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Research article

Sex-specific alterations in creatine metabolism in cellular compartments of peripheral blood leukocytes in type 1 diabetes

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Abstract: The creatine (Cr)/creatine kinase (CK) system plays a crucial role in cellular energy metabolism, glucose control, and the immune response. This study aimed to investigate this system in the peripheral blood leukocytes (PBL) of Armenians with type 1 diabetes (T1D) who received insulin therapy. A total of 270 Armenian participants were enrolled in the study and were divided into two age groups: Group I - included children and preadolescents; and Group II - included adolescents and young adults. Within each group, the participants were further categorized based on sex and disease duration: Recent-onset T1D (RO-T1D), and Long-term T1D (LT-T1D). A group of healthy individuals that were matched for age and sex served as controls. The glycemic control (GC) was assessed using the glycated hemoglobin (HbA1c) test. In girls of Group I with LT-T1D, both the Cr levels and CK activity were significantly reduced in both the cytoplasm and mitochondria of the PBL, despite having a good GC. In contrast, age-matched boys showed a relatively stable Cr metabolism. Beginning at puberty, only girls with LT-T1D and a poor GC showed decreased Cr levels and a reduced CK activity, which was most pronounced in the mitochondria. In boys of Group II, the cytoplasmic Cr levels decreased by half, regardless of the GC or disease duration, while the mitochondrial Cr levels decreased by 55% only in boys with LT-T1D and a poor GC. Their CK activity decreased by 80% in both cellular compartments, regardless of the GC and duration of diabetes. Notably, changes in the plasma were less specific. Our findings indicate age- and sex-dependent changes in Cr metabolism in the cytoplasm and mitochondria of PBL in Armenians with T1D, which are influenced by the glycemic status and the disease duration. Further extensive studies in this area may provide insights into potential strategies to control

metabolism in T1D to counteract autoimmunity and immune dysregulation, and may also serve as a basis for the development of targeted therapeutic interventions.

Keywords: creatine; creatine kinase; cytoplasm; leukocyte; mitochondria; type 1 diabetes

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-producing pancreatic β-cells, disrupted immune function, and systemic metabolic abnormalities [1]. The incidence and prevalence of T1D are increasing worldwide, including within Armenia [2,3]. Metabolic disturbances, especially in leukocytes, which are directly involved in autoimmune processes and the destruction of β-cells, can play a decisive role in the pathogenesis of T1D [4,5].

In T1D, there is a significant shift in energy metabolism due to insulin deficiency. The creatine (Cr)/creatine kinase (CK) system is known to play a significant role in the cellular energy metabolism, and it helps transfer high-energy phosphates from mitochondria, where ATP is generated, to the cytoplasm, where ATP is used [6]. Moreover, this system plays an important role in the immune response and glucose management, and its involvement in the pathophysiology of T1D has become a subject of increasing interest [7,8]. Our work presents data on sex- and age-related changes in the Cr/CK system observed in the cytoplasm and mitochondria of leukocytes and blood plasma of Armenians with T1D, depending on the glycemic status and the duration of diabetes.

2. Materials and methods

2.1. Participants

The study was approved by the Ethics Committee of Yerevan State Medical University named after Mkhitar Heratsi (IRB Expert Conclusion № 1-10/2020). This study involves 270 Armenians aged 4 to 22 years, patients with T1D from the endocrinology department of Muratsan University Hospital (Yerevan, RA), and healthy individuals matched by sex and age. Informed consent was obtained from all participants (parental consent was obtained for all minors, in accordance with Good Clinical Practice standards and the WMA Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects) [9].

2.2. Study criteria and procedures

For the T1D diagnosis, at least two types of antibodies — those against insulin and glutamic acid decarboxylase — tested positive (Radioligand assays for the determination of autoantibodies to Glutamate acid decarboxylase (GAD65Ab) and Tyrosine phosphatase IA2 in human serum; DIA source Anti-CAD65 RIA KIT (DIA source / ImmunoAssays S.A. Belgium) Catalog Number KIMP 2070; DIA source Anti-IA2RIA KIT (DIA source / ImmunoAssays S.A. Belgium) Catalog Number KIMP 2050; RK-400CTACE210301 Insulin KIT [I-125] IRMA (Catalog Number: RK-400CT) LLC “Institute of Isotopes” H-1121 Budapest, Hungary).

The exclusion criteria included macro- and micro-vascular diseases, acute or chronic liver, kidney, or cardiac diseases, malignancy, arterial hypertension, and pregnancy. All procedures were performed in the Endocrinology Department of Muratsan University Hospital (Yerevan, RA). All patients

received multiple daily insulin injections and were monitored according to a standard medical protocol [10].

All participants were examined early in the morning, on an empty stomach, while avoiding caffeinated beverages, cigarettes, and intense physical activity the previous evening.

2.3. Measurement of glycated hemoglobin

The glycated hemoglobin (HbA1c) was assessed using the turbidimetric inhibition immunoassay for HbA1c (Accu-Chek A1c Hemoglobin test for use with BMC Hitachi reagents, that measure glycated hemoglobin, Tina-quant Hb A1c Assay, k#934070 (Boehringer Mannheim Corp., Indianapolis, IN)) on a Roche Hitachi 912 analyzer at the clinical laboratory of Muratsan University Hospital [11].

To isolate the leukocytes and blood plasma, freshly obtained peripheral venous blood was placed in 3.9% sodium citrate ($C_6H_9Na \cdot 5H_2O$) as an anticoagulant, mixed with 6% dextran (Mr 100,000) in a 0.9% NaCl solution, and incubated at 37 °C for 60 minutes to remove the red blood cells by gravity sedimentation. Plasma containing leukocytes and platelets was decanted and centrifuged at 56 g for 5 minutes to pellet the leukocytes, which were washed twice before use. Then the plasma was further separated from the platelets by centrifuging the resulting supernatant at 2,012 g for 20 minutes at 4 °C.

2.4. Isolation of cytoplasmic and mitochondrial fractions from leukocytes

The leukocytes were suspended in a 20 mM HEPES buffer with 0.25 M sucrose (pH 7.4), homogenized, and centrifuged at 81 g for 10 minutes at 4 °C. The pellet containing nuclei and cellular debris was discarded, and the supernatant was centrifuged at 6,764 g for 20 minutes at 4 °C to isolate the cytoplasm in the supernatant and mitochondria in the pellet, which was washed twice, resuspended in the buffer used and homogenized before use.

2.5. Creatine kinase (CK) Assay

The CK activity was assessed by the content of Cr formed from phosphocreatine [12]. The obtained samples of cytoplasmic and mitochondrial fractions of PBL and blood plasma were incubated for 1 hour at 37 °C in a reaction mixture containing 20 mM HEPES buffer (pH 7.4), 5.5 mM phosphocreatine, and 0.06% ADP. The reaction was initiated by adding samples to the reaction mixture and terminated by the subsequent addition of 0.5 N NaOH and 10% $ZnSO_4 \cdot 7H_2O$. Following a centrifugation (12,578 g, 3 minutes), the protein-free supernatants were sampled and analyzed for the Cr content.

2.6. Creatine measurement

Protein-free samples were incubated for 20 minutes with 1% diacetyl and 1% α-naphthol (prepared with 16% Na_2CO_3 and 6% NaOH), and the Cr was measured at an absorbance of 536 nm against a blank control containing all reagents minus the sample [12].

2.7. Protein assay

The Folin-Ciocalteu phenol reagent was used for the protein analysis, and the protein was measured at an absorbance of 656 nm [13]. Bovine serum albumin was used as a protein standard.

2.8. Statistical analysis

Data are presented as mean (M) ± standard error of the mean (SEM). The Shapiro-Wilk normality test for each group was computed using Prism-GraphPad. All data were analyzed using a one-way analysis of variance (ANOVA) followed by the Holm-Sidak post hoc test (SigmaStat 3.5 for Windows). Significance is considered at $P < 0.05$.

3. Results

3.1. Population study

A total of 270 Armenian participants were enrolled in the study and divided into two age groups: Group I. included children and preadolescents; and Group II. included adolescents and young adults. Within each group, the participants were further categorized based on sex and disease duration: Recent-onset T1D (RO-T1D and Long-term T1D (LT-T1D). A group of healthy individuals that were matched for age and sex served as controls. The clinical data and information on the patients with T1D and healthy volunteers are summarized in Tables 1 and 2.

Table 1. Clinical data from children and preadolescents with T1D and healthy controls.

Variable	RO-T1D	LT-T1D	Healthy controls
Number of individuals	47	43	46
Boys/Girls	24/23	23/20	24/22
Age (years), median (range)	9.4 [5.3–11.5]	9.3 [4.5–11.8]	9.5 [4.0–11.5]
Age of debut (years), median (range)	8.0 [4.5–11.2]	6.1 [1.7–10.2]	-
Diabetes duration (years), median (range)	0.5 [0.01–1.0]	5.3 [1.6–9.1]	-
HbA1c (%), median (range)	8.0 [5.6–13.4]	7.3 [6.6–12.7]	4.7 [4.0–5.3]
BMI (kg/m^2), median (range)	17.4 [14.9–22.1]	17.6 [15.1–21.0]	17.5 [15.1–23.0]
Cholesterol (mg/dL), median (range)	169 [141–192]	175 [145–197]	155 [137–175]
Triglycerides (mg/dL), median (range)	89 [71–105]	97 [72–117]	79 [67–99]
Creatinine clearance (mL/min), median (range)	115 [95–128]	113 [89–133]	117 [99–133]

*Note: T1D – type 1 diabetes; RO-T1D – recent onset T1D; LT-T1D – long-term T1D.

Table 2. Clinical data from adolescents and young adults with T1D and healthy controls.

Variable	RO-T1D	LT-T1D	Healthy controls
Number of individuals	44	43	47
Boys/Girls	23/21	23/20	22/25
Age (years), median (range)	15.0 [12.0–22.1]	14.5 [12.2–20.7]	15.5 [12.2–21.5]
Age of debut (years), median (range)	13.9 [10.2–21.2]	10.4 [1.4–20.9]	-
Diabetes duration (years), median (range)	0.6 [0.1–1.0]	5.9 [1.8–9.9]	-
HbA1c (%), median (range)	8.9 [5,7–16.0]	8.5 [5.9–12.4]	4.8 [4.1–5.4]
BMI (kg/m^2), median (range)	19.0 [14.7–23.7]	19.7 [16.6–23.8]	20.6 [17.9–23.7]
Cholesterol (mg/dL), median (range)	168 [141–193]	177 [143–199]	159 [133–179]
Triglycerides (mg/dL), median (range)	85 [65–107]	92 [69–115]	81 [63–107]
Creatinine clearance (mL/min), median (range)	113 [94–127]	111 [91–132]	116 [98–129]

*Note: T1D – type 1 diabetes; RO-T1D – recent onset T1D; LT-T1D – long-term T1D.

Data in both tables referred to: http://op.niscpr.res.in/index.php/IJBB/article/view/36729

Melkonyan, A.M., Guevorkyan, A.G., Alchujyan, N.Kh., Hovhannisyan, M.R., Movsesyan, N.H., Hayrapetyan, H.L., Kevorkian, G.A. and Aghajanova, Y.M., Sex and age-related changes in L-arginine metabolism in peripheral blood leukocytes in young caucasians with type 1 diabetes mellitus. Indian Journal of Biochemistry and Biophysics (IJBB), 2020; 57(3), 339-350. doi: 10.56042/ijbb.v57i3.36729

3.2. Creatine metabolism in PBL cellular compartments of children and preadolescents with T1D

Achieving HbA1c targets of < 7% has been shown to reduce microvascular complications; however, the American Diabetes Association raised the lower glycemic target range consistent with HbA1c goals for the prevention of hypoglycemia, to which young children with T1DM are especially vulnerable [14]. Therefore, when measuring the glycemic control (GC) using the glycated hemoglobin (HbA1c) test, a HbA1c concentration below 7.5% was considered a good GC and a concentration above 7.5% was considered a bad GC.

Figure 1 shows that in girls of Group I, regardless of the duration of diabetes ($P > 0.05$), the Cr level in the cytoplasm of the PBL decreased with a good GC by 55% to 4.39 nmol/mg protein (95% CI: 3.66 – 5.11; $P < 0.001$) and to 2.93 nmol/mg protein with a poor GC ($P = 0.015$). Additionally, it decreased in the mitochondria by 45% to 5.64 nmol/mg protein (95% CI: 4.68 – 6.6; $P < 0.001$) with a good GC and to 3.5 nmol/mg protein ($P < 0.001$) with a poor GC compared to the healthy controls. In boys of Group I, the level of Cr in the cellular compartments of the PBL was generally within the normal range, with the exception for individuals with RO-T1D, in whom it decreased in the mitochondria by 44% to 4.1 nmol/mg protein (95% CI: 3.2–5.0; $P < 0.001$) regardless of the GC ($P > 0.05$) compared to the healthy controls.

Changes in the CK activity were also observed in the PBL cellular compartments (Figure 2). In girls of Group I with RO-T1D, regardless of the GC ($P > 0.05$), the activity of cytoplasmic CK (cCK) decreased by 77% to 4.09 nmol Cr/h/mg protein (95% CI: 3.0–5.19; $P < 0.001$) and mitochondrial CK (mCK) by 75% to 4.95 nmol Cr/h/mg protein (95% CI: 3.64–6.27; $P < 0.001$) compared to the healthy girls. Girls with LT-T1D and a poor GC showed a similar decrease in both cellular compartments, whereas girls with a good GC had a smaller decrease in the CK activity in both cellular compartments, with a mean of 7.8 nmol Cr/h/mg protein (95% CI: 6.84–8.76; $P < 0.001$ for cytoplasm; $P = 0.013$ for mitochondria).

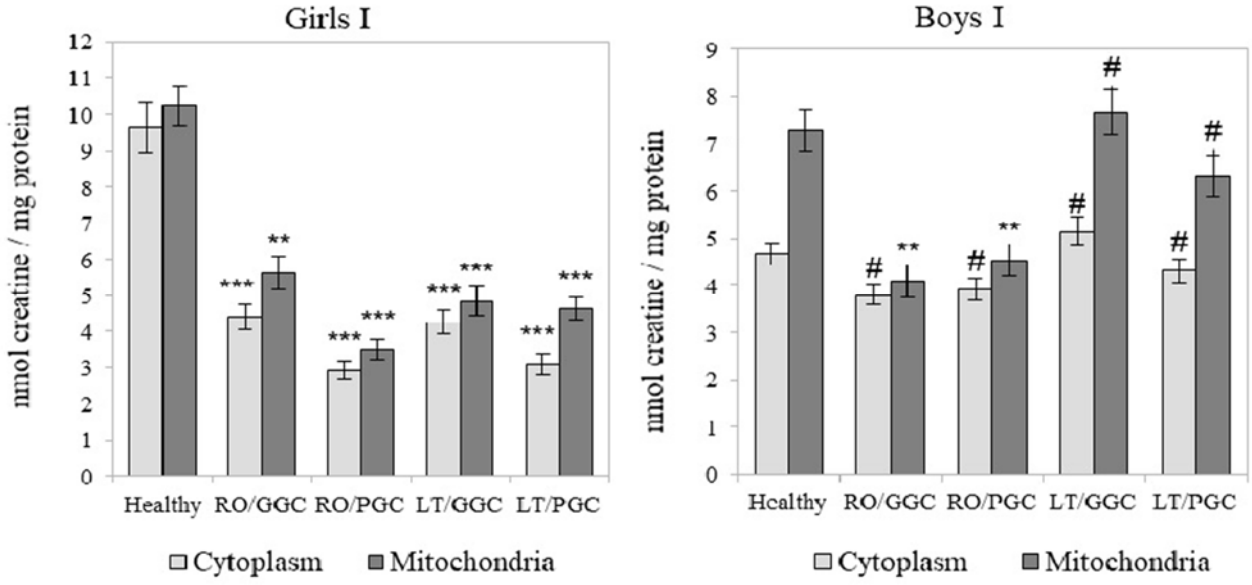

Figure 1. Sex-related changes in subcellular creatine levels in leukocytes depending on glycemic status and duration of T1D in children and preadolescents. RO – recent-onset T1D; LT – long-term T1D; GGC – good glycemic control; PGC – poor glycemic control. Data are M ± SEM. # P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. healthy controls.

In boys of Group I with RO-T1D, the CK activity decreased in the cytoplasm and mitochondria by approximately 24% to an average 8.66 nmol Cr/h/mg protein (95% CI: 7. 91–9.41; P = 0.004 for cytoplasm; P = 0.013 for mitochondria), regardless of the GC (P > 0.05).

In boys with LT-T1DM, the cCK activity decreased by 22% with a good GC (P = 0.008) and by 59% to 4.58 nmol Cr/h/mg protein (95% CI: 4.05–5.11; P < 0.001) with a poor GC. The activity of mCK decreased by 19% with a good GC (P = 0.048) and by 36% to 7.23 nmol Cr/h/mg protein (95% CI: 6.8–7.66; P < 0.001) with a poor GC compared to the healthy controls.

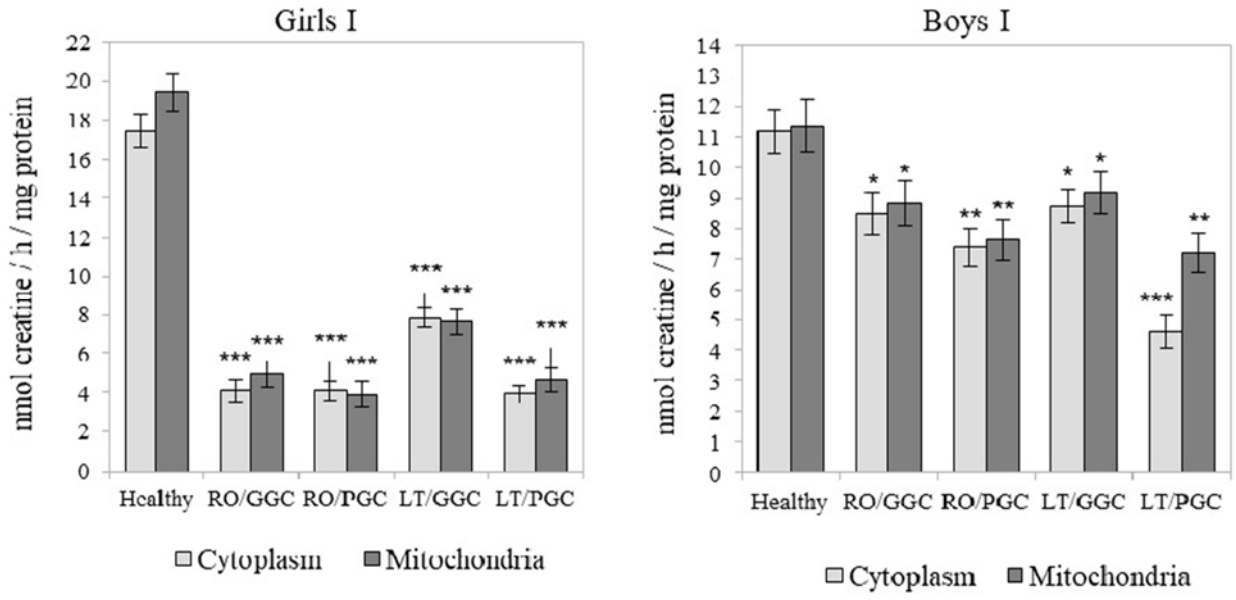

Figure 2. Sex-related changes in subcellular activity of leukocyte creatine kinase depending on glycemic status and duration of T1D in children and preadolescents. RO – recent-onset T1D; LT – long-term T1D; GGC – good glycemic control; PGC – poor glycemic control. Data are M ± SEM. # P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. healthy controls.

3.3. Creatine metabolism in PBL cellular compartments of adolescents and young adults with T1D

In girls of Group II, the Cr level in both cellular compartments of the PBL was dependent on the glycemic status (P < 0.001), and decreased exclusively in girls with LT-T1D and a poor GC, namely by 31% to 7.31 nmol/mg protein in the cytoplasm (95% CI: 6.39–8.23; P = 0.026) and by 66% to 4.46 nmol/mg protein in mitochondria (95% CI: 3.89–5.03; P < 0.001) compared to the healthy controls (Figure 3).

In boys of Group II, the Cr level decreased in the cytoplasm by 60% to 7.34 nmol/mg protein (95% CI: 6.28–8.39; P < 0.001), regardless of the T1D duration and the glycemic status (P = 0.22 for RO-T1D, and P = 0.5 for LT-T1D), whereas it decreased by 67% to 4.34 nmol/mg in mitochondria (P < 0.001) only in individuals with LT-T1D and a poor GC compared to the healthy controls.

Figure 4 shows the changes in the CK activity in the PBL cellular compartments. In girls of Group II with RO-T1DM, the activity of cCK decreased by 38% to 6.99 nmol Cr/h/mg protein with a good GC (95% CI: 5.76–8.23; P < 0.001) and by 71% to 3.34 nmol Cr/h/mg protein with a poor GC (P < 0.001). In girls with LT-T1D and a good GC, the cCK activity was within normal limits, while it decreased by 22% with a poor GC (P = 0.017) compared to the healthy controls. In girls with RO-T1D, the activity of mCK decreased regardless of the GC (P = 0.59) by 43% to 10.56 nmol Cr/h/mg protein (95% CI: 8.87–12.24; P < 0.001). In girls with LT-T1D and a good GC, it also decreased up to 11.13 nmol Cr/h/mg protein, while it decreased by 67% with a poor GC (P < 0.001).

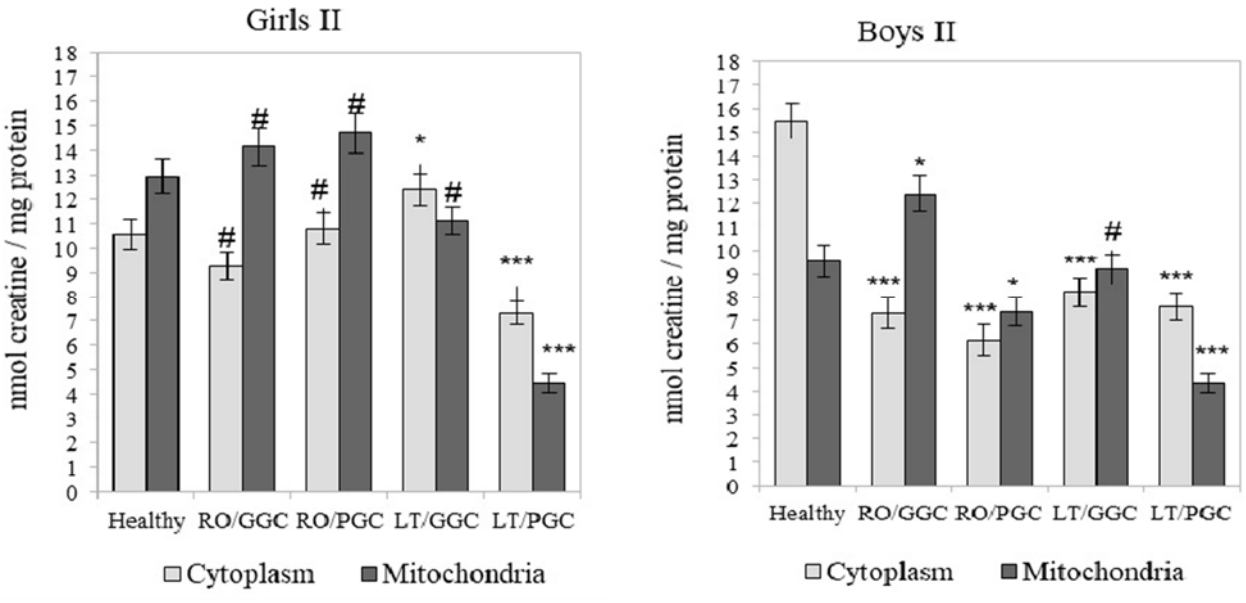

Figure 3. Sex-related changes in subcellular creatine levels in leukocytes depending on glycemic status and duration of T1D in adolescents and young adults. RO – recent-onset T1D; LT – long-term T1D; GGC – good glycemic control; PGC – poor glycemic control. Data are M ± SEM. # P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. healthy controls.

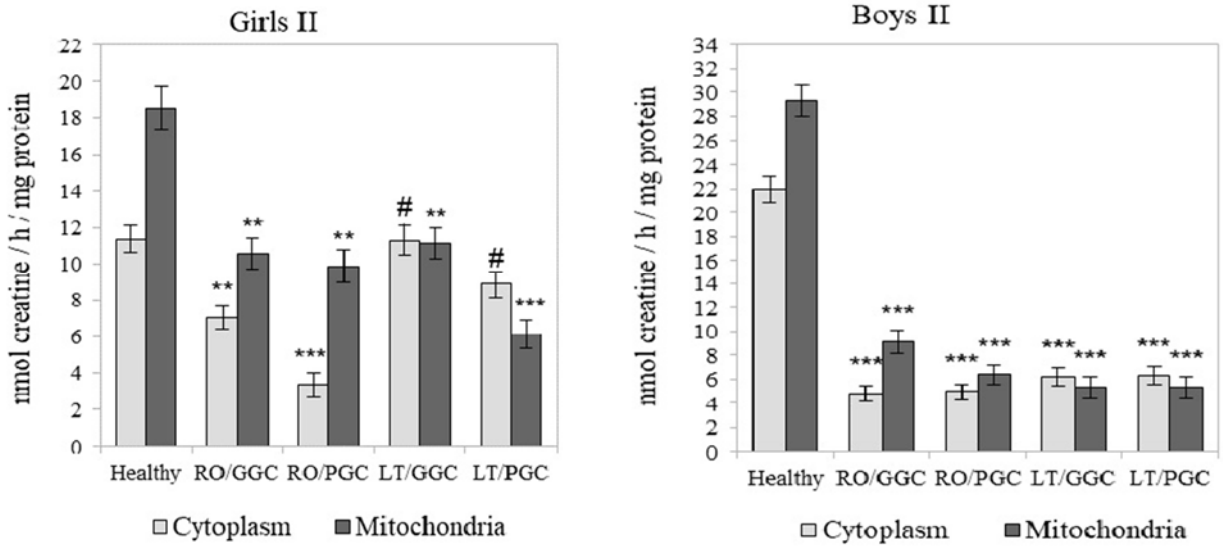

Figure 4. Sex-related changes in subcellular activity of leukocyte creatine kinase depending on glycemic status and duration of T1D in adolescents and young adults. RO – recent-onset T1D; LT – long-term T1D; GGC – good glycemic control; PGC – poor glycemic control. Data are M ± SEM. # P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. healthy controls.

In boys of Group II with RO-T1D, the activity of cCK decreased, regardless of the GC (P = 0.9), by 78% to 4.84 nmol Cr/h/mg protein (95% CI: 3.63–6.06; P < 0.001), and by 71% (P < 0.001) in individuals with LT-T1D. In boys with RO-T1D and a good GC, the activity of mCK decreased by 69%

to 9.09 nmol Cr/h/mg protein (95% CI: 8.11–10.07; P < 0.001) and by 78% with a poor GC (P < 0.001); alternatively, in boys with LT-T1D, it decreased regardless of the GC (P = 0.98) by approximately 82% (P < 0.001) compared to the healthy control group.

3.4. Creatine metabolism in blood plasma of Patients with T1DM

In patients of both sexes in Group I, the level of Cr in the plasma was within the normal limits, regardless of the duration of diabetes and the GC (P = 0.049 for RO-T1D, P = 0.39 for LT-T1D) (Figure 5). However, changes in the plasma CK activity were observed. In girls of Group I with RO-T1D, the CK activity decreased independently of the GC (*P* = 0.9) by 58% to 0.57 nmol Cr/h/mg protein (95% CI: 0.55–0.6; P < 0.001). In girls with LT-T1D and a good GC, it similarly decreased to 0.51 nmol Cr/h/mg protein, and by 42% to 0.79 nmol Cr/h/mg protein with a poor GC (P < 0.001) compared with healthy controls. In boys of Group I with RO-T1D, the plasma CK activity decreased by an average of 54% to 0.42 nmol Cr/h/mg protein regardless of the GC (P = 0.12). In boys with LT-T1D and a good GC, it decreased by 83% (P < 0.001) and by 38% with a poor GC (P < 0.001) compared to the healthy controls.

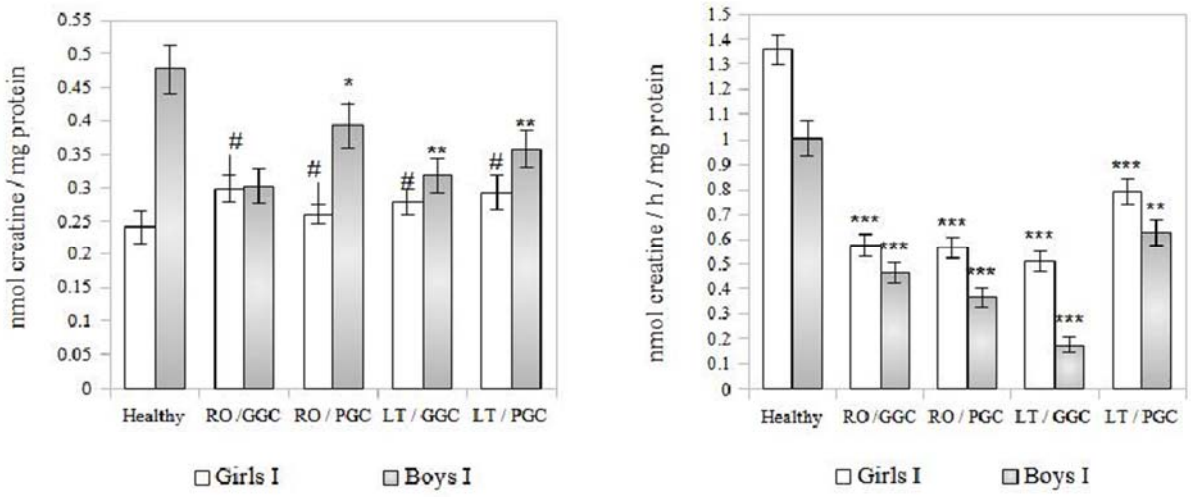

Figure 5. Sex-related changes in blood plasma creatine levels and creatine kinase activity depending on glycemic status and duration of T1D in children and preadolescents. RO – recent-onset T1D; LT – long-term T1D; GGC – good glycemic control; PGC – poor glycemic control. Data are M ± SEM. # P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. healthy controls.

Figure 6 shows changes in the Cr metabolism in the blood plasma depending on sex in patients of Group II. In girls, the plasma Cr level decreased by an average of 41% to 0.57 nmol/mg protein (95% CI: 0.51–0.63; P < 0.01) regardless of the GC (P > 0.05) and the diabetes duration (P > 0.05) compared to the healthy controls.

In boys of Group II with RO-T1D and a good GC, the plasma Cr level decreased by 52% to 0.51 nmol/mg protein (95% CI: 0.47–0.56; P < 0.001) and by 68% with a poor GC (P = 0.003). In boys with LT-T1D, a similar decrease was observed, though independent of the glycemic status (P > 0.05), compared to the healthy controls.

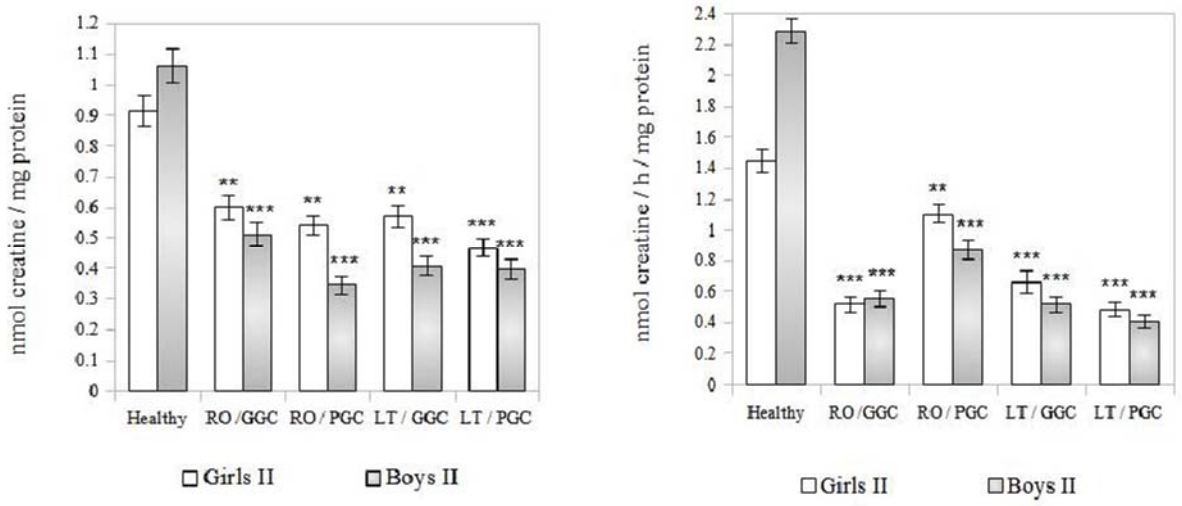

Figure 6. Sex-related changes in blood plasma creatine levels and creatine kinase activity depending on glycemic status and duration of T1D in adolescents and young adults. RO – recent-onset T1D; LT – long-term T1D; GGC – good glycemic control; PGC – poor glycemic control. Data are M ± SEM. # $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. healthy controls.

In boys of Group II with RO-T1D and a poor GC ($P < 0.001$), the CK activity decreased by 69% to 0.71 nmol Cr/h/mg protein (95% CI: 0.67–0.76; $P < 0.001$) and by 76% to 0.55 nmol Cr/h/mg protein with a good GC (95% CI: 0.49–0.61; $P < 0.001$); moreover with LT-T1D, there was an average decrease to 0.46 nmol Cr/h/mg protein regardless of the glycemic status ($P = 0.14$).

4. Discussion

The Cr/CK system plays a key role in the temporal and spatial buffering of energy in cells with high and fluctuating energy demands, which is reliant on cytosolic and mitochondrial CK isoforms. The molecular structure and functions of these isoforms have been comprehensively reviewed in previous studies [6,15]. Specifically, mitochondrial CK is linked to ATP export via the adenine nucleotide transporter, thus enabling phosphocreatine release into the cytosol. Cytosolic CK regulates the phosphocreatine/Cr, and ATP/ADP balance within the cell, with a subset closely associated with the glycogenolytic/glycolytic pathway. Additionally, the various roles of Cr metabolism in cancer cell survival and immune system function have been discussed, as well as the therapeutic potential of studying its alterations that contribute to metabolic diseases, including diabetes [16]. Additionally, the Cr/CK system is involved in the regulation of glycogenolysis, glycolysis, insulin resistance, and mitochondrial activity, which is independent of energy metabolism [17]. Sex and age differences underlie metabolic differences between human populations and influence the immune system and the incidence and outcome of autoimmune diseases, especially in young adults [18,19]. Our study focused on the changes in Cr metabolism in the cellular compartments of circulating leukocytes in Armenians with T1D.

In children and preadolescent girls in Group I, the Cr level in PBL significantly decreased in both the cytoplasm and mitochondria, almost independently of the glycemic status and the duration of T1D. Notably, Cr is known to promote glucose uptake by enhancing the abundance and translocation of

insulin-stimulated glucose transporter type 4, thereby potentially lowering the Hb1Ac levels and improving the glycemic status in T2D patients [20]. In girls with RO-T1D, the CK activity in both cellular compartments decreased by more than two-thirds, regardless of the GC. In contrast, in girls with LT-T1D and a good GC, the CK activity decreased to a lesser extent (by approximately 55%) without significant changes in the Cr levels, thus suggesting the involvement of Cr-independent mechanisms that affect the CK activity (see below).

In contrast to girls, in boys of age Group I, the Cr levels in the PBL cellular compartments was within normal limits, except in the mitochondria of RO-T1D patients, where it decreased; alternatively, the Cr levels were within normal limits in boys with LT-T1D, possibly due to insulin therapy. Additionally, the CK activity in boys remained within normal limits, though it decreased in the cytoplasm of boys with LT-T1D under poor GC conditions, probably due to Cr-independent mechanisms that affected the GC and CK activity.

In contrast to the PBL, the plasma Cr level in Group I patients did not show significant changes, thus suggesting that the decrease in Cr in the PBL may be due to impaired extracellular Cr transport via its transporter SLC6A8, which is expressed in leukocytes [21]. This transporter might be suppressed in Group I girls, who showed a notably greater decrease in the Cr levels compared to the boys. It's worth noting that SLC6A8 is inhibited by prepubertal estrogen in healthy individuals, while testosterone enhances its activity [22]. Moreover, at the average ages of 7.7 and 9.4 years, the estrogen levels in girls are about eight times higher than in boys [19]. Therefore, even low estrogen concentrations may contribute to sex differences in the PBL Cr levels in children and preadolescents with T1D. Most notably, a depletion of intracellular Cr^{2+} by SLC6A8 ablation maintains the activation of anti-inflammatory M2 macrophages while suppressing and downregulating pro-inflammatory M1 macrophages, thus suggesting their role in leukocyte phenotyping [23]. Moreover, both the Cr transporter SLC6A8 and the cytosolic brain-type CK support CD8+ T cell expansion in response to infection by maintaining their homeostasis and effector function independent of their effects on cellular energetics [24]. In turn, mitochondrial CK plays a crucial role in the protective effect of Cr against the opening of the mitochondrial permeability transition pore, thus preventing apoptosis; additionally, it participates in the recycling of ADP, thus preventing the formation of reactive oxygen species at elevated glucose concentrations [25,26].

In contrast to girls in Group I, girls in Group II showed decreased Cr levels in both PBL cellular compartments only in LT-T1D individuals with a poor GC, thus suggesting that long-term insulin therapies with a successful GC can maintain the Cr levels within normal limits in this age group. Additionally, the activity of the CK isoforms decreased in both cellular compartments of the PBL in girls with RO-T1D, whereas it was within the normal limits in the cytoplasm of girls with LT-T1D, although reduced in the mitochondria, especially with a poor GC.

Sex differences observed in the Cr metabolism in the PBL cellular compartments in adolescents and young adults may be related to elevated levels of sex hormones that act through their receptors on leukocytes. In contrast to boys in Group I, boys in Group II showed a decrease in the level of Cr and the CK activity in the cytoplasm of the PBL, regardless of the glycemic status and the T1D duration. In the mitochondria, the Cr levels were generally within normal limits and decreased only in LT-T1D individuals under poor GC conditions, while the CK activity was significantly reduced regardless of the GC, diabetes duration, and Cr content. Notably, in healthy young men, Cr supplementation reduces the counter-insular hormone levels and increases the circulating IGF-1 levels, thus promoting effects similar to those of insulin-like hormones [27]. Cr triggers protein kinase Bα-induced GLUT-4

translocation and facilitates glycogen synthesis [28]. In patients with T1D, a positive correlation between male testosterone and insulin resistance, which affects peripheral glucose use and depends on the duration of diabetes, is often observed during puberty [29].

In girls, estrogen appears to directly inhibit cytokine secretion, thereby providing anti-inflammatory effects and supporting pancreatic β-cell function, thus potentially improving glucose levels [30]. Estrogen interacts with insulin signaling via sirtuin 1, mammalian target of rapamycin, and phosphoinositide 3-kinase to coregulate autophagy and mitochondrial metabolism [31]. However, its influence on two common autoimmune diseases that predominate in women varies: estrogen has a protective effect in multiple sclerosis, but it exacerbates systemic lupus erythematosus [32]. Estrogen receptor-independent mechanisms may also play a dual role, thereby exerting both pro- and anti-inflammatory effects through mechanisms influenced by microenvironments, cell types, etc. [33]. Estrogens can act independently of receptors; however, even in this case, they can play a dual role, acting as both pro- and anti-inflammatory agents, depending on various factors, including mechanisms influenced by the microenvironmental factors, cell types, etc.

Notably, L-arginine is involved in the synthesis of endogenous Cr [34]. Hyperglycemia-induced oxidative stress results in the upregulation and increased activity of arginase isoforms [35,36]. As a consequence, the increased hydrolysis of L-arginine by arginase limits the Cr levels and the activity of cCK and mCK, which can be inhibited by free radicals, particularly those produced by arginase [37,38]. Moreover, ornithine produced by arginase can inhibit both the mCK, and L-arginine by glycine amidinotransferase, thus preventing the synthesis of Cr [36]. These processes can lead to immune cell dysfunction and/or endothelial dysfunction and play a crucial role in the onset and development of diabetes and its complications, thus affecting insulin release, insulin resistance, immune response, and the development of oxidative stress [39]. Changes in the Cr/CK system can be associated with sex- and age-related activation of arginase isoforms, which we studied in conjunction with the creatine metabolism in the same participants in T1D [40]. This is supported by the low Cr levels found in the vitreous body of Japanese patients with proliferative diabetic retinopathy, and Cr supplementation has been shown to reduce retinal neovascularization [41]. Conversely, arginase deficiency in urea cycle disorder raises the plasma Cr levels in patients of both sexes aged 0–25 years [42].

Arginase contributes to "uncoupling" nitric oxide synthase by depleting intracellular L-arginine, thus leading to the preferential formation of superoxide over nitric oxide, and the subsequent synthesis of peroxynitrite (ONOO (-)), which is a powerful oxidizing and nitrating agent, very reactive at physiological pH, and is involved in the production of autoantigens and autoantibodies in autoimmune disorders [43]. In particular, peroxynitrite may inhibit the CK activity [44]. CKs have a highly conserved active site containing a cysteine residue essential for their activity which undergoes endogenous posttranslational oxidative modification in all isoforms [45]. Hydrogen peroxide can irreversibly inactivate creatine kinase by targeting the active site cysteine [46]. In T1D, a drop in the catalase activity may stimulate these processes [47]. In turn, Cr can scavenge charged radicals, such as peroxynitrite and superoxide anion, with a direct antioxidant effect, as shown in mammalian cell lines expressing Cr transporters [48]. Note, Cr supplementation reduces an elevation in the blood glucose, serum urea, and alanine aminotransferase in serum and protects against islet depletion and restores antioxidant enzymes and the hydrogen peroxide activity to control the levels in streptozotocin-induced diabetes [49]. Additionally, Cr can act as an indirect antioxidant and have antiapoptotic effects, while phosphocreatine can interact with and protect cell membranes [50]. In a rat model of chronic acidosis, Cr supplementation was shown to directly scavenge reactive oxygen species, reverse the

chronic downregulation glucose transporter 2, and functionally strengthen the jejunal epithelium, thus making it more resistant to acidosis [51]. Notably, Cr supplementation is considered safe and beneficial for children and adolescents, thus supporting potential therapeutic applications in diabetes [52].

In Group II, a persistent decrease in the plasma Cr levels was observed in both girls and boys, regardless of the GC and the diabetes duration, which differed from Group I. At the same time, the CK activity decreased in both groups, thus showing differences depending on sex and age. It can be assumed that a decrease in the level of Cr and the CK activity can occur not only in leukocytes, but also in the organs and tissues in T1D, which is reflected in the plasma and may contribute to the development of oxidative stress and an impaired glucose control.

Cr plays a key role in homeostasis, and a decrease in its level that accompanies various hereditary and non-hereditary diseases almost always indicates a more severe phenotype, being either an etiological factor or a secondary symptom [53]. A marked decrease in the serum CK levels, accompanied by an increase in the level of C-reactive protein, was detected in Fukuyama congenital muscular dystrophy, as well as in Duchenne and Becker muscular dystrophies as rare complications of autoimmune and/or autoinflammatory diseases [54,55]. However, sex- and age-related changes in the plasma creatine metabolism are less specific than in the PBL cellular compartments, in which the creatine/creatine kinase system is involved in their reprogramming, their protection from apoptosis, oxidative stress, and mitochondrial dysfunction, as well as other processes that may contribute to the pathophysiology of T1D. These changes may influence metabolic regulation and require the development of decision boundaries that take these factors into account to assist in medical decision making and nutritional therapy strategies in T1D.

The limitations of the study included the sample size, the lack of CK isoform gene expression analyses, and potential confounding factors such as the vitamin D status, the severity of diabetic ketoacidosis at the diagnosis, the insulin dosing regimens, insulin resistance, diet, and the presence of comorbid conditions such as autoimmune thyroiditis, hypothyreosis, and others. Additionally, the use of concomitant medications during insulin therapy may influence the results.

5. Conclusions

In conclusion, the creatine/creatine kinase system was down-regulated in a sex- and age-dependent manner in both the cytoplasm and mitochondria of PBL in Armenian children / preadolescents and adolescents / young adults with T1D who received insulin therapy. This depends on the glycemic status and the disease duration and may indicate specific regulatory changes. This emphasizes the need for expanded, detailed studies to develop individualized treatment strategies that consider sex and age-related differences in creatine metabolism within leukocyte cellular compartments.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

NA, EA and MH are the guarantors who controlled the study. EA, AM, and AG: clinical data and information of patients, their recruitment and treatment. AA, MH, NA, and AM: experiments, data acquisition, data and statistical analysis. NA, EA, MH and AG: conceptualization, methodology, investigation, data curation and writing the manuscript. All authors approved the final version of the manuscript.

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