregulate Fas expression on endothelial cells, increasing their susceptibility to Fas-mediated apoptosis [11].

Cytokeratin-18 is a cytoskeletal protein found in pseudostratified and simple epithelia [12]. Several studies have shown that CK-18 is one of the main keratin types in pancreatic islet cells [13]. During apoptosis of epithelial cells, cytokeratin is cleaved twice by activated caspases, generating caspase-cleaved cytokeratin-18 (cCK-18). A neo-epitope is exposed at the c-terminal end of cCK-18, which is recognized by M30, a specific monoclonal antibody [14]. Serum cCK-18 is used as a prediction marker of development of liver disorders in T2DM even at early stage [15, 16]. Elevated cCK-18 fragments may also reflect endothelial cell apoptosis which is involved in vascular complications [17].

Intercellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein expressed on the surface of endothelial cells, macrophages and activated leukocytes [18, 19]. It is encoded by the *ICAM-1* gene, which is considered a candidate gene for susceptibility to diabetes mellitus. In addition, ICAM-1 is involved in T-lymphocytes activation and leukocyte-endothelial cell interaction. Studies on isolated human and animal beta cells show that exposure to high glucose levels and inflammatory cytokines increases ICAM-1 expression and beta-cell apoptosis [20].

The aim of the present study was the investigation of the possible role of the apoptotic markers Apo1/Fas and cCK-18, as well as of the adhesion molecule ICAM-1, in T1DM in children and adolescents. Also, the investigation of the association between these markers and biochemical parameters.

Materials and methods

Forty-nine (49) children and adolescents with T1DM, 53.1% male, and 49 age-matched controls, 49.0% male, were studied. The participants were recruited from the Outpatient Clinics of Paediatric Endocrinology of the University General Hospital of Patras, Greece. T1DM was diagnosed based on the criteria of the American Diabetes Association (ADA) [21]. Among the patients with T1DM, 30% were on insulin pump therapy and the remaining 70% on Multiple Dose Injection (MDI) therapy. None of the patients had diabetic complications. The study was approved by the Research Ethics Committee of the University General Hospital of Patras, Greece (IRB number: 02.09.15/353) and was in accordance with the principles of the Helsinki Declaration in 1975, as revised in 1983. Written informed consent was obtained from the parents of the participating children and adolescents.

Somatometric measurements, such as weight and height, were obtained and the Body Mass Index (BMI) was calculated. Since it has been reported that overweight and obesity are confounding factors for the measured biomarkers [22], the participants were divided into two groups, one group of BMI ≤ 85%, which according to the CDC criteria represents normal-weight children and adolescents, and one group of BMI > 85%, which according to the CDC represents children and adolescents with overweight or obesity [23].

White cell count and biochemical parameters, including glucose concentrations, lipid profile {total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides}, renal function tests (urea, creatinine), liver function tests {serum glutamic-oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), gamma-glutamyl transferase (GGT)}, c-reactive protein (CRP), uric acid, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and glycated haemoglobin (HbA1c), were measured by standard laboratory methods in the laboratory of the University General Hospital of Patras.

Apo1/Fas, cCK-18 and ICAM-1 were measured using the Elisa technique in all the patients and the controls. For APO1/Fas, the enzyme-linked immunosorbent Elisa kit assay (ELISA kit Human Fas/TNFRSF6/CD95 NBP1-91,190 Novus Biologicals, Centennial, Colorado USA) was used. The MC30 ELISA was used to detect caspase-cleaved CK18 produced during the early stages of apoptosis, (VLVbio Hästholmsvägen, Sweden) and for ICAM-1, the Sandwich Elisa kit was used (ELISA kit

OriGene Technologies, Inc. EA100244, Rockville, Maryland USA).

For Apo1/Fas the intra-assay coefficient of variation (CVs) was 4.5% and the calculated inter-assay CV was 3.1%. For cCK-18 (M30) the intra-assay CV was < 10% and the calculated inter-assay CV was < 10%. For ICAM-1, the intra-assay CV was 5.1% and the calculated inter-assay CV was 5.8%.

Statistical analysis

All values were expressed as mean ± SD. Data was tested for normality using Shapiro–Wilk and Kolmogorov–Smirnov tests. Since data presented no normal distribution, Mann–Whitney's U-test Kruskal–Wallis tests were used to compare values of Apo1/Fas, cCK-18 and ICAM-1 levels between children and adolescents with T1DM and controls. Correlation between the apoptotic markers ICAM-1 levels, the anthropometric measurements and the biochemical parameters were analysed for participants with T1DM and controls using Pearson correlation coefficient. The statistical analysis was performed using IBM SPSS Statistics 23 (SPSS, Chicago, IL, USA), while the significance level was set at 5%.

Results

The age of the participants with T1DM varied between 5 and 19 years (mean 11.65 years old), and that of the controls between 7 and 17 years (mean 12.98 years old). The duration of the diabetes in the T1DM group was 8.41 ± 4.33 years.

The anthropometric and laboratory parameters of the participants are shown in Table 1.

Table 1 Demographic characteristics and laboratory parameters of children and adolescents with T1DM and controls. The values are presented in mean ± standard deviation (SD)

Parameters	T1DM	Controls	*p*-value
Age	12.97 (4.2)	11.65 (2.23)	0.06
BMI%	55.83 (26.72)	75.37 (26.11)	**0.001**
	≤ 85 = 43	≤ 85 = 24	
	> 85 = 4	> 85 = 24	
HbA1c %	8.84 (2.59)	5.9 (0.79)	**0.001**
HDL mg/dL	55.71 (11.78)	60.65 (15.03)	0.089
LDL mg/dL	88.80 (19.30)	104.22 (21.46)	**0.001**
CHOL mg/dL	161.48 (22.26)	175.12 (31.91)	**0.021**
TRG mg/dL	85 (45.51)	81.18 (57.61)	0.726
ALT U/L	16.31 (9.11)	16.38 (6.43)	0.969
AST U/L	22.09 (7.59)	26.69 (8.40)	**0.014**
GGT U/L	12.42 (3.64)	17.63 (3.37)	**0.001**
Urea mg/dl	30.01 (9.83)	28.50 (6.32)	0.788
Creatinine mg/dl	0.74 (0.15)	0.75 (0.10)	0.787
CPK U/L	119.71 (25.60)	129.68 (29.80)	0.650
Uric Acid mg/dl	3.64 (0.82)	3.93 (1.46)	0.403

Data were analysed using Student's t-test

BMI% Body mass index %, *T1DM* type 1 diabetes mellitus, *HbA1c* haemoglobin A1c, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *CHOL* cholesterol, *TRG* triglycerides, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transferase, *CPK* creatine phosphokinase

Apo1/Fas

Apo1/Fas concentrations were significantly higher in the children and adolescents with T1DM (Mann–Whitney U = 1553, $p = 0.001$) (Fig. 1a).

cCK-18

cCK-18 was significantly higher in the children and adolescents with T1DM (Mann–Whitney U = 1725.50, $p = 0.001$) (Fig. 1b).

ICAM-1

ICAM-1 was significantly higher in the children and adolescents with T1DM (Mann–Whitney U = 1314, $p = 0.001$) (Fig. 1c).

Apo1/Fas, cCK-18 and ICAM-1 according to sex and weight status

No statistically significant differences were observed in the three markers between males and females (Table 2), both in the group of children with T1DM and in the controls (Table 2).

Children and adolescents with T1DM and the controls were divided into two groups according to the BMI%: i) normal-weight children (BMI% ≤ 85), ii) overweight children and children with obesity (BMI > 85%). No statistically significant differences were observed in Apo1/Fas, cCK-18 and ICAM-1 between controls with BMI ≤ 85% and controls with BMI > 85% (Table 3).

The Mann–Whitney U-test was performed in the participants with BMI% ≤ 85 and statistically significant differences in the three markers (Apo1/Fas, cCK-18 and ICAM-1) were confirmed between the children with T1DM and the control group (Table 4).

Significantly higher concentrations were observed in Apo1/Fas, cCK-18 and ICAM-1 in normal-weight children with T1DM (BMI ≤ 85%) compared to controls with BMI > 85% (Table 5).

Pearson Correlations

The correlations between each of the three studied parameters (Apo1/Fas, cCK-18, ICAM-1) and the biochemical markers in children and adolescents with T1DM and controls, are shown in Table 6.

In the control group statistically significant positive correlations were observed between ICAM-a and SGPT

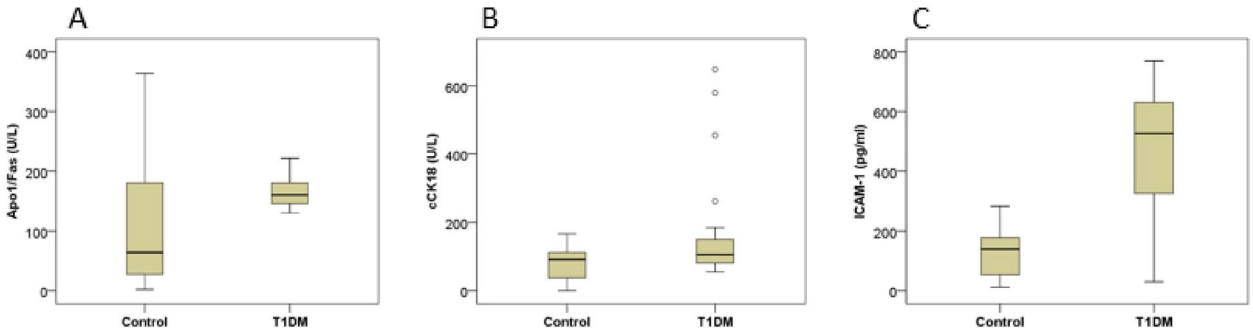

Fig. 1 Apo1/Fas, cCK-18 and ICAM-1 concentrations in children and adolescents with T1DM and the controls. Comparison between controls and children with T1DM in the relative protein expression of: (**a**) Apol/Fas: 112,33 U/L (± 105,69) vs 165,28 U/L (± 24,05) ($p = 0{,}001$), (**b**) Cleaved cytokeratin-18 (cCK18): 74,47 U/L (± 48,54) vs 140,30 U/L (± 117,94) ($p = 0{,}001$) and (**c**) ICAM-1: 127,88 pg/ml (± 80,31) vs 472,48 pg/ml (± 212,69) ($p = 0{,}001$)

Table 2 The three studied markers (Apo1/Fas, cCK-18 and ICAM-1) in children and adolescents with T1DM and the controls according to the sex

	Control				T1DM		
	Apo1/FasU/L	**cCK-18**	**ICAM-1**		**Apo1/Fas**	**cCK-18**	**ICAM-1**
Male *N* = 24 Female *N* = 25	U = 234.00 *p* = 0.666	U = 289.50 *p* = 0.833	U = 146.00 *p* = 0.857	Male *N* = 26 Female *N* = 23	U = 396.00 *p* = 0.052	U = 336.0 *p* = 0.459	U = 208.0 *p* = 0.791

Table 3 The studied markers (Apo1/Fas, cCK-18, ICAM-1) in control children and adolescents according to the BMI%

Control					
	BMI% ≤ 85 *N* = 24		**BMI% > 85 *N* = 24**		
	Mean	**SD**	**Mean**	**SD**	***p*-value**
Age	10,96	2,01	12,33	2,32	0.062
Apo1/FasU/L	108,72	97,40	118,13	117,29	0.888
ICAM1	131,65	89,43	122,22	67,13	0.907
cCK18	80,35	53,55	67,46	43,94	0.316

Table 4 The three studied markers (Apo1/Fas, cCK-18 and ICAM-1) in normal-weight (BMI ≤ 85%) children and adolescents with T1DM and controls

	Control (BMI% ≤ 85)		**T1DM (BMI% ≤ 85)**		
	Mean	**SD**	**Mean**	**SD**	***p*-value**
Age	10,96	2,01	12,38	3,79	0.064
Apo1/FasU/L	108,72	97,40	169,24	23,26	**0.018**
ICAM1	131,65	89,43	467,26	219,31	**< 0.001**
cCK18	80,35	53,55	149,11	132,57	**0.032**

Table 5 The three studied markers (Apo1/Fas, cCK-18 and ICAM-1) in normal-weight (BMI ≤ 85%) children and adolescents with T1DM and in controls with BMI > 85%

	Control (BMI% > 85)		**T1DM (BMI% ≤ 85)**		
	Mean	**SD**	**Mean**	**SD**	***p*-value**
Age	12,33	2,32	13,27	4,13	0.220
Apo1Fas	118,13	117,29	166,26	24,36	**0.004**
ICAM1 × 5	122,22	67,13	473,41	216,54	**< 0.001**
MC30	67,46	43,94	143,18	122,09	**< 0.001**

T1DM Type 1 diabetes mellitus, *BMI* Body mass index, *Apol/Fas, cCK-18* (caspase-cleaved cytokeratin-18), *ICAM-1* (Intercellular Adhesion Molecule 1); Analysis was performed using Mann–Whitney U-test

($R = 0.413$, $p = 0.014$), LDH ($R = 0.380$ $p = 0.029$) and between cCK18 and CPK ($R = 0.441$, $p = 0.009$). Also negative correlations were observed between Apo1/Fas and glucose ($R = -0.305$, $p = 0.042$), Uric acid ($R = -0.376$, $p = 0.026$), cholesterol ($R = -0.339$, $p = 0.023$), LDL ($R = -0.412$, $p = 0.005$), creatinine ($R = -0.387$, $p = 0.022$), as well as between ICAM-I and creatinine ($R = -0.393$, $p = 0.019$). Regarding the children with TD1M a statistically significant positive correlation was found between Apo1/Fas and Cholesterol ($R = 0.375$, $p = 0.013$), LDL

Table 6 Correlations between Apo1/Fas, cCK-18, ICAM-1 and biochemical markers

		Control			T1DM		
		Apo1Fas	**ICAM-1**	**cCK-18**	**Apo1Fas**	**ICAM-1**	**cCK-18**
Apo1/Fas U/L	R	1	0.153	0.253	1	-0.012	-0.055
	p-value	-	0.379	0.093	-	0.938	0.710
ICAM-1 pg/ml	R	0.153	1	0.215	-0.012	1	0.146
	p-value	0.379	-	0.215	0.938	-	.357
cCK-18 U/L	Pearson Correlation	0.253	0.215	1	-0.055	0.146	1
	p-value	0.093	0.215	-	0.710	0.357	-
BMI%	Pearson Correlation	-0.074	0.003	-0.179	-0.094	-0.065	0.125
	p-value	0.631	0.988	0.224	0.531	0.689	0.403
Urea mg/dL	Pearson Correlation	-0.133	-0.307	-0.136	-0.035	0.059	-0.014
	p-value)	0.446	0.073	0.429	0.814	0.709	0.922
Glucose mg/dL	Pearson Correlation	**-0.305**[a]	-0.022	0.144	0.250	-0.388	-0.287
	P-value	**0.042**	0.902	0.325	0.350	0.190	0.282
Uric Acid mg/dL	Pearson Correlation	**-0.376**[a]	-0.283	-0.090	0.054	0.024	0.071
	p-value	**0.026**	0.099	0.601	0.807	0.926	0.748
Cholesterol mg/dL	Pearson Correlation	**-0.339**[a]	-0.148	-0.181	**0.375**[a]	0.000	0.140
	p-value	**0.023**	0.395	0.212	**0.013**	1.000	0.370
HDL mg/dL	Pearson Correlation	0.105	0.328	-0.064	0.213	-0.226	0.057
	p-value	0.492	0.054	0.662	0.175	0.192	0.719
LDL mg/dL	Pearson Correlation	**-0.412**[b]	-0.265	-0.175	**0.469**[b]	0.096	0.133
	p-value)	**0.005**	0.125	0.230	**0.003**	0.606	0.426
Triglycerides mg/dL	Pearson Correlation	-0.193	-0.295	-0.012	-0.046	0.093	-0.101
	p-value	0.205	0.086	0.932	0.766	0.583	0.515
Creatinine mg/dL	Pearson Correlation	**-0.387**[a]	**-0.393**[a]	-0.046	-0.170	0.074	0.054
	p-value	**0.022**	**0.019**	0.791	0.252	0.647	0.719
SGOT U/L	Pearson Correlation	0.014	-0.079	0.138	0.045	0.076	0.282
	p-value	0.935	0.653	0.421	0.781	0.660	0.074
SGPT U/L	Pearson Correlation	0.149	**0.413**[a]	0.313	-0.041	-0.086	0.107
	p-value	0.393	**0.014**	0.063	0.797	0.618	0.505
GGT U/L	Pearson Correlation	-0.220	-0.091	0.021	0.126	-0.221	-0.217
	p-value	0.204	0.603	0.903	0.470	0.249	0.210
ALP U/L	Pearson Correlation	0.258	-0.067	-0.173	0.149	-0.245	-0.339
	p-value	0.134	0.704	0.312	0.661	0.468	0.308
LDH U/L	Pearson Correlation	0.270	**0.380**[a]	0.269	0.365	-0.061	0.241
	p-value	0.129	**0.029**	0.124	0.374	0.885	0.565
CPK U/L	Pearson Correlation	-0.035	0.043	**0.441**[b]	-0.485	0.182	-0.413
	p-value)	0.847	0.814	**0.009**	0.270	0.696	0.357

Table 6 (continued)

		Control			T1DM		
		Apo1Fas	**ICAM-1**	**cCK-18**	**Apo1Fas**	**ICAM-1**	**cCK-18**
HbA1c %	Pearson Correlation	-0.048	0.033	0.217	0.043	**-0.339**[a]	-0.169
	p-value	0.896	0.919	0.477	0.786	**0.046**	0.283

T1DM Type 1 diabetes mellitus, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *SGOT* serum glutamic-oxaloacetic transaminase, *SGPT* Serum Glutamic Pyruvic Transaminase. γ-glutamyl transferase, HbA1c haemoglobin A1c. All significant values are highlighted in bold

[a]. Correlation is significant at the 0.05 level (2-tailed)

[b]. Correlation is significant at the 0.01 level (2-tailed)

c. Cannot be computed because at least one of the variables is constant

Table 7 Differences in Apo1/Fas, cCK-18 and ICAM1 according with HbA1c levels

	HbA1C < = 6.5 (a)	**6.5 < HbA1C < = 8 (b)**	**HbA1C > 8 (c)**	**Adj. Sig**
	Mean (sd)	**Mean (sd)**	**Mean (sd)**	
Apo1Fas U/L	98.63 (68.84)	163.32 (24.15)	150.71 (47.06)	(a)-(b), $p=$**0.046** (a)-(c), $p=$**0.019** (b)-(c), $p=1.000$
cCK-18 U/L	85.39 (43.18)	186.16 (103.04)	136.43 (142.87)	(a)-(b), $p=$**0.001** (a)-(c), $p=0.429$ (b)-(c), $p=$**0.026**
ICAM-1 pg/ml	655.92 (117.64)	357.87 (194.67	467.13 (205.64)	(a)-(b), $p=$**0.005** (a)-(c), $p=0.081$ (b)-(c), $p=0.403$

HbA1c Glycosylated haemoglobin, *sd* standard deviation. Analysis was performed using Kruskal–Wallis analysis

($R=0.469$, $p=0.003$). Also negative correlations were observed between ICAM-1 and HbA1C ($R=$-0.339, $p=0.046$).

HbA1c levels and studied markers

A statistically significant increase in Apo1/Fas ($p=$0,046) and cCK-18 ($p=0.001$) concentrations and a decrease in ICAM-1 concentrations ($p=0.005$) were found in patients with HbA1c above 6,5% (Table 7).

Diabetes duration and studied markers

A statistically significant positive correlation was found between the duration of diabetes and cCK-18 ($R=0.393$, $p=0.001$) and a negative correlation between the duration of diabetes and ICAM-1 ($R=$-0.611, $p=0.001$).

Stratification of the patients according to diabetes duration showed a statistically significant increase in cCK-18 ($p=0.008$) in patients with diabetes duration more than 5 years compared to those with less than 5 years of diabetes duration (Table 8).

Logistic regression analysis

For control-T1DM distinction, logistic regression analysis was performed and showed that only GGT and ICAM-1 had a statistically significant effect ($\chi2(2)=55.011$, $p=0.000$), with a classification success rate of 90.6%, The relative probability of T1DM decreases by 32.3% for every one-unit increase in GGT and increases by 1.1% for every one-unit increase in ICAM.

Table 8 Apo1/Fas, ICAM-1 and cCK18 concentrations in patients with diabetes duration of less or more than 5 years

Duration	**Apo1/Fas U/L**	**ICAM-1 U/L**	**cCK18 U/L**
≤ 5 years $N=14$	167,17	443,68	100,28
> 5 years $N=35$	164,52	483,99	156,29
p-Value	0,62	0,83	**0.008**

Discussion

The present study demonstrates a positive relationship between the apoptotic markers Apo1/Fas and cCK-18, and T1DM in children and adolescents.

It is believed that activation of Fas expressed on β-cells results in their death and, subsequently leads to diabetes [24]. It is presumed that Fas/FasL interaction on infiltrating T cells results in Fas engagement on the surface

of β-cells leading to their apoptosis [25]. Nonetheless, deletion of the Fas gene in β-cells did not protect them from autoimmune destruction [26]. Clarifying whether Fas-mediated apoptosis mediates cytotoxicity of diabetogenic CD8 T cells still remains a challenge. Non-obese diabetic (NOD) mice bearing homozygous loss-of-function mutations in Fas and FasL were found to be protected from autoimmune diabetes, which has implicated the Fas pathway in the diabetogenic process. In NOD mice, β-cells have been found to upregulate Fas during the natural course of diabetes, which suggests that Fas-mediated apoptosis of the β-cells may represent one of the predominant mechanisms for insulin deficiency and development of diabetes [25]. In this context, the finding of the current study of a positive association of Apo1/Fas with T1DM further supports its involvement in the diabetogenic process. It should be noted though that this association can only be perceived as an indication of the involvement of Apo1/Fas in the diabetogenic process, since the pancreatic micro-environment and immune dysregulation is not straightforwardly represented by serum changes of antibodies against apoptotic markers. More research is needed at a basic and animal-model level in order to prove this hypothesis. Of interest, the positive correlation between Apo1/Fas and HbA1c found in the present study is an additional indication of the potential implication of Apo1/Fas in the development of long-term diabetes-related complications.

In addition, an alternative possible role of Apo1/Fas is implied by a recent survey conducted in a healthy paediatric population, according to which Apo1/Fas had a protective role against the predisposing factors for metabolic syndrome and atherosclerosis. Specifically, Apo1/Fas was negatively correlated with glucose, cholesterol, uric acid, LDL and triglycerides leading to the achievement of endothelial homeostasis [5]. Similarly, in the present study a negative correlation was found between Apo1/Fas and glucose, uric acid, cholesterol and LDL in the control group, which strengthens the hypothesis of a possible protective role of Apo1/Fas in healthy children. In contrast, a positive correlation was found between Apo1/Fas and total cholesterol and LDL in children and adolescents with T1DM, which may suggest disrupted function of Apo1/Fas in this population and loss of its protective role.

Cholesterol is an essential component of the plasma membranes of eukaryotic cells for the regulation of membrane permeability and receptor function [27]. Whereas membrane cholesterol depletion inhibits ligand-induced apoptosis and Apo1/Fas clustering [28], cholesterol excess changes the biophysical properties of the keratinocyte membrane, thus altering Apo1/Fas action, and induces conformational changes of the receptor [29].

Although cholesterol content in the cell membrane could not be measured, the association between serum lipid concentrations and Apo1/Fas was investigated in the current study, since serum lipids are related to membrane lipid composition in some cell types [30]. The finding of a positive correlation between Apo1/Fas and total cholesterol and LDL in children and adolescents with T1DM is in agreement with previous literature reports which support the association between cholesterol and apoptosis. It has been described that prolonged diet-induced hypercholesterolemia in the pig results in rapid cellular turnover [31]. It has also been reported that oxidized LDL induces apoptosis in cultured endothelial cells, smooth muscle cells, fibroblasts, macrophages and beta cells. This is particularly relevant considering the inflammatory environment present in T1D [32, 33]. In addition, a positive relationship between serum lipids and Apo1/Fas has been described in healthy adults [34, 35].

Furthermore, increased levels of cCK-18, a serological marker of epithelial apoptosis, and particularly hepatocyte apoptosis, have been found in adult patients with T2DM, T1DM and T1DM with insulitis, suggesting a more extensive epithelium and enterocyte damage in these patients [36]. Since several studies have demonstrated that CK-18 serum concentrations in children correlate with liver impairment caused by Non-Alcoholic Fatty Liver Disease (NAFLD) [37–39], the finding of the present study of increased cCK-18 levels in association with diabetes duration, may suggest the beginning and progression of liver damage in this population as a result of chronic low-grade inflammation and activated apoptosis [17]. To the best of our knowledge, only one previous study has reported the relationship between cCK-18 and T1DM in a paediatric population. According to this study, CK-18 levels were found lower in children and adolescents with T1DM [40]. In their attempt to explain why they failed to demonstrate an elevation of serum CK-18 concentrations in paediatric patients with T1DM as a sign of hepatic impairment or a beginning NAFLD caused by chronic systemic low-grade inflammation, the authors hypothesized that low CK-18 levels may be due to the short duration of T1DM in their population. The opposite finding of the current study may be explained by the longer duration of T1DM in our study population compared to the study by Nurten et al. [41] (8.41 ± 4.33 vs 4.7 ± 3.4 years) or by differences in glucose regulation and the metabolic profile of the patients included in the two studies. Interestingly, the results of the present study demonstrated that cCK-18 is positively correlated with HbA1c, thus it appears that it may increase with worsening of metabolic control. This may provide a possible mechanism through which cCK-18 is implicated in liver

impairment in children and adolescents with T1DM. Further research though, in this field is imperative.

Furthermore, another important finding of the present study is the increased ICAM-1 concentrations in children and adolescents with T1DM. Also, logistic regression analysis showed that the relative probability of T1DM increased by 1.1% for each one-unit rise in ICAM. This has also been reported by Rostampour et al., who concluded that ICAM-1 could be used for the identification of early atherosclerosis in children and adolescents with T1DM [41]. Similarly, Macrovecchio et al. found increased levels of ICAM-1 in youth with obesity and T1DM compared to controls, with similar levels between the two groups, which according to the authors confirm a similar increased cardiovascular risk associated with these two conditions. In the same study, ICAM-1 was also independently associated with eGFR and albumin excretion rate (AER), supporting an association between renal and endothelial dysfunction [18]. Of interest, the present study demonstrated a negative correlation between ICAM-1 and the diabetic state (HbA1c levels). Similarly, Noda et al. have shown that ICAM-1 decreased in advanced type 2 diabetes (HbA1c > 10%) in the Nile grass rat (NGR) when compared with that in normal or moderately diabetic animals, possibly because of endothelial dysfunction [42]. In addition, prevention of the onset of diabetes has been detected in NOD mice using monoclonal antibodies against ICAM-1 or after genetic deletion of the *ICAM-1* gene [43]. Several studies have shown a genetic association of the *ICAM-1* gene (K469E polymorphism) with T1DM and diabetic nephropathy in Romanian and Japanese populations [44, 45]. Other studies, however, have not found such a correlation in Danish, Finnish and British Caucasian populations [46, 47], which implies an important role of the genetic background in the pathogenesis of T1DM. It has also been reported that increased ICAM-1 levels in adult patients with T1DM are associated with a relative risk of developing microalbuminuria [48]. As a result, ICAM-1 has been proposed as a potential biomarker for the prediction of diabetes and diabetic nephropathy [19].

Furthermore, the association of *ICAM-1* and *HMGA1* (High Mobility Group AT Hook 1) gene variants with retinopathy in type 2 diabetes mellitus has been reported among Chinese individuals [49]. Single-nucleotide polymorphisms such as rs5498 E469K (A/G) and rs1799969 R241G (A/G) have been associated with diabetic retinopathy and diabetic nephropathy [19, 50]. The *ICAM-1* 469AG genotype has also been associated with adult-onset T1DM [45], and the *ICAM-1* rs5498 polymorphism with T1DM [51]. The exact role of ICAM-1 in the development of diabetes and DN remains vague. A suggested mechanism involves binding of ICAM-1 to leukocyte adhesion protein-1 (LFA-1), hence more lymphocytes from blood are transferred into cells in glomeruli and peritubular capillaries of the nephron in the kidney. This may result in injury of kidney glomeruli and tubules, leading to protein excretion in urine [19].

ICAM-1 has also been associated with increased all-cause mortality and cardiovascular morbidity in adult patients with T1DM and diabetic nephropathy [52]. The finding of the current study of increased levels of ICAM-1 in a paediatric population with T1DM may be the first step for identifying novel prediction biomarkers for early detection of diabetes and diabetes-related complications in children. If future research findings confirm such an association, identifying children and adolescents at risk will be of major importance as it may offer the opportunity to reverse or even prevent these complications. ICAM-1 may also serve as a potential therapeutic target, as targeting ICAM-1 pathways may attenuate inflammation, endothelial dysfunction and diabetes-associated complications [53].

Interestingly, all the three markers studied differed significantly between the children and adolescents with T1DM and controls, regardless of the sex or the weight status of the participants. Also, all the markers were significantly higher in children adolescents with poor glycaemic control compared to those with optimal glycaemic control.

Some limitations of the present study need to be acknowledged. The sample size was relatively small. The results obtained from power analysis with 49 participants per group ($n1 = n2 = 49$) are: $a = 0.05$, $(1\text{-}\beta) = 0.8$, Effect size = 0.52. Also, although there was no statistically significant difference in the age between the T1DM and the control groups, the age of the children was not completely matched. Finally, considerably more children in the control group had overweight or obesity than the T1DM group, which might have influenced the results shown in Tables 3 and 5.

Conclusions

The present study investigated the possible association between three non-invasive serological markers and T1DM in children and adolescents. All three markers were increased in children and adolescents with T1DM, particularly in those with poor glycaemic control. According to existing data, these markers may be involved in the pathogenesis of T1DM and T1DM-associated complications. However, their usefulness as predictive markers of long-term complications deserves further investigation by large-scale studies.

Abbreviations

T1DM Type 1 diabetes mellitus
T2DM Type 2 diabetes mellitus

cCK-18	Cytokeratin-18
ICAM-1	Intercellular adhesion molecule-1
BMI	Body mass index
Treg	Regulatory T-cells
ADA	American Diabetes Association
NAFLD	Non-Alcoholic Fatty Liver Disease
AER	Albumin excretion rate
NGR	Nile grass rat
HMGA1	High Mobility Group AT Hook 1
LFA-1	Leukocyte adhesion protein-1
LDL	Low-density lipoprotein
HDL	High-density lipoprotein liver function tests {serum glutamic-oxaloacetic SGOTtransaminase
SGPT	Serum glutamate pyruvate transaminase
GGT	Gamma-glutamyl transferase
CRP	C-reactive protein
ALP	Alkaline phosphatase
LDH	Lactate dehydrogenase
CPK	Creatine phosphokinase
HbA1c	Glycated haemoglobin

Acknowledgements
The publication fees of this manuscript have been financed by the Research Council of the University of Patras.

Authors' contributions
E.K. and A.P.R.G. made substantial contributions to the conception and design of the study. E.K., M.E.K., A.I. and I.D. participated in the acquisition and analysis of the study. E.K., M.F., I.D., B.E.S. and A.P.R.G. were involved in the interpretation of data. E.K., M.E.K. and A.P.R.G. have drafted the work. A.I., M.F., I.D. and B.E.S. have substantively revised the draft. All authors read and approved the final manuscript and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature. All authors reviewed the manuscript.

Funding
No funding was received.

Availability of data and materials
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
The study was in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments and it was approved by the Research Ethics Committee of the University Hospital of Patras. To partake in the study, the parents of the participating children and adolescents gave their informed consent in a written format and children and adolescents their informed assent, while participation was voluntary, and anonymity was assured.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 27 November 2023 Accepted: 2 July 2024
Published online: 02 August 2024

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