# Serological markers of intestinal barrier impairment do not correlate with duration of diabetes and glycated hemoglobin in adult patients with type 1 and type 2 diabetes mellitus.

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## ABSTRACT

**Background and aims:** Growing evidence suggests that diabetes mellitus is associated with impairment of the intestinal barrier. However, it is not clear so far if the impairment of the intestinal barrier is a consequence of prolonged hyperglycemia or the consequence of external factors influencing the gut microbiota and intestinal mucosa integrity. Aim of the study was to perform an estimation of relationship between serological markers of impairment of the intestinal barrier: intestinal fatty acid-binding protein (I-FABP), cytokeratin 18 caspase-cleaved fragment (cCK-18), and soluble CD14 (sCD14) and markers of prolonged hyperglycemia, such as the duration of diabetes mellitus and glycated hemoglobin (HbA1c) via a correlation analysis in patients with diabetes mellitus. **Methods:** In 40 adult patients with type 1 diabetes mellitus and 30 adult patients with type 2 diabetes mellitus the estimation has been performed. **Results:** Statistically significant positive correlation was found between cCK-18 and HbA1c ($r = 0.5047$, $p = 0.0275$) in patients with type 1 diabetes mellitus with fading insulitis (T1D). In patients with type 1 diabetes mellitus with ongoing insulitis (T1D/INS) and in patients with type 2 diabetes mellitus (T2D), no statistically significant positive correlations were found between serological markers of intestinal barrier impairment (I-FABP, cCK-18, sCD14) and duration of diabetes or levels of HbA1c. Similarly, in cumulative cohort of patients with T1D/INS and patients with T1D we revealed statistically positive correlation only between HbA1c and cCK-18 ($r = 0.3414$, $p=0.0311$). Surprisingly, we found statistically significant negative correlation between the duration of diabetes mellitus and cCK-18 ($r = -0.3050$, $p = 0.0313$) only in cumulative group of diabetic patients (T1D, T1D/INS, and T2D). **Conclusion:** Based on our results, we hypothesize that the actual condition of the intestinal barrier in diabetic patients is much more dependent on variable interactions between host genetic factors, gut microbiota, and environmental factors rather than effects of long-standing hyperglycemia (assessed by duration of diabetes mellitus or HbA1c).

## 1. Introduction

There is a growing body of publications showing that intestinal barrier impairment and increased intestinal permeability ("leaky gut") are present in immune-mediated disorders (such as type 1 diabetes mellitus) and metabolic disorders (such as type 2 diabetes mellitus). However, it is not clear if the impairment of the intestinal barrier present in these disorders is causally associated with their onset and development or is a consequence of pathophysiological events and factors like prolonged hyperglycemia (as in the case of diabetes).

Systematic studies in both humans and animal models have shown some evidence that increased intestinal permeability can be a significant factor in the development of type 1 (T1D) and type 2 (T2D) diabetes mellitus. In T1D (as with other autoimmune disorders), it is assumed that the loss of intestinal barrier function precedes the onset of diabetes. Pronounced changes in the composition and function of the microbiota can alter intestinal barrier function [1,2]. Increased translocation of microbial and food antigens from the intestinal lumen into the systemic circulation is thought to overstimulate the immune system, and (in predisposed individuals) activate islet-reactive T cells; these cells then promote autoimmune destruction of insulin-secreting beta-cells in the pancreas, which can lead to T1D [3–10].

In T2D, intestinal dysbiosis, together with increased intestinal permeability, facilitates the translocation of highly immunogenic microbial components (e.g., lipopolysaccharide) into the intestinal lamina propria and then into the systemic circulation; these substances can induce chronic low-grade systemic inflammation, leading to metabolic disorders such as insulin resistance and T2D [11–15].

*Vice versa*, it is also documented that long-standing hyperglycemia (in both types of diabetes mellitus) can generate histomorphological and functional changes of the small intestinal mucosa (i.e., a proliferation of the villi and crypts, ultrastructural changes in microvilli, transcriptional reprogramming of intestinal epithelial cells, and changes in the integrity of tight and adherence junctions) leading to intestinal barrier dysfunction and increased intestinal permeability [16–19].

The goal of this study was to verify the hypothesis if the impairment of the intestinal barrier observed in patients with T1D and T2D could be explained as a consequence of higher glycated hemoglobin (HbA1c) and/or duration of diabetes mellitus.

In our previous study [20], the intestinal barrier function was assessed by examining the following serological markers: (a) marker of enterocyte damage, i.e., intestinal fatty acid-binding protein (I-FABP), (b) marker of epithelial apoptosis, i.e., cytokeratin 18 caspase-cleaved fragment (cCK-18), and (c) marker of activation of systemic innate immunity (related

to lipopolysaccharide translocation), i.e., soluble CD14 (sCD14). We confirmed impairment of the intestinal barrier in patients with both types of diabetes mellitus by finding significantly elevated serum levels of I-FABP and cCK-18 compared to healthy controls. A trend toward higher sCD14 levels (but without statistical significance) was also seen in both types of diabetes mellitus.

Based on our previous findings, the present study tried to find a relationship between the above-mentioned serological markers of intestinal barrier dysfunction and the duration of diabetes mellitus and HbA1c (in both types of diabetes mellitus).

## 2. Materials and Methods

The correlation included patients with type 1 diabetes mellitus with ongoing insulitis (T1D/INS), patients with type 1 diabetes mellitus with fading insulitis (T1D), and patients with type 2 diabetes mellitus (T2D). Characteristics of cohorts of diabetic patients, including the levels of serological markers of intestinal barrier function are shown in Table 1.

Table 1. **Characteristic of patients with diabetes mellitus and healthy controls.**

| Cohorts | Controls | T1D/INS | T1D | T2D |
|---|---|---|---|---|
| Number of subjects | 41 | 20 | 20 | 30 |
| Age: mean/range | 39.4/18-81 | 53.5/20–87 | 47.1/ 20–78 | 66.3/41–84 |
| Gender: ratio F/M | 19/22 | 7/13 | 9/11 | 12/18 |
| Duration of diabetes: mean/range (years) | – | 15.03/0.5–41 | 18.65/0.5–41 | 14.21/0.5–24 |
| HbA1c: mean/range (%) | 3.346/2.8–3.8 | 9.7/5.4–13.2 | 8.59/4.3–16.7 | 8.85/3.7–14.7 |
| cCK-18: mean ±SD (pM) | 137.2 ± 86.3 | 204.1 ± 109.5 | 200.5 ± 84.3 | 355.4 ± 287.5 |
| I-FABP: mean ± SD (ng/ml) | 0.8 ± 0.7 | 1.4 ± 0.7 | 1.5 ± 0.8 | 1.1 ± 0.4 |
| sCD14: mean ± SD (µg/ml) | 1.8 ± 1.2 | 2.6 ± 2.4 | 3.1 ± 3.0 | 2.1 ± 1.6 |
| GAD antibodies | 2.7 ± 2.1 | 120.8 ± 23.4 | 1.8 ± 0.5 | 1.2 ± 0.5 |
| IA−2 antibodies | 2.0 ± 0.4 | 40.3 ± 20.1 | 0.7 ± 0.4 | 0.5 ± 0.5 |

Controls, healthy controls; T1D/INS, type 1 diabetes mellitus with ongoing insulitis; T1D, type 1 diabetes mellitus with fading insulitis; T2D, type 2 diabetes mellitus; HbA1c, glycated hemoglobin (%); cCK-18, cytokeratin 18 caspase-cleaved fragment (pM); I-FABP, intestinal fatty acid-binding protein (ng/ml); sCD14, soluble CD14 (µg/ml). The data originates from our previous study [20].

Ongoing insulitis was characterized by the presence of circulating autoantibodies against beta-cell antigens (i.e., antibodies to tyrosine phosphatase-like insulinoma antigen 2 and antibodies to glutamic acid decarboxylase). Exclusion criteria included altered hepatic and renal

function, gastrointestinal disease, cardiovascular disease, severe dyslipidemia, thyropathy, cancer history, recent infection, smoking, and alcoholism.

Pearson correlation coefficients were used for statistical analyses. The null hypothesis stated that there was no linear dependence between the pairs of correlated parameters; the alternative hypothesis stated that pairs of correlated parameters had a linear dependence. Values with $p < 0.05$ were considered statistically significant. The results of the correlations are shown in the correlation matrix. The statistics were calculated using STATISTICA 12 software and using GraphPad Prism$^{TM}$ 5. Capillarys 2 Flex Piercing (C2FP) capillary electrophoresis was used for the measuring of HbA1c [21]. All the tests were performed in the same sample from individual patients. The tests were performed in at least three independent experiments and the presented value represents the mean of test results in a single sample.

## 3.    Results

Firstly, we performed correlation analysis between I-FABP (indicating enterocyte damage), cCK-18 (apoptotic marker) and sCD14 (indicator of activation of systemic innate immune response to Gram-negative bacteria or their components i.e. lipopolysaccharide, suggesting their mucosal translocation from lumen of gastrointestinal tract to immunocompetent cells in submucosa) and HbA1c in healthy individuals. No statistically significant correlations among the mentioned markers were found in healthy individuals, as documented in Table 2.

Table 2. **Correlation matrix of healthy individuals dataset (n=41).**

| Variables | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|
| HbA1c (%) | r=−0.1466 p=0.3603 | r=−0.1436 p=0.3704 | r=−0.02352 p=0.8840 |
| cCK-18 (pM) | | r=−0.06430 p=0.6896 | r=−0.04432 p=0.7832 |
| I-FABP (ng/ml) | | | r=0.2998 p=0.0569 |

r = correlation coefficient, p = statistical significance of the test (no statistically significant correlation, p>0.05), n = number of healthy controls

3.1.   Results in type 1 diabetes mellitus with ongoing insulitis (T1D/INS)

3.1.1. In T1D/INS (defined by the seropositivity of GAD-antibodies or/and IA−2-antibodies), no statistically significant relationship were found between the tested serological markers of intestinal barrier impairment and apoptosis (I-FABP, cCK-18), and sCD14 and markers of long-standing hyperglycemia (the duration of diabetes and HbA1c). The results of the correlations are shown in the correlation matrix (Table 3).

Table 3. **Correlation matrix of T1D/INS dataset (n=20).**

| Variables | HbA1c (%) | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|---|
| Duration of T1D/INS (years) | r=−0.3449 p=0.107 | r=0.0661 p=0.7646 | r=−0.2758 p=0.214 | r=−0.3024 p=0.2734 |
| HbA1c (%) | | r=0.1431 p=0.5149 | r=0.0258 p=0.9091 | r=−0.3483 p=0.2033 |
| cCK-18 (pM) | | | r=0.3332 p=0.130 | r=0.2124 p=0.447 |
| I-FABP (ng/ml) | | | | r=0.1315 p=0.654 |

r = correlation coefficient, p = statistical significance of the test (no statistically significant correlation, p>0.05)

Nevertheless, the distribution of T1D/INS patients into subgroups (1/ positivity only for GAD-antibodies; 2/ positivity for IA−2-antibodies; 3/ positivity of both GAD- and IA−2-antibodies) revealed statistically significant negative correlation between the levels of GAD-antibodies in patients seropositive both for GAD- and IA−2-antibodies (6 out of 20 T2D/INS) and cCK-18 (p=0.0146) and I-FABP (p=0.0040). However, statistically significant positive correlation was found between GAD-antibodies and sCD14 (p=0.0390) in patients seropositive both for GAD- and IA−2- antibodies (Table 4).

Table 4. **Correlation analysis between measured parameters in subgroups of patients with T1D/INS divided according to seropositivity for individual autoantibodies.**

*Statistically significant negative correlation between GAD-antibodies and I-FABP, and cCK-18.*
*Statistically significant positive correlation between GAD-antibodies and sCD14.*

| Measured parameters | GAD Ab[1] | IA−2 Ab[2] | GAD Ab[3] | IA−2 Ab[3] |
|---|---|---|---|---|
| Duration of T1D/INS (years) | r=−0.2904 | r=−0.4678 | r=−0.1936 | r=−0.6119 |
| | p=0.3357 | p=0.2898 | p=0.7132 | p=0.1967 |
| HbA1c (%) | r=0.5036 | r=−0.1071 | r=0.3788 | r=0.1400 |
| | p=0.0794 | p=0.8397 | p=0.4590 | p=0.7914 |
| cCK-18 (pM) | r=0.06214 | r=−0.01693 | r=−0.8997 | r=−0.02140 |
| | p=0.8402 | p=0.9713 | p=0.0146* | p=0.9679 |
| I-FABP (ng/ml) | r=−0.1949 | r=0.2584 | r=−0.9479 | r=0.2709 |
| | p=0.5235 | p=0.5758 | p=0.0040** | p=0.6036 |
| sCD14 (µg/ml) | r=−0.3369 | r=−0.4753 | r=0.8342 | r=−0.5058 |
| | p=0.2603 | p=0.2810 | p=0.0390* | p=0.3060 |

[1] Patients positive only for GAD-antibodies (Ab) (number of patients, n=13 (out of 20))
[2] Patients positive for IA−2-antibodies (Ab) (n=7 (out of 20)); 6 of those patients were also positive for GAD- antibodies
[3] Patients positive both for GAD and IA−2-antibodies (Ab) (n=6 (out of 20))
r = correlation coefficient, p = statistical significance of the test
statistically significant correlation (*, $p \leq 0.05$; **, $p \leq 0.01$)
no statistically significant correlation ($p > 0.05$)

3.1.2. In T1D patients, the statistically significant positive relationship was between cCK-18 and HbA1c (p = 0.0275). Interestingly, statistically significant negative correlation between I-FABP and sCD14 (p = 0.036) was found in these patients. The results of the correlations are shown in the correlation matrix (Table 5).

Table 5. **Correlation matrix of T1D dataset (n=20).**
*Statistically significant positive correlation between HbA1c and cCK-18.*

| Variables | HbA1c (%) | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|---|
| Duration of T1D (years) | r=−0.4373 | r=−0.3547 | r=−0.2961 | r=0.2099 |
| | p=0.061 | p=0.1362 | p=0.2183 | p=0.3884 |
| HbA1c (%) | | r=0.5047 | r=0.2399 | r=−0.041 |
| | | p=0.0275* | p=0.3226 | p=0.8676 |
| cCK-18 (pM) | | | r=0.1778 | r=0.0194 |
| | | | p=0.467 | p=0.937 |
| I-FABP (ng/ml) | | | | r=−0.4842 |
| | | | | p=0.036* |

r = correlation coefficient, p = statistical significance of the test
statistically significant correlation (*, p≤0.05)
no statistically significant correlation (p>0.05)

3.1.3. The correlation analysis of a cumulative cohort of patients with T1D/INS and patients with T1D showed statistically positive correlation between HbA1c and cCK-18 (p=0.0311), and statistically significant negative correlation between I-FABP and sCD14 (p=0.0066).

Table 6. **Correlation matrix of a cumulative group T1D/INS and T1D patients dataset (n=40).**

*Statistically significant positive correlation between HbA1c and cCK-18.*

| Variables | HbA1c (%) | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|---|
| Duration of T1D (years) | r=−0.4207 | r=−0.09489 | r=−0.1794 | r=0.06102 |
| | p=0.0069** | p=0.5603 | p=0.2680 | p=0.7084 |
| HbA1c (%) | | r=0.3414 | r=0.09219 | r=0.007526 |
| | | p=0.0311* | p=0.5716 | p=0.9632 |
| cCK-18 (pM) | | | r=0.1491 | r=−0.01912 |
| | | | p=0.3586 | p=0.9068 |
| I-FABP (ng/ml) | | | | r=−0.4228 |
| | | | | p=0.0066** |

r = correlation coefficient, p = statistical significance of the test
statistically significant correlation (*, $p \leq 0.05$; **, $p \leq 0.01$)
no statistically significant correlation ($p > 0.05$)

### 3.2. Results in type 2 diabetes mellitus (T2D)

In T2D, a statistically significant negative relationship was found between the duration of diabetes and cCK-18 (p = 0.021), as shown in the correlation matrix (Table 7).

Table 7. **Correlation matrix of T2D dataset (n=30).**

*Statistically significant negative correlation between the duration of T2D and cCK-18.*

| Variables | HbA1c (%) | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|---|
| Duration of T2D (years) | r=−0.5552 | r=−0.4267 | r=0.2686 | r=−0.1571 |
| | p=0.002** | p=0.021* | p=0.167 | p=0.4248 |
| HbA1c (%) | | r=0.198 | r=−0.0259 | r=0.0798 |
| | | p=0.3125 | p=0.8978 | p=0.6866 |
| cCK-18 (pM) | | | r=−0.1425 | r=−0.0358 |
| | | | p=0.470 | p=0.857 |
| I-FABP (ng/ml) | | | | r=−0.1426 |
| | | | | p=0.478 |

r = correlation coefficient, p = statistical significance of the test
statistically significant correlation (*, p≤0.05; **, p≤0.01)
no statistical significant correlation (p>0.05)

The relationship between apoptotic marker (cCK-18), marker of enterocyte damage (I-FABP), sCD14 (indicator of activation of systemic innate immune response to Gram-negative bacteria) and duration of diabetes and HbA1c (markers of long-standing hyperglycemia) was analyzed in subgroups of T2D patients cured only by metformin (13 out of 30) (Table 8). Here, we found statistically significant positive correlation between the duration of diabetes and I-FABP (p= 0.0229).

Table 8. **Correlation matrix of T2D cured only by metformin (n=13 (out of 30)).**

*Statistically significant positive correlation between the duration of the T2D and I-FABP*

| Variables | HbA1c (%) | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|---|
| Duration of T2D (years) | r=−0.6407 | r=−0.4188 | r=0.6230 | r=−0.1691 |
| | p=0.0183* | p=0.1544 | p=0.0229* | p=0.5807 |
| HbA1c (%) | | r=0.1951 | r=−0.4557 | r=0.1072 |
| | | p=0.5230 | p=0.1176 | p=0.7274 |
| cCK-18 (pM) | | | r=−0. 3946 | r=−0.4805 |
| | | | p=0.1821 | p=0.0965 |
| I-FABP (ng/ml) | | | | r=−0.1050 |
| | | | | p=0.7327 |

r = correlation coefficient, p = statistical significance of the test
statistically significant correlation (*, p≤0.05)
no statistically significant correlation (p>0.05)

The relationship between the mentioned markers was analyzed in subgroups of T2D patients cured by metformin and statins (Atorvastatin, 20 mg/day or Fluvastatin, 80 mg/day; (totally 13 out of 30). Surprisingly, we observed significant statistically positive correlation between the duration of T2D and sCD14 (p=0.0099) (Table 9).

Table 9. **Correlation matrix of T2D cured by metformin & statins (n=13 (out of 30)).**

*Statistically significant positive correlation between the duration of T2D and sCD14.*

| Variables | HbA1c (%) | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|---|
| Duration of T2D (years) | r=−0.1969 | r=−0.4309 | r=0.05741 | r=0.6844 |
| | p=0.5190 | p=0.1416 | p=0.8522 | p=0.0099** |
| HbA1c (%) | | r=0.4163 | r=−0.3040 | r=0.1655 |
| | | p=0.1570 | p=0.3126 | p=0.5889 |
| cCK-18 (pM) | | | r=−0.1915 | r=0.1174 |
| | | | p=0.5308 | p=0.7025 |
| I-FABP (ng/ml) | | | | r=−0.03372 |
| | | | | p=0.9129 |

r = correlation coefficient, p = statistical significance of the test.
statistically significant correlation (**, p≤0.01)
no statistically significant correlation (p>0.05)

The relationship among I-FABP, cCK-18 and sCD14, and markers of long-standing hyperglycemia (duration of diabetes and HbA1c) was also assessed by the correlation analysis in a cohort of all diabetic patients, which included patients with T1D/INS, T1D and T2D (a cumulative cohort of patients with diabetes mellitus, DM). The results of the correlation analysis are presented in the correlation matrix (Table 10). In this cumulative group of diabetic patients we surprisingly demonstrated statistically negative correlation between the duration of diabetes mellitus and cCK-18 (p=0.0313).

Table 10. **Correlation matrix of DM (T1D, T1D/INS, T2D) patients dataset (n=50).**

*Statistically significant negative correlation between the duration of DM and cCK-18.*

| Variables | HbA1c (%) | cCK-18 (pM) | I-FABP (ng/ml) | sCD14 (µg/ml) |
|---|---|---|---|---|
| Duration of DM (years) | r=−0.4842 p=0.0004*** | r=−0.3050 p=0.0313* | r=0.08054 p=0.5782 | r=−0.07097 p=0.6263 |
| HbA1c (%) | | r=0.1810 p=0.2084 | r=−0.1543 p=0.2846 | r=0.1464 p=0.3102 |
| cCK-18 (pM) | | | r=−0.1071 p=0.4590 | r=0.002219 p=0.9878 |
| I-FABP (ng/ml) | | | | r=−0.1787 p=0.2142 |

DM, diabetes mellitus, r = correlation coefficient, p = statistical significance of the test
statistically significant correlation (*, p≤0.05; ***, p≤0.001)
no statistically significant correlation, p>0.05

## 4. Discussion

Long-standing hyperglycemia plays a crucial role in the development of diabetic complications based on its detrimental impact on endothelial cells, podocytes, proximal tubular cells, cardiomyocytes, and neuronal cells [22–24]. In the last decade, scientific interest has also focused on the relationship between hyperglycemia and the function of intestinal barrier [19,25–30].

The intestinal barrier is an extremely complex and dynamic system [31,32] that can be investigated using a variety of experimental methods. However, any kind of testing of intestinal barrier dysfunction usually reflects only certain aspects of the complex pathophysiological mechanisms involved. In a clinical setting, evaluating intestinal barrier function is a difficult task. Clinical studies testing intestinal barrier function often use (1) detection of microbial products in the circulation, (2) serological surrogate markers of enterocyte damage and

apoptosis, or (3) measurement of intestinal permeability via perorally administered sugars that require paracellular and transcellular transport across the intestinal epithelium [33].

In our clinical study, we compared serological markers of intestinal barrier impairment (I-FABP, cCK-18, and sCD14) with the markers of long-standing hyperglycemia (the duration of diabetes and HbA1c) in patients with T1D and T2D. We demonstrated a statistically significant positive correlation between cCK-18 and HbA1c in patients with T1D but only those with fading insulitis. Nevertheless, in the subgroup of T1D/INS patients seropositive both for GAD- and IA−2-antibodies we found statistically significant negative correlation between GAD-antibodies and I-FABP, and cCK-18. A statistically significant positive correlation between GAD-antibodies and sCD14 was revealed in this subgroup of T1D/INS patients. The results of the correlation analysis in a cohort of all patients suffering from T1D/INS suggested that neither GAD- nor IA−2-antibodies have influenced the levels of apoptotic marker cCK-18, marker of enterocyte damage I-FABP, and the indicator of activation of innate immune response to Gram-negative bacteria sCD14. Nevertheless, the correlation analysis in cumulative group of T1D and T1D/INS patients suggests association between an excessive glycation of hemoglobin during long-term hyperglycemia and an increasing apoptotic status in organism. Moreover, correlation analysis of data in the subgroup of T1D/INS characterized by seropositivity for GAD- and IA−2-antibodies revealed a statistically significant negative correlation between the levels of GAD-antibodies and the levels of I-FABP, and cCK-18. The pathogenic role and clinical significance of GAD-antibodies are not exactly known; obviously, they might be involved in a destructive process of pancreatic islets. With certainty, it could be said that the occurrence of autoantibodies depends on the presence of immunogenic autoantigen, spatially accessible to the immune system. Indeed, the presence of a significantly positive correlation between seropositivity in GAD-antibodies and sCD14 suggests systemic activation of the innate immune response to Gram-negative bacteria or their components i.e. lipopolysaccharide. The assumption for the activation of the innate immune is mucosal translocation of Gram-negative bacteria or their molecules from lumen of gastrointestinal tract to immunocompetent cells in submucosa.

In our study, we showed a negative statistically significant correlation between cCK-18 levels and the duration of T2D. A simple mechanistic explanation seems unlikely since the rate of epithelial cell apoptosis (tested using cCK-18) would not systematically decrease with longer durations of T2D. One possible explanation is that antidiabetic medication (metformin) may have a beneficial effect on the status of the intestinal barrier [34]. In fact, 26 of our 30 patients with T2D were treated with metformin. Nevertheless, the influence of treatment on the levels

of I-FABP, cCK-18, and sCD14 and the duration of diabetes and HbA1c was statistically analyzed in the subgroups of T2D patients characterized by treatment only with metformin or treated with metformin plus statins. Interestingly, we found a statistically significant positive correlation between the duration of the T2D and I-FABP treated with metformin. Indeed, possible intestinal damage may occur with the extended duration of T2D. Although, several studies documented that metformin and other antidiabetic or hypolipidemic drugs may improve the status of the intestinal barrier in patients with T2D [34-36]. In the subgroup of T2D patients cured by metformin plus statins, we found a significantly positive correlation between the duration of T2D and sCD14. Thus, this finding likewise suggests possible impairment of mucosal barrier enabling penetration of bacterial components into the mucous layer and stimulation of mucosal immune system. In T2D patients, immunological abnormalities may occur and in this respect spontaneously diabetic Torii rats may contribute to elucidation of T2D pathophysiology [37].

Although, the serum levels of sCD14 were not statistically significantly elevated in the group of T2D patients, we found a relation between the duration of T2D and elevation of sCD14 in the subgroup of T2D patients treated by a combination of metformin and statins. Large standard deviation of sCD14 values in T2D patients may be the reason for the absence of statistical difference between the patient's group and healthy controls [20]. Patients treated with atorvastatin usually suffer from advanced atherosclerosis, which may lead to subclinical gut ischemia and increased gut mucosa permeability contributing to translocation of Gram-negative bacteria and/or their components, i.e. lipopolysaccharide, in T2D patients [38]. Vascular abnormalities are a frequent complication in diabetes mellitus; vascular diseases are principal causes of co-morbidity and mortality in diabetic patients [39].

To complete our study we performed the correlation analysis between cCK-18, I-FABP, sCD14 and duration of diabetes and HbA1C in a cumulative group of all diabetic patients (including T1D/INS, T1D, and T2D patients). We showed a statistically significant negative correlation between the duration of the disease and cCK-18 in the group of all diabetic patients. These results may suggest that the shorter is the duration of diabetes, the more intense is glycation of hemoglobin and the higher levels of apoptotic marker cCK-18 are then reflected in the longer duration of the disease. These facts could be explained by successful therapy of these patients, irrespectively the type of diabetes mellitus. Correlation analysis of the whole (cumulative) group of diabetic patients (including T1D/INS, T1D, and T2D) suggests that the shorter is the duration of diabetes, the more intense is the glycation of hemoglobin and elevation of apoptotic marker cCK-18.

Currently, serum I-FABP levels are considered to be convenient non-invasive clinical biomarkers for evaluating intestinal barrier dysfunction [29,33,40–42]. To our best knowledge, there are no other studies evaluating I-FABP in patients with T1D. In T2D, studies evaluating the levels of I-FABP, in the context of long-standing hyperglycemia, have shown conflicting results [27–29]. Lalande et al., in a study that included 154 nondiabetic men and 67 men diagnosed with T2D, found significantly elevated I-FABP plasma levels in patients with T2D, particularly in those with inadequate glycemic control (where plasma I-FABP levels correlated with fasting glucose ($r = 0.25$; $p < 0.05$)); however, no statistical analysis of correlations between plasma I-FABP levels and HbA1c was performed. Interestingly, insulin resistance (assessed using HOMA-IR) had no significant impact on plasma I-FABP levels in men without diabetes [27]. A study by Verdam et al. found, in 40 severely obese patients with chronic hyperglycemia, a correlation between plasma I-FABP levels and HbA1c levels ($r(s) = 0.33$, $P = 0.005$) [28]. Wang et al. found that serum I-FABP level was positively associated with the duration of hyperglycemia and glycemic variability ($r = 0.362$, $P < 0.001$) in 122 hospitalized diabetics. Furthermore, serum I-FABP levels were higher in patients with diabetic retinopathy than in those without diabetic retinopathy, which led the authors to conclude that dysfunction of the intestinal barrier increases with the progression of diabetes [29]. Nevertheless, the use of HbA1c as a marker of long-term diabetes control has its clinical limitation [43].

A recent publication demonstrated the relationship between hyperglycemia and the function of the intestinal barrier [19]. In an *in vitro* model of cultured intestinal epithelial (Caco-2) cells from hyperglycemic mice, it was demonstrated that hyperglycemia affects intestinal barrier function via an alteration of cell-cell junctions and by causing global reprogramming of epithelial transcriptome. The reprogramming involves expression of genes associated with intracellular glucose metabolic pathways and the expression of genes associated with the maintenance of epithelial barrier function. Moreover, the authors demonstrated, in 27 healthy humans, the relationship between glycemia and intestinal barrier function based on a positive correlation between HbA1c serum levels and the serum levels of pattern recognition ligands (e.g., ligands for toll-like receptors 2, 3, 4, 5, 7, and 9, and NOD receptors 1 and 2), which were used as markers of microbial products in the circulation; however, no diabetic patients were studied. Both the animal and human findings lead the authors to hypothesize that hyperglycemia *per se* drives intestinal barrier dysfunction and increases translocation of microbial products from the intestinal lumen into the systemic circulation [19].

In contrast, an opposite conclusion came from a recent study performed on several mouse models of type 1 diabetes mellitus and mice rendered hyperglycemic without inflammation

[26]. The study confirmed changes in intestinal epithelial cells, impairment of intestinal barrier function, and dysbiosis. Anti-inflammatory treatment restored intestinal mucosa and immune cell function, restored protective commensal microbiota, and decreased the incidence of diabetes. These findings lead the authors to hypothesize that intestinal barrier dysfunction and dysbiosis were primarily linked to inflammation rather than hyperglycemia in type 1 diabetes mellitus [26].

Besides, recent extensive research is revealing the key role of the microbiota in regulating the intestinal barrier [12,44,45]. A large number of environmental stimuli can influence the composition and function of the microbiota, e.g. dietary factors, bile acids, emulsifiers and other intraluminal components, drugs (such as antibiotics, nonsteroidal anti-inflammatory drugs, aspirin, proton pumps inhibitors, steroids, and estrogens), allergens, inflammation of any origin, the gastrointestinal blood supply, and stress [5,12,13,31–33,46]. Changes in the composition of the microbiota and subsequent changes in intestinal barrier function are involved in the etiopathogenesis of immune-mediated and metabolic diseases, including T1D and T2D [5–7,9,12,14,15].

Therefore, our results may also reflect interactions between a large set of variables that can affect the function of the intestinal barrier in diabetic patients, i.e. the actual condition of the intestinal barrier may not be explained mechanistically as a simple consequence of prolonged hyperglycemia. Despite the relatively small sample sizes for all our groups of diabetes patients, our results nonetheless cannot support the hypothesis that long-standing hyperglycemia *per se* habitually weakens the status of the intestinal barrier in patients with diabetes mellitus regardless of the type. Further research is needed to elucidate the mechanisms driving intestinal barrier dysfunction in diabetes patients and find potential targets for therapeutic interventions. Besides the traditional glucose-lowering therapeutic approaches, strengthening the function of the intestinal barrier and modulating the microbiota may represent a new goal in the treatment for both types of diabetes mellitus [47,48].

## 5. Conclusion

The relationship between impairment of the intestinal barrier and long-standing hyperglycemia is still enigmatic. Based on the results of our study, we hypothesize that the actual condition of the intestinal barrier in patients with either type of diabetes mellitus is influenced more by interactions between host genetic factors, gut microbiota, and environmental factors than by long-standing hyperglycemia.

**Conflicts of Interest**

The authors declare that there is no conflict of interest regarding the publication of this article.

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