THE INFLAMMATORY AND HEMOSTATIC CARDIOVASCULAR RISK MARKERS DURING ACUTE HYPERGLYCEMIC CRISIS IN TYPE 1 AND TYPE 2 DIABETES

INFLAMATORNI I HEMOSTATSKI KARDIOVASKULARNI MARKERI RIZIKA
U TOKU AKUTNE HIPERGLIKEMSKE KRIZE U TIPU 1 I TIPU 2 DIJABETESA

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Summary

Background: We analyzed cardiovascular inflammatory (C-reactive protein (CRP), interleukin 6 (IL-6)), haemostatic (homocysteine) risk markers in lean and obese patients at admission and acute hyperglicemic crisis (AHC) resolving, involving diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic state (HHS).
Methods: In that context, we included group A: N=20 obese, B: N=20 lean patients with DKA; C: N=10 obese, D: N=10 lean patients with HHS; E: N=15 obese, F: N=15 lean controls. CRP, IL-6, homocysteine were determined by ELISA.
Results: Our results showed that CRP, IL-6, and homocysteine levels decreased in all groups: (A: $p<0.001$; B: $p<0.001$, C: $p<0.05$; D: $p<0.001$ mg/L), (A: $p<0.001$ B: $p<0.001$, C: $p<0.001$, D: $p<0.01$ pg/mL), (A: $p<0.001$, B: $p<0.001$; C: $p<0.05$, D: $p=0.001$ μmol/L), respectively, at resolving AHC. However, CRP persisted higher ($p<0.001$, $p<0.01$), IL-6 lower ($p<0.05$, $p<0.001$), while homocysteine levels turned out to be similar to controls.
Conclusions: AHC is associated with increased inflammatory and hemostatic cardiovascular risk markers. Also, insulin therapy in AHC has had more pronounced favorable effect on IL-6 and homocystein than on CRP.

Keywords: acute hyperglycemic crisis, cardiovascular risk markers, inflammatory markers, haemostatic markers

Kratak sadržaj

Uvod: Analizirali smo kardiovaskularne inflamatorne (C-reaktivni protein (CRP), interleukin 6 (IL-6)) i (homocistein) hemotatske markere rizika u negojaznih i gojaznih pacijenata pri prijemu i razrešenju akutne hiperglikemijske krize (AHK), uključujući dijabetesnu ketoacidozu (DKA) i hiperosmolarno hiperglikemijsko stanje (HHS).
Metode: U tom kontekstu uključili smo grupu A: N=20 gojaznih, B: N=20 negojaznih bolesnika sa DKA; C: N=10 gojaznih, D: N=10 negojaznih bolesnika sa HHS; E: N=15 gojaznih, F: N=15 negojaznih kontrola. Nivo CRP, IL-6 i homocistein određeni su ELISA metodom.
Rezultati: Naši rezultati su pokazali niže nivoe CRP, IL-6 i homocisteina nakon rešavanja AHK u poređenju sa prijemom u svim grupama: (A: $p<0,001$; B: $p<0,001$, C: $p<0,05$; D: $p<0,001$ mg/L), (A: $p<0,001$ B: $p<0,001$; C: $p<0,05$, D: $p=0,001$ mmol/L), (A: $p<0,001$, B: $p<0,001$, C: $p<0,001$, D: $p<0,01$ pg/mL) nakon rešavanja AHK. Međutim, nivo CRP je ostao viši ($p<0,001$, $p<0,01$), IL-6 niži ($p<0,05$, $p<0,001$), dok je nivo homocisteina sličan u poređenju sa kontrolama.
Zaključak: AHK su povezane sa povišenim nivoom inflamatornih i hemostatičnih kardiovaskularnih markera rizika. Takođe, terapija insulinom u AHK ima značajno povoljniji efekat na nivo IL-6 i homocisteina, nego na nivo CRP.

Ključne reči: akutna hiperglikemijska kriza, inflamatorni markeri, hemotatski markeri

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Introduction

Acute hyperglicemic crisis (AHC) involving diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic state (HHS) are the most severe hyperglycemic metabolic impairments in patients with type 1 diabetes (T1D) and type 2 diabetes (T2D) (1).

Also, previous studies have shown that repeated hyperglycemic crises increase the cardiovascular risk markers. Previously, it was reported that novel cardiovascular risk biomarkers might be classified as follows: inflammatory (C-reactive protein (CRP), interleukin 6 (IL-6), etc), haemostatic (homocysteine, etc) and other markers. On the other hand, acute hyperglycemia is associated with inflammation and accelerates the inflammatory immune response (2). Simultaneously, it has been shown that hyperglycemia can induce proinflammatory cytokines genes in T cells and acute phase reactants, CRP and IL-6 (3, 4). In addition, T2D and obesity are linked with increased levels of CRP and IL-6 (2, 5–9). Furthermore, elevated plasma homocysteine levels were associated with cardiovascular complications in diabetes (10–12). However, opposite findings regarding homocysteine levels in T2D have been demonstrated: increased (13), unchanged (14) or decreased (15) levels, respectively. In that context, the first study evaluated the level of inflammatory and haemostatic cardiovascular risk markers in AHC reported increase of these markers at admission in emergency care unit (16).

Therefore, the aim of this study was to analyze the changes in cardiovascular inflammatory (CRP and IL-6) and haemostatic (homocysteine) risk markers in lean and obese patients with AHC.

Materials and Methods

Patients/Research design

The study included 90 subjects, 60 were diagnosed with DKA or HHS, whereas the control group consisted of 30 weight-matched control subjects. All included subjects were grouped as follows: 1) 20 obese patients with DKA; 2) 20 lean patients with DKA; 3) 10 obese patients with HHS; 4) 10 lean patients with HHS; 5) 15 obese control subjects and 6) 15 lean control subjects. The study included patients of both genders, 35 to 70 years of age. The study did not include either patients with manifested infection or any known precipitating illness for DKA or HHS, or those with overt cardiovascular disease. DKA was defined on the basis of glycaemia > 13.9 mmol/L, pH level < 7.3, bicarbonate > 18 mmol/L, anion gap > 15 and positive ketonouria. HHS was defined on the basis of glycaemia > 22.4 mmol/L, pH level > 7.3 and bicarbonate > 18 mmol/L. The criterion for the resolving of hyperglycemic state was the reduction of glycaemia 13.9 mmol/L, elevation of the pH level > 7.3, of bicarbonate >18 mmol/L, with

anion gap normalisation (1). Each participant was subjected to:

a) anamnestic interview with defining of anthropometric measures: body weight (BW) and height (H), while body mass index (BMI) was calculated using the following formula: BMI $(kg/m^2)=$BW $(kg)/H^2$ (m^2);

b) physical examination with measuring of body temperature;

c) Laboratory analyses: glycaemia, using the glucose oxidase test (Beckman Instruments, Fullerton, CA, USA), glycated hemoglobin (HbA1c) levels were determinated using turbidimetricimmunoassay for HbA1c (Boehringer Mannheim, Mannheim, Germany), pH of deoxigenated blood, bicarbonate, electrolytes, and other standard laboratory analyses in accordance with the criteria. All analyses were carried out during the same day and blood samples drawn by the same study nurse after a 12 h overnight fast and were stored at −70 °C until assayed. The presence of diabetes was defined in accordance with the WHO definition, while the presence of obesity was determined on the basis of BMI≥30 kg/m² (17, 18).

The study included the following procedures:

1. Determining the levels of CRP (mg/L) and homocysteine (μmol/L) by the ELISA method;

2. Determining the level of proinflamatory cytokine IL-6 (pg/mL) using the ELISA method (ALPCO, Salem, NH, USA)

The high-sensitive C-reactive protein (hs-CRP) serum concentrations were measured using commercial assays on Roche Cobas 6000 automated analyzer. Reference range for hs-CRP was 0–5 mg/L. Homocysteine serum concentrations were measured using commercial assay on ADVIA Centaur System automated analyzer (Siemens, Manheim, Germany). Reference range for homocysteine for patients younger than or equal to 70 years was 4 to 14 μmol/L, and for patients older than 70 years 6 to 20 μmol/L. The coefficients of variation of the assays were all 5%. The instrument calibrations for the assays were performed as recommended by the manufacturers and were within the specifications. The study was conducted in the Metabolic Unit of the Emergency Centre of the Clinical Centre of Serbia, with the permission of the Ethical Committee of the Faculty of Medicine, University of Belgrade, after patients or next of kin gave the informed consent to participate in the study.

All the patients were treated by intravenous insuline infusion according to the standard protocol (bolus dose 0.2 IU/kg BW, followed by 0.1 IU/kg BW/h)(1).

Blood samples for defining the level of CRP, IL-6 and homocysteine were taken during the DKA and HHS at admission and 24 hours after the beginning of insulin therapy, upon resolving AHC.

Statistics

Data are presented as mean ± SD. Data were tested for normal distribution using Kolmogorov–Smirnov test. The appropriate descriptive and analytical methods (absolute and relative numbers, t test, Wilcoxon test, Mann-Whitney test) were used and the results were presented in the form of figures and tables. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software (Advanced Statistics, version 20.0), Chicago, IL. The P<0.05 criterion was used in the testing procedure to calculate the significant difference level.

Results

The total number of investigated patients was 60, among which 20 lean (between 23 to 49 yrs) and 20 obese DKA patients (between 30 to 53 yrs), and 10 lean (between 65 to 70 yrs) and 10 obese (between 58 to 70 yrs) HHS patients. The control groups consisted of 15 lean and 15 obese subjects, aged 35 to 70, both males and females. BMI values ranged from 21.3 to 45.2 kg/m^2. None of the subjects reported simptoms or signs of infective diseases or other illness. Summary of baseline clinical characteristics of all participants included in the study is shown in *Table I*. Laboratory parameters of all patients at admission and after resolving AHC as well as baseline values for control subjects are presented in *Table II*. After insulin therapy was administrated, during the first 24h, levels of glycemia, bicarbonates and pH were corrected at resolving AHC, DKA or HHS.

Inflammatory cardiovascular risk markers in acute hyperglicemic crises in diabetes

In order to evaluate the status of cardiovascular risk markers in AHC, we determined the level of two parameters, CRP and IL-6, in obese and lean patients with DKA and HHS.

Table I Clinical characteristics of lean and obese patients with diabetic ketoacidosis (DKA) or hyperosmolar hyperglycemic state (HHS) and control subjects.

Parameters	Lean DKA	Obese DKA	Lean HHS	Obese HHS	Lean control	Obese control
Patients (n)	20	20	10	10	15	15
Age (yrs)	39 ± 1.23	43.1 ± 1.29	68 ± 0.71	63.7 ± 1.15	45 ± 3.05	47.13 ± 1.91
Sex (M/F)	7/13	6/14	3/7	5/5	4/11	7/8
BMI (kg/m^2)	22.84 ± 0.88	31.69 ± 0.19	21.31 ± 0.47	31.33 ± 0.31	28.09 ± 0.30	45.52 ± 0.93
HbA1c (%)	10.89 ± 0.34	10.75 ± 0.41	7.99 ± 0.21	8.16 ± 0.22	5.49 ± 0.12	5.8 ± 0.08
Hb (mmol/mol)	90.35 ± 5.45	93.9± 4.53	63.84± 2.34	65.69± 2.36	36.51 ±1.30	39.9± 0.86

Data are means ± SE.

Table II Laboratory parameters of lean and obese patients with diabetic ketoacidosis (DKA), or hyperosmolar hyperglycemic state (HHS) and control subjects at admission and after resolving AHC.

	Obese DKA		Obese HHS		Control
Parameters	Admission	Resolving AHC	Admission	Resolving AHC	
Glycemia (mmol/L)	34.08 ± 3.60	12.79 ± 1.21*	27.00 ± 1.28	13.88 ± 0.79*	4.83 ± 0.13
pH	7.06 ± 0.02	7.34 ± 0.01*	7.38 ± 0.02	7.42 ± 0.01#	7.40 ± 0.01
HCO$_3$ (mmol/L)	5.24 ± 0.54	17.63 ± 0.77*	22.91 ± 0.96	24.33 ± 0.22	21.55 ± 0.33
	Lean DKA		Lean HHS		Control
Glycemia (mmol/L)	37.26 ± 3.71	12.75 ± 0.89*	30.91 ± 2.53	14.04 ± 0.70*	4.65 ± 0.24
pH	7.02 ± 0.03$	7.34 ± 0.02*$	7.41 ± 0.01	7.42 ± 0.01	7.41 ± 0.01
HCO$_3$ (mmol/L)	4.90 ± 0.57$	21.88 ± 0.49*$	21.61 ± 0.46	24.46 ± 0.21*	20.91 ± 0.26

Data are means ± SE. *P<0.01 vs. admission value of each group, [#]P<0.05 vs. admission value of each group, P<0.01 vs. obese HHS value, [a]P<0.01 vs. obese HHS value, [b]P<0.01 vs. obese DKA value, [$]P<0.01 vs. lean HHS value
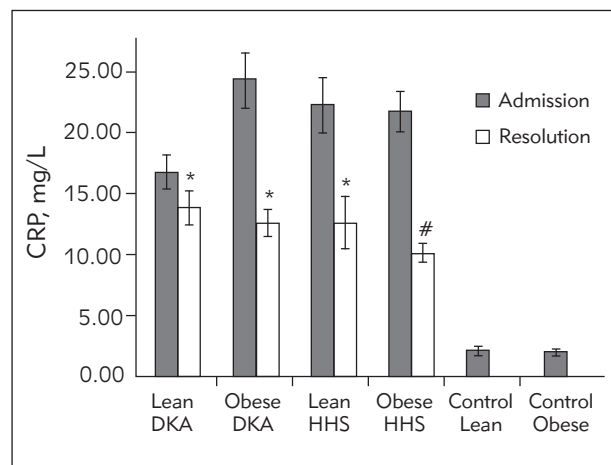
Figure 1 The levels of C-reactive protein (CRP) measured in plasma in obese and lean patients with diabetic ketoacidosis (DKA), or hyperosmolar hyperglycemic state (HHS), and control subjects at admission and after resolving AHC. The bar graphs show the means ± SD for each of the patient groups. The levels of CRP were significantly lower at resolving AHC vs admission in both lean and obese patients with DKA and HHS. The levels of CRP were significantly higher in obese and lean patients with DKA and HHS, vs control subjects (*p<0.001, # p<0.05).

Figure 2 The level of interleukin 6 (IL-6) measured in plasma in obese and lean patients with diabetic ketoacidosis (DKA) or hyperosmolar hyperglycemic state (HHS), and control subjects at admission and after resolving AHC. The bar graphs show the means ± SD for each of the patient groups. The levels of IL-6 was significantly decreased at resolving AHC vs admission in both lean and obese patients with DKA and HHS. Moreover, the resolving AHC levels of IL-6 in lean patients HHS did not differ vs control subjects, while all other groups had lower levels vs control subjects.

When we analyzed the level of cardiovascular risk marker CRP, we found that the level of CRP was significantly decreased at resolving AHC in comparison to moment of admission in both lean and obese patients with DKA and HHS (lean DKA: 16.77 ± 6.12 vs. 13.86 ± 6.28, p 0.001; obese DKA: 24.26 ± 10.16 vs. 12.63 ± 4.88, p 0.001; lean HHS: 22.25 ± 7.09 vs 12.64 ± 6.74, p 0.001; obese HHS: 21.7 ± 5.03 vs 10.26 ± 2.48, p 0.05, mg/L, respectively) (*Figure 1*). Yet, the resolving AHC levels of CRP, in both obese and lean patients with DKA and HHS were significantly higher compared with control subjects (obese controls 2.14 ± 0.93, lean controls 2.20 ± 1.59)(HHS p<0.01, DKA p<0.001) (*Figure 1*).

In addition, we determined interleukin 6 (IL-6) in obese and lean patients with diabetic ketoacidosis (DKA), or hyperosmolar hyperglycemic state (HHS), and control subjects at admission and after resolving AHC.

We found that the levels of IL-6 were significantly decreased at resolving AHC in comparison to moment of admission in both lean and obese patients with DKA and HHS (lean DKA: 37.73 ± 11.24 vs. 28.70 ±9.45, p 0.001; obese DKA: 53.94 ± 89.38 vs. 33.31 ± 83.79, p 0.001; lean HHS: 107.23 ± 86.29 vs. 75.79 ± 45.14, p 0.01; obese HHS: 84.73 ± 98.66 vs 36.60 ± 39.81, p 0.001, pg/mL, respectively) (*Figure 2*). Still, the resolving AHC levels of IL-6, did not differ significantly among groups, and were lower compared with control subjects (obese control 79.70 ± 6.86, lean control 78.06 ± 4.85
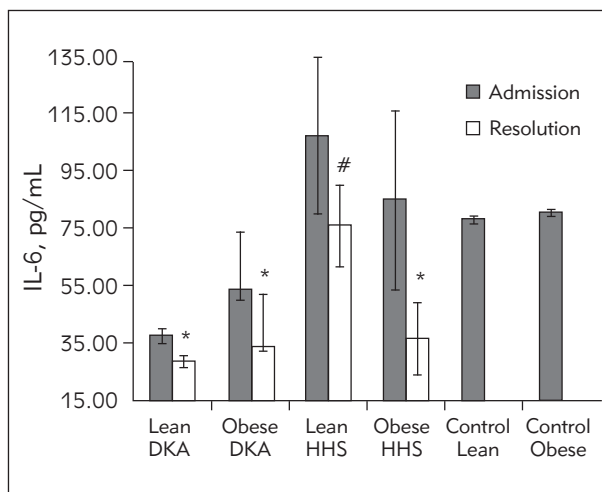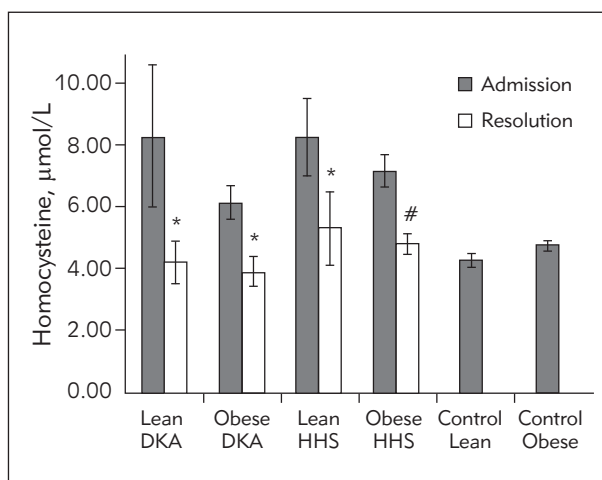

Figure 3 The level of homocysteine measured in plasma in obese and lean patients with diabetic ketoacidosis (DKA) or hyperosmolar hyperglycemic state (HHS), and control subjects at admission and after resolving AHC. The bar graphs show the means ± SD for each of the patient groups. The levels of homocysteine were significantly decreased at resolving AHC vs admission in both lean and obese patients with DKA and HHS. Moreover, the resolving AHC levels of homocysteine, in both obese and lean patients with DKA and HHS did not differ vs control subjects.

pg/mL) (HHS obese p<0.001, DKA p<0.05) (*Figure 2*), whereas in lean patients with HHS there was no significant difference compared with control subjects (p>0.05).

Haemostatic cardiovascular risk marker in acute hyperglicemic crises in diabetes

When we evaluated the level of third investigated cardiovascular risk marker homocysteine, we found that the level of homocysteine was significantly decreased at resolving AHC than at admission in both lean and obese patients with DKA and HHS (lean DKA: 8.25 ± 10.32 vs. 4.19 ± 3.02, p 0.001; obese DKA: 6.14 ± 2.41 vs. 3.88 ± 2.17, p 0.001; lean HHS: 8.25 ± 3.92 vs. 5.31 ± 3.75, p=0.001; obese HHS: 7.16 ± 1.62 vs. 4.79 ± 1.07, p 0.05, μmol/L, respectively) (*Figure 3*). Moreover, the resolving AHC levels of homocysteine in both obese and lean patients with DKA and HHS did not differ compared to control subjects (obese controls 4.74 ± 0.59 lean controls 4.26 ± 0.77 μmol/L) (p>0.05) (*Figure 3*).

Discussion

We have analyzed the level of cardivascular risk markers (CRP, IL-6, homocystein) before and 24 hours after the onset of AHC and adequate insulin therapy in obese and lean patients with DKA and HHS, without overt infections, cardiovascular disease or trauma. Our results have shown that in patients with AHC, the levels of inflammatory and hemostatic markers of cardiovascular risk were increased at admission, while after the management of hyperglycemic crisis with intensified insulin therapy, these values of these markers significantly decreased, implying the decrease of the cardiovascular risk. The obtained results are in accordance with the previous findings, suggesting the beneficial antiinflamatory effect of insulin (5, 19–21).

It is well known that not only in chronic hyperglycemia but also in intermittent hyperglycemic crisis, the levels of inflammatory and haemostatic cardiovascular risk biomarkers were impaired (22). However, previously it was demonstrated in animal models that the repetitive acute hyperglycemia represents a risk factor for initiation and progression of atherosclerosis independently of other metabolic factors, such as insulin resistance and dislipidemia (23).

When we evaluated the levels of inflammatory cardiovascular risk marker CRP, we found that the level of CRP was significantly decreased at resolving AHC than at admission in both lean and obese patients with DKA and HHS, but still higher than the level determined in control subjects.

Previously, it has been shown that the level of CRP was associated with T2D via insulin resistance (24), and that it correlated with HbA1c in non-diabetics (25), while higher HbA1c in diabetes was found to be associated with elevation of CRP (19). Our patients had unsatisfactory metabolic control before acute decompensation, suggesting chronic state of hyperglycemia and long-term low-grade inflama-

tion, probably leading to the slowest recovering of CRP levels. Some other data suggested that the level of CRP started to fall only after 5 days after insulin therapy had been initiated in intensive care unit (9). Also, this marker might reflect the long term cardiovascular risk still existing after resolving of the AHC.

In addition, we measured IL-6 levels in obese and lean patients with DKA, or HHS, and in control subjects at admission and after resolving AHC. We found that the levels of IL-6 were significantly decreased at resolving AHC than at admission, in both lean and obese patients with DKA and HHS, following the implementation of insulin therapy which exerted a potent IL-6 lowering effect. Still, the IL-6 levels at resolving of AHC levels in lean HHS patients were similar to control subjects, whereas in all other groups, they were lower than in control subjects.

It is well known that acute hyperglycemia can significantly increase the inflammatory cytokines in the peripheral circulation, and that it plays important roles in immune system activation in diabetes (1, 26, 27). Previously, it was documented that the patients with DKA have the activation of T-cells caused by increased level of oxidative stress (3, 28). Moreover, circulating levels of IL-6 were increased, but there was downregulation of antiinflammatory cytokine production in T2D. Furthermore, circulating blood cells have the capacity to produce cytokines in diabetes which contribute to the augmented acute-phase response, but the main source of the increased plasma IL-6 concentrations may be from non-circulating cells (29). Additionally, previous studies have shown that higher IL-6 can affect the signaling pathways of the insulin receptor in adipose tissue (30).

Simultaneously, further investigations have shown that IL-6 was significantly elevated in patients with hyperglycemic crises and were significantly decreased after insulin treatment, although levels in remission remained higher than in controls, in contrast to our results (31, 32). We speculate that this difference may be due to the previously described heterogeneity of IL-6 sources and effects. Increased levels of IL-6 levels in patients with AHC may decrease expression of PPAR-γ (29), interfering with phosphorylation of insulin receptors in peripheral tissue, affecting insulin signaling, (32) and increasing the expression of cell adhesion molecules that are involved in endothelial cell damage and increased risk for cardiovascular event. Moreover, IL-6 released from monocytes during the state of hyperglycemia could upregulate PKC, through p38MAPK and NF- B, resulting in increased mRNA and protein for IL-6, thus amplifying its own effect (33).

On the other hand, previous data suggested the effect of obesity on the level of IL-6, implicating that obese patients with T2D have higher levels of IL-6 than nonobese T2D patients. Also, insulin therapy tends to counter this marker of inflammation, but the

response is delayed in obese diabetics. Additionally, after 24h of insulin administration to nonobese diabetic patients, a high-magnitude decrease of IL-6 was detected (34). In our study, lean HHS had higher levels of IL-6, at resolution of ACS, than other groups, i.e. their responce on insulin therapy was blunted, irrespective the absence of obesity. Interestingly, lean HHS patients had the highest level of IL-6 among the groups innitially, at admision. It might be that acute hyperglycemia in this group compared to obese HHS, has domminant influence on inflammation, and consequently on IL-6 levels, which could be the background for its delayed responce to insulin therapy. Moreover, in some investigations it was suggested that the IL-6 gene has functional variants, some of which are associated with high IL-6 circulating levels and cardiovacular risk in patient with proinflammatory states (35).

When we analysed the level of third investigated cardiovascular risk marker homocysteine, we found that the levels of homocysteine were lower at resolving AHC than at admission in both lean and obese patients with DKA and HHS. Moreover, the resolving AHC levels of homocysteine, in both obese and lean patients with DKA and HHS, did not differ compared to control subjects. These data are not in line with the previously published results (16, 36), where homocysteine levels were not reduced promptly by intensified insulin therapy. On the other hand, it is reported that the level of homocysteine is influenced by many factors, including different drugs (37), which might devaluate its role as a marker of cardiovascular risk.

In addition, the compared groups differ in gender due to the fact that the absolute number of women is higher than men, but without statistical difference among groups. In that context, the current literature suggests different findings, higher levels of CRP and IL-6 in women (38) together with the absence of difference in the level of homocysteine between the sexes (39, 40). On the other hand, we analyzed patients in states of acute metabolic decompensation, and we did not include either patients with manifested infection or any known precipitating illness for DKA or HHS, or those with overt cardiovascular disease, which would increase the level of our markers. Moreover, the scope of our study was not the difference in levels of these markers between sexes.

Finally, there are some limitations of our study, regarding the relatively small number of patients in investigated groups. However, the statistical significances among the groups that we obtained were based on non-parametric tests, which are more precise than parametric test, for small samples (41). Future direction in our investigation will be to include more patients in each group which will be the added value of our research.

Our study confirmed that AHC, which involved in our investigation DKA and HHS, is associated with increased levels of inflammatory and hemostatic cardivascular risk markers, irrespective of diabetes type or presence of obesity. We speculate that repetitive AHC may play important role, amplyfing the risk factor for cardiovascular diseases. In addition, the normalization of them can be obtained by intensive insulin therapy, which appears to be based on the strong antiinflammatory effect of insulin. Moreover, our results imply that insulin therapy in these metabolic impairements has stronger beneficial effect on IL-6 and homocystein, than on CRP.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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