KenKyu

Diabetes, Obesity & Metabolic Disorders Open Access 4:10-15 (2018)

Research

Composition of intestinal microbiome and barrier function in Type 2 diabetic patients and nondiabetic controls

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Received February 28, 2018; **Accepted** March 24, 2018; **Published** March 26,2018

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Abstract

Background: Relationships between composition of intestinal microbiome (IMB) and function of intestinal barrier in Type 2 diabetic patients without cardiovascular complications (CVCs) have hardly been investigated. We examined the composition of IMB and serum zonulin concentration as a parameter of intestinal permeability in these patients and a control group.

Methods: Demographic characteristics, nutritional habits, routine lab results, serum zonulin concentration and composition of IMB assessed by 16S rRNA sequencing of faecal samples were collected from 32 diabetic patients and 19 nondiabetic controls. Bacteria abundance of both groups was compared at different classification levels. Abundance of bacteria known (a) to produce butyrate and (b) to be associated with onset of CVCs was investigated. Relationship between bacterial type abundance and zonulin concentration was assessed by logistic regression.

Results: Compared with controls, diabetic patients showed a higher *Firmicutes/Bacteroidetes* Ratio (3.87 ±1.06 vs 4.89 ± 1.12; p=0.024) and a higher prevalence of *Synergistetes* (10.5 % vs 37.5 %). In tendency there was a higher prevalence of bacteria associated with the occurrence of CVCs in the diabetes group. Zonulin concentration was significantly higher in the diabetic group compared to the controls (66.2 ± 11.6 vs 42.2 ± 9.5 ng/ml; p = 0.001). Regression analysis failed to reveal any significant relationship between zonulin concentration and abundance of bacteria.

Conclusion: Compared with controls, diabetic patients without manifest CVCs exhibit only moderate differences regarding the composition of IMB. The tendentially higher prevalence of bacteria associated with occurrence of CVCs and the elevated permeability of intestinal barrier may, however, be indicative of a raised risk of CVCs among this group of patients. The failure to detect a relationship between zonulin concentration and the composition of IMB indicates that the function of the intestinal barrier in our group of diabetic patients is primarily influenced by other factors.

Key words: Type 2 –Diabetes; intestinal microbiome; intestinal permeability; zonulin

Introduction

The microbiome of the gastrointestinal tract predominantly consists of millions of bacteria that fulfil a variety of functions: defence against pathogenic bacteria and toxins, modulation of immunological and inflammatory response, digestion of complex carbohydrates, generation of hormones and neurotransmitters, and maintenance of integrity of the intestinal barrier. Uptake of nutrients, metabolic processes, energy household, and immunological and inflammatory processes may thus be influenced by resorptive processes or by the gut-brain axis (1-4). Changes in the composition of intestinal microbiome (IMB) are seen not only as potential causes of intestinal diseases, but also as triggers of a multitude of clinical disorders such as obesity, metabolic disorders, tumour diseases, or neurological diseases (5-8). From metabolic aspect, the relationships between the IMB and diabetes mellitus are of particular interest, since morbidity of diabetes and incidence of related cardiovascular complications (CVCs) are constantly rising. In recent years, a number of changes in the composition of IMB in people suffering from type 2 diabetes have been described; the results of these studies, however, are not uniform (9-14). Furthermore, some authors have also reported a raised permeability of the intestinal barrier in this group of patients (15,16). This disorder facilitates the transport of bacteria, viruses, food allergens and endotoxin (Lipopolysaccharides (LPS)) from intestinal lumen into systemic circulation, which may subsequently result e.g. in inflammatory reactions, insulin resistance, and the development of CVCs (17). The relationships between changes in the composition of IMB and the impairment of the function of the intestinal barrier have not yet been elucidated in patients with type 2 diabetes. In this study we investigated the relationship between composition of IMB and serum zonulin concentration as a parameter of intestinal permeability in a well-characterized group of patients with a long history of type 2 diabetes without manifest CVCs as well as in an apparently healthy control group of subjects of a similar age.

Methods

The study was performed in a population of 32 patients with type 2 diabetes attending the diabetes outpatient clinic of the St. Josefskrankenhaus Heidelberg GmbH and who fulfilled the following inclusion criteria: Type 2 diabetes with a known history of dia¬betes of > 2 years, between 50 and 80 years of age, stable metabolic control, i.e. no change in medication in the previous three months. Exclusion criteria were: Other types of diabetes, acute metabolic imbalance, acute infection, acute or chronic intestinal disorder, antibiotic medication during the previous three months, systemic therapy with cytostatic agents, im¬munosuppressive drugs, cortisone-containing agents or antirheumatic substances, serious diseases such as tumour diseases, liver cirrhosis, cardiac insufficiency (> NYHA II), renal insuf¬ficiency (eGFR< 30 mL/min), and previous surgery within the gastrointestinal tract. Nineteen nondiabetic and apparently otherwise healthy patients served as controls.

The data of all patients were reviewed to record their demographics and their current treatment and complication status. The following laboratory parameters were measured: blood count, HbA1c, glucose, lipids, creatinine, hepatic function parameters, hsCRP, ferritin, insulin and gliadin antibodies (IgA, IgG).The eGFR parameter was calculated according to the CKD-EPI formula. The serum concentration of zonulin was measured using the IKD Zonulin ELISA kit (Immundiagnostik AG, Bensheim, Germany). All subjects were studied in fasting state. The nutritional habits were recorded using a patient questionnaire. Based on examples of different types of nutrition, the subjects were asked (a) how often per week they consume carbohydrate- resp. fat- and protein-containing food; (b) whether and how often they take pre- or probiotic supplements, and (c) whether they consume sweeteners. The details collected in this manner were then taken to calculate the frequency of the uptake of gliadin-containing food products and of fibre. Patients were asked to submit fresh stool samples which were immediately stored at -70°C. The analysis of the IMB was performed by CeMet, Tübingen, Germany. Means, standard deviations (SDs) and relative frequencies were used to summarize demographic characteristics, routine lab results and bacterial type abundances in type 2 diabetes patients and nondiabetic controls. Mean differences between the two groups of patients were assessed by t-tests, Chi square test or Fisher´s exact test were used to compare proportions. In addition, univariate logistic regression was used to identify bacterial types potentially associated with the prevalence of CVCs and to calculate odds ratios with the corresponding 95% confidence intervals. The present study had exploratory character and probability values were not adjusted for multiplicity. Statistical analyses were conducted using SAS version 9.3 (SAS Institute Inc, Cary, NC).

The study was approved by the local ethics committee and written informed consent was obtained from all subjects prior to participation.

Results

The most important demographic findings and laboratory results obtained from the patients with diabetes and controls are given in Table 1. With a mean duration of 12.9 years, the diabetes patients showed already a relatively long duration of disease. There were no significant differences between diabetic patients and controls regarding gender ratio and age. As expected, body mass index (BMI), HbA1c and triglyceride levels were higher in patients with diabetes. Most diabetic patients were taking metformin (75%), and the frequency of treatment with insulin lies at 43.8%, with DPP4 inhibitors/incretin mimetica at 37.5% and/or sulfonylureas at 12.5%. The prevalence of hypertension and dyslipidemia was higher in patients with diabetes than in controls (hypertension 81.3 vs 42.2%; dyslipidemia 43.8% vs 15.8%).

The exploratory survey of the patients' nutritional habits revealed that none of the study subjects was on vegetarian or vegan diet. No patients had been diagnosed as suffering from gliadin intolerance. Tab. 2 shows the intake of carbohydrates and protein-/fat-containing food as well as other substances in controls and diabetic patients. No significant differences were found between the two groups regarding food intake of carbohydrates, fat and protein. The frequency of gliadin intake or of fibre-rich food was somewhat higher in diabetic patients; however, the difference was only of borderline significance regarding fibre intake. The proportion of patients consuming sweetener or dietary supplements was not significantly different between controls and diabetic patients.

The composition of the microbiome at the phylum level is given in Tab. 3. Compared with controls, diabetic patients displayed a higher Firmicutes/Bacteroides ratio. However, no further significant differences could be detected between the two groups at this level (Tab. 2). The only noticeable finding was the detection of bacteria of the Synergistetes phylum in 12 (=37.5%) of diabetic patients, albeit in low abundance. In controls, this phylum could be found only in 2 persons (10.5%; p=0.12). Similar findings were obtained from order level downwards (not shown).

Characteristic	Controls (n=19)	Diabetic patients (n=32)	p-value*
Female proportion (%)	52.6	59.4	0.64
Age (Years)	64.8 ±9.5	66.8 ±6.3	0.37
Diabetes duration (Years)		12.9 ±5.7	
BMI (kg/m^2)	28.8 ± 4.7	32.1 ±5.7	**0.04**
hsCRP (mg/L)	2.26 ±4.7	3.0 ±3.04	0.34
HbA1c (%)	5.6 ±1.8	7.34 ±0.97	**0.0001**
HbA1c (Mmol)	37.71 ±0.25	56.6 ±10.7	**0.0001**
Triglycerides (mg/dL)	115.5 ± 65.6	172.7 ±71.3	**0.007**
Cholesterol (mg/dL)	206.3 ±67.2	189.9 ±40.2	0.14
HDL cholesterol (mg/dL)	63.2 ±32.4	51.6 ±10.8	**0.001**
LDL cholesterol (mg/dL)	129.3 ±13.2	119.1 ±35.9	0.29
eGFR (ml/min)	82.7 ±25.9	82.6 ±18.1	0.98
Haemoglobin (g/dL)	14.6 ±13.8	14.2 ±1.2	0.28

* bold denotes $p < 0.05$

Table 1: Demographic and laboratory characteristics of the diabetic and the control group

Nevertheless, we investigated the abundance of some selected bacterial species that show special functions to detect trends as the case may be. First, we assessed the abundance of some known butyrate-forming bacteria that are essential for the metabolism of the intestinal epithelium. As shown in Tab. 4, no significant differences could be detected between controls and diabetic patients. The abundance of four bacterium species is somewhat higher, three species somewhat lower in diabetic patients.

Furthermore, we investigated the abundance of bacteria that are described in the literature as being associated with arteriosclerotic complications or that produce trimethylenamine (TMA), which is known to have atherogenic effects after oxidation in the liver into TMA-O (18). As shown in Tab. 5, the average abundance was not significantly different between the two groups, as expected. However, in seven of nine assessed bacteria, the proportion was somewhat higher in diabetic patients than in controls.

Characteristic	Controls (n=19)	Diabetic patients (n=32)	p-value
Meals with carbohydrates (n meal/week)	15.1 ±4.4	16.6 ±4.8	0.262
Meals with gliadin-containing food (n meal/week)	7.7 ±2.7	9.1 ±3.1	0.108
Meals with protein/fat (n meal/week)	13.1 ±5.1	13.9 ±4.3	0.312
Meals with fibres (n meal/week)	11.8 ±4.6	14.8 ±5.6	0.056
Intake of pre-/probiotics n (%)	12 (63.2)	27 (84.4)	0.075
Intake of sweeteners n (%)	9 (47.4)	13 (40.6)	0.772

Table 2: Nutritional habits of diabetic patients and controls

Phylum	Controls (n=19)	Diabetic patients (n=32)	p-value*
Firmicutes	73.41 ±13.82	75.51 ±11.62	0.58
Bacteroidetes	18.97 ±12.98	15.42 ±11.21	0.31
Firm./Bact. Ratio	3.87 ±1.06	4.89 ±1.12	**0.024**
Verrucomicrobia	0.89 ±1.13	1.35 ±1.34	0.37
Proteobacteria	1.25 ±2.18	1.30 ±1.75	0.65
Actinobacteria	0.82 ±0.82	1.32 ±1.55	0.2
Synergistetes		0.01 ±0.03	0.58

* bold denotes p < 0.05, the arrow represents average differences

Table 3: Abundance of bacteria (in %) at the phylum level in diabetic patients and controls

Bacteria name	Controls (n=19)	Diabetic patients (n=32)	p-value
Roseburia (%)	0.03 ±0.03	0.03 ±0.05	
Faecalibacterium (%)	5.98 ±4.17 ↑	4.88 ±4.25	0.37
Eubacterium (%)	2.36 ±2.62	3.96 ±3.16 ↑	0.07
Akkermansia (%)	0.89 ±1.33	1.35 ±2.04 ↑	0.39
Bifidobacterium (%)	0.51 ±0.84	0.74 ±0.87 ↑	0.36
Ruminococcus (%)	6.88 ±6.41 ↑	6.09 ±4.44	0.61
Butyrvibrio (%)	0.02 ±0.07	0.05 ±0.19 ↑	0.51
Coprococcus (%)	1.71 ±2.58 ↑	0.85 ±1.39	0.13
Grand average (%)	2.30 ± 2.68	2.24 ±2.37	0.77

Table 4: Abundance of butyrate-producing bacteria (in %) in diabetic patients and controls

*probability value, the arrows represent average differences even if statistically not significant

Intestinal barrier function

The mean zonulin concentrations as a parameter for the function of the intestinal barrier were significantly higher in diabetic patients than in controls (66.2 ± 11.6 vs 42.2 ± 9.5 ng/ml; p = 0.001). There was no association between patient serum zonulin concentrations and the abundance of selected bacterial species at the phylum and class levels (Table. 6) as well as at lower classification levels (not shown).

Bacteria name (Bibliography)	Controls (n=19)	Diabetic patients (n=32)	p-value*
Proteobacteria (%) (20)	1.25 ± 2.18	1.3 ±1.75 ↑	0.93
Desulfovibrionaceae (%) (35, 36)	0.12 ± 0.13	0.19 ±0.33 ↑	0.38
Enterobacteriaceae (%) (35, 37)	0.84 ± 2.21 ↑	0.82 ±1.67	0.97
Veillonellaceae (%) (38)	0.41 ± 0.66	0.64 ±1.06 ↑	0.4
Prevotellaceae (%) (39)	0.96 ± 2.14	1.11 ±3.83 ↑	0.88
Oscillobacter (%) (35)	0.57 ± 0.65 ↑	0.53 ±0.74	0.85
Collinsella (%) (40)	0.08 ± 0.08	0.11 ±0.11 ↑	0.31
Streptococcus (%) (39)	0.36 ± 0.63	0.38 ±0.65 ↑	0.91
Lactobacillus (%) (41)	0.05 ± 0.09	0.25 ±0.73 ↑	0.24
Grand average (%)	**0.52 ± 0.41**	**0.59 ± 0.42**	**0.55**

*probability value, the arrows represent average differences even if statistically not significant

Table 5: Abundance of bacteria (in %) that have been associated with cardiovascular complications (CVCs) and/or produce TMA (genus/family classification level with the exception of phylum Proteobacteria)

Discussion

Our study shows that composition of IMB in people with a relatively long history of type 2 diabetes but without manifest CVC exhibit only moderate differences compared with controls of a similar age. Nevertheless, this group of patients exhibited elevated zonulin levels as a parameter of a raised intestinal permeability.

Results of taxonomy-based analysis of people with type 2 diabetes generally exhibit a dysbiosis compared with nondiabetic controls, the described changes in the composition of IMB microbiome are not uniform. Some authors, for example, have described a decrease of *Firmicutes* and/or *Clostridia* species in association with type 2 diabetes, while others have not been able to confirm these observations (9-14). The findings regarding the abundance of *Bacteroidetes species* and *Lactobacillus species* are also contradictory (9,11,14). In this investigation, the relative abundance of *Firmicutes* and *Bacteroides* between diabetic patients and controls did not differ, although the *Firmicutes/Bacteroides* ratio was significantly higher in the diabetes group. Since there is a positive correlation between the *Firmicutes/Bacteroides* ratio and BMI, this finding can be ascribed to the higher BMI of the diabetic patients compared with the controls (19). A further typical feature of intestinal

dysbiosis in connection with diabetes is the raised abundance of *Proteobacteria*, a bacterial species that is associated with the occurrence of CVCs (13,20,21). In this study, however, no difference in the abundance of Proteobacteria was found between the two groups investigated. The evaluation of the bacterial species that have previously been associated with the occurrence of CVCs also failed to reveal any significant differences (Tab. 6). One remarkable aspect, however, was that the abundance of seven of the nine species under investigation was slightly higher in the diabetes patients than in the controls. This finding may be indicative of a raised risk of the emergence of CVCs in this group of patients. In a recent study in diabetic patients with CVCs, for example, we detected a in some cases significantly raised abundance of the bacteria listed in Tab. 6 compared with patients without CVCs (22). However, future prospective studies are mandatory to clarify the predictive role of the intestinal microbiome on CVCs development.

The detection of Synergistetes in approximately 38% in the diabetes patients is a surprising result. Synergistetes are oral bacteria that are of relevance regarding inflammations in this area i.e. gingivitis or peridonditis (23). One reason for this may be the known frequent occurrence of these infections in people with type 2 diabetes, an aspect that, however, was not investigated in the scope of this study (24). A further reason may be the shift in the gastric pH due to medication with H2 blockers: a quarter of the diabetes patients, but none of the controls had been taking H2-blockers for their gastric symptoms.

A decrease in the abundance of butyrate-producing bacteria counts as a further typical finding regarding dysbiosis in association with type 2 diabetes (9,10,12). These bacteria have important functions in the metabolism of the intestinal wall, the provision of energy, and the regulation of metabolic processes. However, our study did not reveal any significant differences between the two study groups in this regard, not even as a trend (Table. 5). The reasons for the different results of the taxonomic investigations in the separate studies are complex. The patient groups investigated in the literature differ in a number of factors that may influence the composition of the intestinal microbiome, e.g. the ethnic or geographic origin of the subjects – aspects that play a major role in their lifestyle or nutritional habits – or differences in the clinical status of the diabetes patients regarding their age, metabolic disposition, cardiovascular complications, or therapy. Our study involved only "Caucasian" diabetes patients without manifest CVCs and with a relatively long history of diabetes, who were not significantly different from the subjects in the control group in numerous factors such as age, gender, renal function, or nutritional habits. Three quarters of the diabetes patients were under treatment with metformin, which according to recent studies has an impact on the composition of the intestinal microbiome, in particular regarding the abundance of *Akkermansia* (25,26). This bacterial species plays a major role in the metabolism of the mucin layer of the intestine and thus also influences the function of the intestinal barrier (27). Differing results have been recorded regarding the abundance of this species in association with type 2 diabetes (9,11). In our study, the abundance of *Akkermansia* was slightly higher in the diabetic patients, albeit without attaining statistical significance. This can be ascribed to the broad use of metformin, since this agent – as described above – promotes in particular the abundance of *Akkermansia species.*

A further characteristic feature of the modified intestinal function in patients with diabetes that has been reported are the elevated serum levels of zonulin, as an indication of an enhanced permeability of the intestinal barrier (15,16). Zonulin is formed by the intestinal wall in reaction to a variety of stimuli and induces a relaxation of the so-called tight junctions, with the result that the intestinal barrier is made permeable for a number of substances, for example toxic components of foodstuffs, various foreign antigens, bacteria, or bacterial toxins (28). This in turn triggers the activation of the intestinal immune system and of chronic inflammatory processes that may lead to a variety of clinical manifestations. In this connection, several study groups have described significantly higher zonulin concentrations in patients with coronary heart disease than those found in patients with a healthy coronary status (29, 30). Studies into the factors influencing the formation of zonulin have shown that besides bacterial toxins other dietary constituents – e.g. gluten – lead to an enhanced secretion of this agent (31). Furthermore, correlations have also been found between the zonulin concentration and various clinical factors such as age, BMI, blood glucose, HbA1c, parameters of chronic inflammation, or renal function (15, 16, 32, 33). The extent to which these associations are causally related is still unclear. The correlations between the composition of the IMB and the function of the intestinal barrier in patients with type 2 diabetes have as yet hardly been investigated. A Spanish research team recently described a negative correlation between zonulin concentrations and the abundance of *Faecalibacteria* in diabetes patients with coronary heart disease as well as a positive correlation between zonulin concentrations and the abundance of *Prevotella* and *Rikenellaceae* (30). Animal experiments have yielded results indicating correlations between the abundance of *Bifidobacteria* and *Lactobacilli* and intestinal-barrier function, albeit without clarifying the underlying mechanisms (34). The patients investigated in our study exhibited no signs of acute or chronic intestinal disease that may explain the elevated zonulin concentrations. Since the composition of the IMB did not significantly differ between the diabetic patients and the nondiabetic controls, this may be an indication that the IMB does not constitute a major factor of influence for the function of the intestinal barrier among this population. As mentioned above, multiple diet and clinical factors are associated with zonulin concentration. In the present study diabetic patients showed a significantly higher BMI and HbA1c levels than nondiabetic controls possibly explaining the difference in zonulin concentration as marker of impaired barrier function.

Stirp	Slope	95%	CI	p-value
Phylum_Actinobacteria	13.941	-1.497	42.849	0.34
Phylum_Bacteroidetes	0.1201	-0.246	0.486	0.51
Phylum_Firmicutes	-0.1306	-0.433	0.1722	0.39
Phylum_Proteobacteria	0.1512	-2.021	23.233	0.89
Phylum_Verrucomicrobia	0.7429	-1.114	25.994	0.43
ratio_Firm_Bact	-0.0965	-0.199	0.0062	0.06
Class_Actinobacteria	18.107	-3.222	68.435	0.47
Class_Bacilli	23.933	-0.231	50.178	0.07
Class_Bacteroidea	0.1182	-0.251	0.487	0.52
Class_Betaproteobacteria	30.797	-11.848	180.074	0.68
Class_Clostridia	-0.1703	-0.447	0.1061	0.22
Class_Coriobacteria	24.164	-2.644	74.767	0.34
Class_Deltaproteobacteria	24.673	-11.806	167.401	0.73
Class_Erysipelotrichia	19.585	-8.346	122.634	0.71
Class_Gammaproteobacteria	-0.037	-2.285	22.109	0.97
Class_Negativicutes	0.6521	-1.048	23.519	0.45
Class_ Verrucomicrobiae	0.7411	-1.116	25.981	0.43

Table 6: Association between serum zonulin concentrations and abundance of selected bacterial species at phylum and class level in diabetic patients

The main limitations of this study are the relatively low number of investigated patients and the observational study design. Some details are lacking for the nutritional habits of the subjects, in particular regarding

alcohol consumption or smoking, which may also have an impact on the composition of the intestinal microbiome. The effect of antidiabetic therapy on GMB will be investigated separately.

Conclusion

Compared with the nondiabetic controls, diabetes patients without manifest CVC showed only modertate differences in the composition of IMB. They did, however, in tendency show a raised abundance of bacteria that are associated with the occurrence of CVCs. In the context of an enhanced permeability of the intestinal barrier, these findings may be an indication of a raised risk of CVCs among these patients. The absence of a correlation between zonulin concentration and the composition of the IMB demonstrates that the function of the intestinal barrier in our population of diabetes patients is not primarily influenced by the composition of the intestinal microbiome, but rather by other factors instead.

Acknowledgement

The study was kindly supported by an unrestricted grant of the Dietmar Hopp Stiftung, St. Leon Rot, Gemany

Conflict of interest statement

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of the study.

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