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THE CONTENT OF PRO-INFLAMMATORY CYTOKINES IL-1β, IL-6, IL-17A AND TNFα IN THE BLOOD OF PATIENTS WITH TYPE 2 DIABETES AFTER THERAPY WITH METFORMIN

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Currently the world society is extremely worried about the global increase in the number of patients with diabetes on our planet. Annually, 4 million people die of this disease, and the cost of its treatment reaches trillions of dollars. A new highly effective oral antidiabetic drug metformin (1,1-dimethylbiguanide hydrochloride) is one of the most common hypoglycemic remedies currently prescribed for the first-line treatment of patients with type 2 diabetes (T2D). However, the mechanism of its curative effect is still not clear. The results of our study showed that metformin treatment of patients with newly diagnosed T2D was followed by pronounced normalization of the increased levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-17A and TNFα), inflammation indexes and lymphocyte's immunophenotype. The obtained data confirm the existing hypothesis about the inflammatory nature of T2D and indicate that the immune system, in particular proinflammatory cytokines, plays a significant role in the mechanism of the curative effect of metformin at T2D.

K e y w o r d s: metformin, immunity, cytokine, type 2 diabetes (T2D).

The global steady increase in the number of patients with diabetes mellitus (DM) around the world, having the pandemic nature, is becoming a tragedy. Over the past 25 years, the number of patients with diabetes on our planet was increased more than 4 times and consisted of 527 million people in 2017, and by 2045 as it is estimated, will reach 627 million, 90% of whom have type 2 diabetes (T2D) [1]. According to the latest data [2], prediabetes is detected in half of people over 65, and a clinically diagnosed disease – in ¼ of them in USA. T2D, along with cardiovascular and oncological diseases, is among the three diseases with the most fatal outcome, leading to an annual death of approximately 4 million people in the world. The society spends colossal funds on its treatment – 352 milliard dollars annually [1].

A vast achievement in the field of diabetes treatment in recent decades was to obtain both high-quality insulin preparations and create the new highly effective hypoglycemic oral drugs. Metformin was especially popular among them [3, 4]. Metformin (1,1-dimethylbiguanide hydrochloride) is one of the most common hypoglycemic remedies, the medicines of choice, currently prescribed for first-line treatment of patients with type 2 diabetes in almost all countries of the world [5]. So, in the UK, metformin in 2013 was used in 83.6% of patients with type 2 diabetes, and in the USA in 2012 – in 83.6% (61.6 million) of patients [6].

According to the recommendations of ADA and EASD for 2019-2020 [7, 8] metformin is preferred as medical mean in the first line of treatment for patients with type 2 diabetes after recommendations for lifestyle changes have not given the desired result. It can be used for a long time, as it is tolerant and not contraindicated with other antidiabetic drugs. Metformin is often used at a later time in the course of T2D as a combination therapy of the second and third lines in a case of insufficient gly-

cemic control: insulin, sulfonylureas, incretins, an SGLT inhibitor, as well as for preventing the conversion of diabetes to clinically diagnosed T2D [9-11].

Metformin is polypotent in its action, i.e. in addition to hypoglycemic action, it also has other important physiological properties, in particular, normalizes the body mass, improves cardiovascular function and, as suggested, can be a geriaprotector, i.e. has a positive effect on longevity, prevents aging [12].

However, despite the wide popularity of metformin as a medical mean for the treatment of T2D, the mechanism of its curative effect is still poorly understood. At the same time, since according to the modern hypothesis, T2D is a highly immunologically heterogeneous polygenetic disease of an inflammatory nature [13, 14], then the question naturally arises – to what extent the therapeutic effect of metformin is due to its effect on immunity and inflammation.

Only a few works have been devoted to the studying the role of the immune system and inflammation in the mechanism of the therapeutic effect of metformin in patients with type 2 diabetes [15, 16], which showed that in patients with T2D treated with metformin, there is a decrease in the level of certain pro-inflammatory cytokines (IL-1β, IL-6 and fractokine chemokine) in PB.

In our previous studies [17, 18] it was found that in patients with newly diagnosed T2DM after metformin therapy, a significant decrease in the level of pro-inflammatory cytokines IL-1β, TNFα, as well as an increase in the level of anti-inflammatory cytokines IL-4 and IL-10 in PB, which was accompanied by a decrease in absolute neutrophil count and NLR index.

This report is a summary of the results of our subsequent more detailed studies on the role of the pro-inflammatory cytokines IL-1β, IL-6, TNFα, as well as the recently described cytokine IL-17A in the mechanism of the anti-inflammatory therapeutic effect of metformin in patients with newly diagnosed T2D.

Materials and Methods

There were examined 52 patients of both sexes with newly diagnosed T2D, 30 (mean age 40-60 years) of whom were selected with a clinically similar course of the disease and without myocardial infarction, stroke, malignant tumors, acute and chronic pulmonary diseases in anamnesis, as well as previously not taking any hypoglycemic or other medications who were prescribed metformin treatment (2 000 mg/day for 3 months). The control group consisted of 42 practically healthy normoglycemic individuals, approximately of the same age and gender (Table 1).

T2D was diagnosed according to the recommendations of the ADA [2]. Body mass index (BMI) was calculated by dividing body weight in kg per square of growth in meters, % glycosylated hemoglobin (HbA1c) by reaction with thiobraburic acid, systolic blood pressure (SBP) was measured by the automatic monitor UA-778 (Japan).

The total number of leukocytes in the PB was determined using a hematological analyzer, and the leukocyte composition – as by the analyzer and in blood smears stained according to Pappenheim using cacodilate buffer (pH 6.85) per 200 identifiable cells. The NLR inflammation index was calculated by dividing the absolute number of neutrophils into the absolute number of lymphocytes.

The number of lymphocytes of different immunophenotype (CD3 + T, CD4 + T, CD8 + T, CD20 + and CD56 + cells) was determined by flow cytometry using a FACStar plus Becton Dickinson laser cytofluorimeter (USA).

The content of pro-inflammatory cytokines (IL-1β, IL-6, IL-17A and TNFα) was determined by ELISA using a Starfax 3 200 vertical beam spectrophotometer from Star (USA) and a kit of reagents from Diaclon (France), Invitrogen (USA).

Statistical processing of the obtained data was carried out by the method of variation statistics using the standard package of statistical calculation with the Libre Office Calc program.

Results and Discussion

Traditional clinical, laboratory and anthropometric studies of a selected group of patients with newly diagnosed T2D showed that, compared with the group of normoglycemic individuals (control), as seen from Table 1, there were the higher indices of BMI ($P < 0.01$), percent HbA1c ($P < 0.01$) in many patients with SBP ($P < 0.05$). At the same time, the examined patients with T2D, before their treatment with metformin, were characterized by a small, but statistically significant ($P < 0.05$) increase in the total number of leukocytes (without a left shift). Similar leukocytosis in patients with type 2 diabetes was also observed in studies conducted in a number of clinical centers on thousands of patients with type 2

diabetes, [19, 20] and is consistent with our previous works [21]. Leukocytosis was due to an increase in the absolute number of neutrophils ($P < 0.01$) and monocytes ($P < 0.01$). NLR index ($P < 0.05$) also confirms the inflammatory nature of T2D in examined patients [13, 22]. After metformin therapy (Table 1), there was an obvious improvement in the clinical condition and well-being of patients, accompanied by normalization of HbA1c ($P < 0.05$), BMI ($P < 0.01$) and stabilization of SBP ($P < 0.05$), which was accompanied by a decrease in the total number of leukocytes ($P < 0.01$), neutrophils ($P < 0.01$) and monocytes ($P < 0.01$), as well as the Neutrophil/Lymphocyte Ratio (NLR) ($P < 0.05$). Especially significant changes in the absolute number of monocytes were observed in women both before and after treatment with metformin. It should be noted that there is also an article in which the possibility of participating the cells of the monocytic macrophage series in the mechanism of metformin action in T2D was shown [23].

When studying the immunophenotype of lymphocytes in the BP of patients with T2D an insignificant increase (CD3 + T cells) in their main subpopulations (CD4 + T cells and CD8 + T cells) was observed before metformin treatment (Fig. 1). Moreover, in all obese women with high BMI and monocytosis, the absolute number of CD4 + T cells had especially significant increase, which is consistent with data from other authors [24, 25].

As a result of metformin treatment, as can be seen from Fig. 1, a slight normalization of the content of CD3 + T cells ($P < 0.05$), subpopulations of CD8 + T cells ($P < 0.05$) and, especially, CD4 + T cells ($P < 0.01$), compared with those before treatment was revealed. However, this decrease did not reach the indices observed in healthy individuals of the control group. The content of CD20 + and CD56 + cells was irregularly changed.

Especially demonstrative were the data on a decrease in the elevated level of pro-inflammatory cytokines in the BP of patients with T2D after metformin therapy. As known, an elevated level of pro-inflammatory cytokines in BP is a convincing biomarker for the development and presence of T2D. In support of this, there are numerous publications [21], based on a survey of a vast number of patients with prediabetes and T2D and their meta-analysis [26]. At the same time, an elevated level of pro-inflammatory cytokines in BP (IL-1β, IL-6, IL-17A and TNFα) is considered a reliable inflammation biomarker [27].

As can be seen from Fig. 2, as a result of a three-month metformin therapy in the examined patients, there was a significant decrease in the level of all the studied pro-inflammatory cytokines, but the degree of their change was largely individual and depended on the sex and the values of HbA1c and BMI.

So, after metformin therapy, the content of IL-1β (Fig. 2, *A*) significantly decreased by 88% ($P < 0.01$) and the degree of its change correlated

Table 1. Clinical, laboratory and anthropometric indicators in the examined groups of healthy normoglycemic people and patients with T2D before and after metformin therapy

Values	Healthy individuals	T2D before metformin use	T2D after metformin use
IBM, kg/m^2	26.29 ± 0.55	34.23 ± 0.91**	30.2 ± 0.85^{++}
HbA1c, %	5.26 ± 0.30	8.21 ± 0.31**	6.66 ± 0.25^{+}
SBP	120.00 ± 3.29	148.0 ± 2.88*	137.0 ± 3.9^{+}
Leukocytes, 10^9/l	5.96 ± 0.31	6.91 ± 0.30*	5.76 ± 0.23^{++}
Neutrophils, 10^9/l	3.40 ± 0.25	4.45 ± 0.24**	3.46 ± 0.18^{++}
Eosinophils, 10^9/l	0.13 ± 0.03	0.15 ± 0.03	0.12 ± 0.02
Basophils, 10^9/l	0.01 ± 0	0.01 ± 0	0.01 ± 0
Monocytes, 10^9/l	0.43 ± 0.05	0.56 ± 0.04*	0.32 ± 0.08^{++}
Lymphocytes, 10^9/l	2.00 ± 0.18	1.95 ± 0.19	1.84 ± 0.09
NLR index	2.02 ± 0.19	2.48 ± 0.19*	2.06 ± 0.19^{+}

Notes: *$P < 0.05$ compared with the group of healthy people; **$P < 0.001$ compared with the group of healthy people; $^{+}P < 0.05$ compared with the group of patients with type 2 diabetes before treatment with metformin; $^{++}P < 0.001$ compared with the group of patients with type 2 diabetes before treatment with metformin

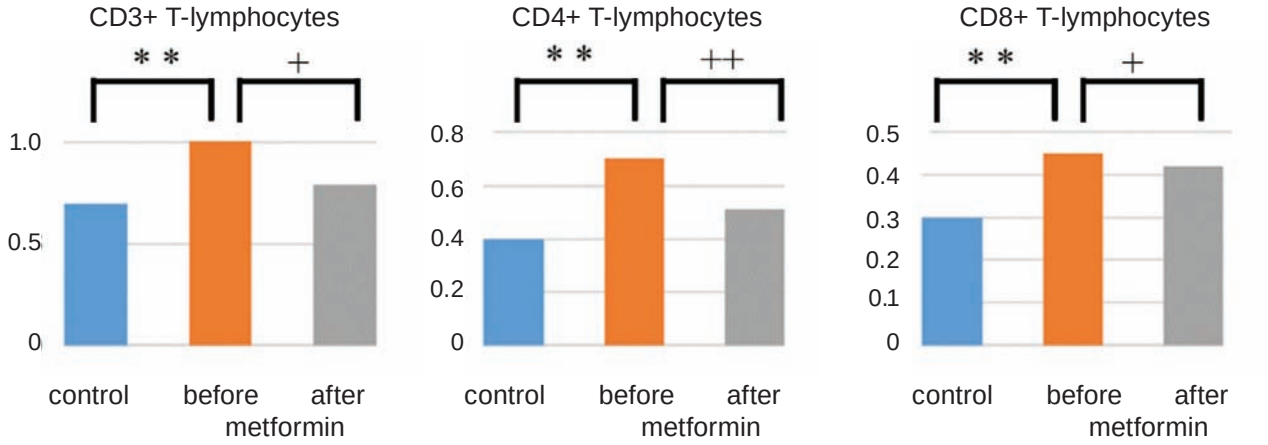

*Fig. 1. Immunophenotype of PB lymphocytes in healthy people and patients with type 2 diabetes before and after metformin therapy. **Differences compared with the control group ($P < 0.05$). $^{+}$Differences compared with the group before treatment with metformin ($P < 0.05$)*

with the values of HbA1c and BMI, accompanied by an improvement in the clinical condition of patients. The obtained results are consistent with recent publications [14, 28, 29], in which it was shown that blockade of IL-1β using its receptor antagonist (IL-1Pa) in patients with metabolic syndrome leads to a decrease in the inflammatory process in pancreatic islets, a reduction of glucose level in BP, SBP, an improvement of vascular complications and prevents the conversion of prediabetes in clinically expressed T2D.

The fact that an increase in the level of IL-1β plays an important role in the pathogenesis of T2D is also confirmed by the curative effect of the IL-1β blockade by monoclonal antibodies neutralizing this cytokine (using the drugs Canacinumab and Gevokizumab) used in the clinic for immunointervention [27, 30, 31].

The results of our studies also found that in the metformin treatment in patients with type 2 diabetes, there is a significant decrease in the pro-inflammatory cytokine IL-6 (Fig. 2, *B*), which is considered the main marker for predicting the development of type 2 diabetes and has an activating effect on the conversion of prediabetes to clinically diagnosed diabetes [32].

In patients with type 2 diabetes treated with metformin, as seen in Fig. 2(*B, C*) we also observed a decrease of another macrophage proinflammatory cytokine – TNF. It is known that TNFα, as well as IL-6, is secreted in large amounts by adipose tissue, and macrophages migrated and recruited into it [25].

The recently discovered pro-inflammatory cytokine IL-17 secreted by Th17 cells also plays a cardinal role in T2D. For the present, there are only a few publications regarding the metformin action in patients with type 2 diabetes, which show an increase in its level in BP in type 2 diabetes [33]. It is believed that the mechanism of its action on inflammation in the islets of Langerhans is somewhat different from the action of Th1 and Th2 cytokines. It has been suggested that it potentiates the destruction of beta cells by the CD8 + T cells [34]. In our studies, it was shown for the first time in the literature (Fig. 2, *D*) that under the influence of metformin therapy in a number of patients with newly diagnosed T2D, a significant normalization of its content in PC is observed similar to other proinflammatory cytokines.

Due to the fact that almost 80% of patients with type 2 diabetes suffer from obesity [35], and adipose tissue, according to modern concepts, is a powerful endocrine organ, and obesity is its inflammation [14], then the question naturally arises – to what extent the increase in the content and activity of inflammatory cytokines in T2D are specific – that is, characteristic for the disease itself or are a consequence of the accompanying obesity, requires a further solution.

T2D and obesity are also closely associated with another most common complication that is cardiovascular diseases, the biomarkers of which in their development and course are also inflammatory cytokines [36, 37]. A meta-analysis of the data ob-

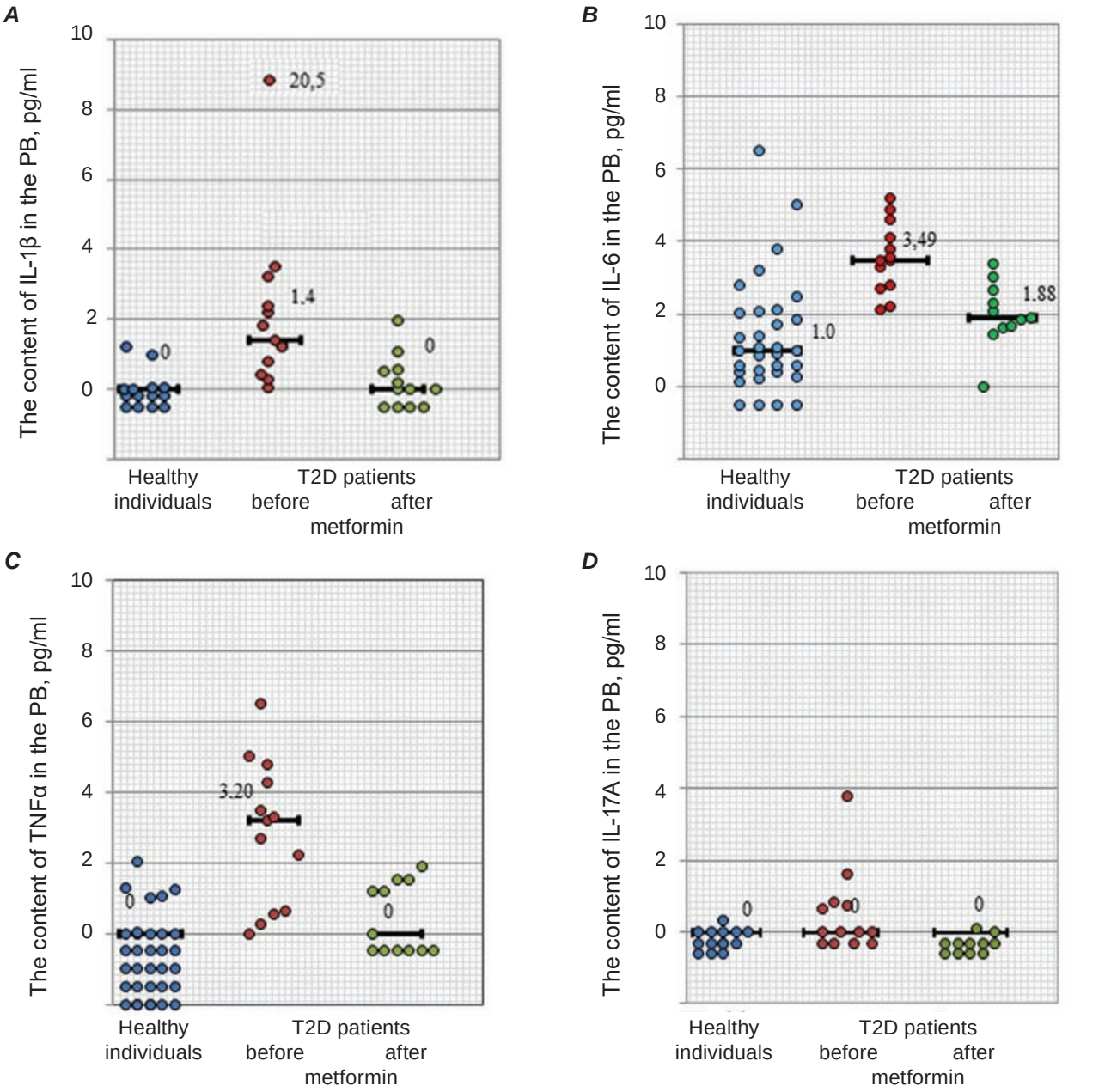

*Fig. 2. The content of IL-1β (**A**), IL-6 (**B**), TNFα (**C**) and IL-17A (**D**) in the PB of healthy individuals (control) and patients with newly diagnosed T2D before and after metformin therapy*

tained from an examination of 2 million individuals shows that cardiovascular complications are also more common in women [38]. Moreover, metformin therapy has a beneficial effect both on obesity and on the cardiovascular system, both in patients with type 2 diabetes and without it [39].

Thus, the carried out studies showed that in patients with type 2 diabetes after metformin therapy, along with a positive clinical effect, a pronounced normalization of the increased level of pro-inflammatory cytokines accompanied by inflammation reversion has been occurred. The data obtained confirm the hypothesis about the inflammatory nature of T2D and show that the immune system, in particular proinflammatory cytokines, plays a significant role in the curative mechanism of metformin.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ВМІСТ ПРОЗАПАЛЬНИХ ЦИТОКІНІВ (ІЛ-1β, ІЛ-6, ІЛ-17А І ФНОα) В КРОВІ ХВОРИХ НА ДІАБЕТ 2-ГО ТИПУ, ЯКІ ЛІКУВАЛИСЯ МЕТФОРМІНОМ

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Наразі світове співтовариство надзвичайно непокоїть питання про глобальне трагічне збільшення кількості хворих на цукровий діабет на нашій планеті. Щорічно смерть від цього захворювання забирає 4 млн. людей, а витрати на його лікування досягають мільярдів доларів. Нещодавно для лікування цукрового діабету 2-го типу (Т2D) почали використовувати антидіабетичний препарат метформін (1,1-диметилбігуанід гідрохлорид), який зараз відносять до ліків, що найчастіше приймають мільйони хворих на Т2D. Однак механізм дії цього препарату ще недостатньо з’ясовано. Результати проведених досліджень показали, що за терапії метформіном у хворих із вперше виявленим Т2D відбувається виражена нормалізація підвищеного рівня прозапальних цитокінів (ІЛ-1β, ІЛ-6, ІЛ-17А і ФНОα), що супроводжується нормалізацією біомаркерів запалення (лейкоцитозу, нейтрофілозу, імуноцитозу) та імунофенотипу лімфоцитів (CD3+T, CD8+T та, особливо, CD4+T-клітин). Одержані дані підтверджують гіпотезу, що існує, про запальну природу Т2D і те, що в механізмі дії метформіну за Т2D істотну роль відіграє імунна система, зокрема прозапальні цитокіни.

Ключові слова: метформін, імунітет, цитокіни, цукровий діабет 2-го типу.

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