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ORIGINAL ARTICLE

Cardiovascular Complications and Composition of the Intestinal Microbiome in Patients with Type 2 Diabetes

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Summary

Background: The composition of the gut microbiome appears to exert an influence on the development of cardiovascular complications (CVCs). We investigate here the relationship between the composition of the gut microbiome and the prevalence of CVCs in patients with type 2 diabetes.

Methods: Demographic characteristics, routine laboratory results, data on the prevalence of CVCs and the composition of the gut microbiome assessed by 16S rRNA sequencing of fecal samples were collected from 60 patients affected by type 2 diabetes for 13 years on average. The relationship between bacterial type abundance, in particular butyrate- and trimethylamine (TMA)-producing bacteria, and the prevalence of CVCs was investigated by t-tests and logistic regression.

Results: 28 patients presented with CVCs and they showed a lower abundance of *Verrucomicrobia* at the phylum level than CVCs-unaffected patients ($p = 0.02$). At the order level, the average proportion of *Coriobacteriales* was higher for patients with (0.29%) versus patients without CVCs (0.13%; $p = 0.001$). The odds ratio of CVCs for patients in the highest quartile of *Coriobacteriales* abundance (> 0.30%) was 9.6 (95% CI 1.86 to 49.5) compared to patients in the lowest quartile (abundance < 0.08%). Logistic regression analysis revealed a 2-fold increased risk of CVCs for each 0.01% increase in *Coriobacteriales* abundance ($p = 0.01$). This association was also found at lower classification levels (species *Collinsella aerofaciens*). Patients with CVCs showed a lower average abundance of butyrate-producing bacteria than patients without CVCs (2.06% vs. 2.24%) and a higher average abundance of bacteria traditionally associated to CVCs (0.72% vs. 0.58%) but mean differences did not reach statistical significance.

Conclusion: *Collinsella*, a TMA-producing bacterium with atherogenic effects, was associated with CVC risk in type 2 diabetes patients. Patients with CVCs also showed lower counts of *Verrucomicrobia* and butyrate-producing bacteria. These findings may reflect an impaired intestinal barrier and should be investigated in future prospective studies.

Keywords

Intestinal microbiome, Diabetes, Cardiovascular complications, *Verrucomicrobia*, Butyrate producing bacteria, TMA producing bacteria

Abbreviations

CVC: Cardiovascular Complication; eGFR: Estimated Glomerular Filtration Rate; LPS: Lipopolysaccharide; PTA: Percutaneous Transluminal Angioplasty; TIA: Transient Ischemic Attack; TMA: Trimethylamine Producing Bacteria; TMAO: Trimethylaminoxide

Introduction

Cardiovascular complications (CVCs) are still the most frequent cause of morbidity and death in patients with type 2 diabetes. The current options for the treatment of the classic risk factors such as dyslipoproteinemia, hypertension, and diabetes have so far not been

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able to alter this situation to a fundamental extent, meaning that new therapeutic approaches must be identified [1,2]. In this connection, in recent years more and more focus has come to rest on the intestinal microbiome. It is apparent that the millions of bacteria in the intestinal tract exert an influence on a multitude of metabolic and immunological processes in the human body, and hence play a major role in the emergence of a variety of health disorders, for example of metabolic and intestinal diseases, neurological disorders, or the development of cancer diseases [3,4]. The microbiome also appears to be of relevance regarding the pathogenesis of cardiovascular disorders: It has been possible to demonstrate, for example, that bacteria or their constituents are capable of passing through a compromised intestinal barrier and thus entering the organism, resulting in metabolic endotoxemia with silent inflammation and insulin resistance [5,6]. A number of research teams have been able to detect bacterial DNA in arteriosclerotic plaques [7-9]. Furthermore, it is known that specific groups of bacteria are capable of producing metabolic products that have atherogenic effects in the human body [10,11]. Little attention has so far been paid to the relevance of these effects for patients with a longer history of type 2 diabetes. Therefore, we performed a descriptive study assess the composition of the intestinal microbiome in this group of patients, some of whom already exhibited cardiovascular complications.

Methods

The study was conducted in a population of 60 patients with known longstanding type 2 diabetes attending the diabetes outpatient clinic of the St. Josefskrankenhaus Heidelberg GmbH. Type 2 Diabetes was formerly diagnosed according to the criteria of ADA, i.e. HbA1c ≥ 6.5% or fasting blood glucose ≥ 126 mg/dl [12]. The main inclusion criteria were: Age between 50 and 80 years, stable diabetes 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, serious diseases such as tumour diseases, liver cirrhosis, cardiac insufficiency (> NYHA II), renal insufficiency (estimated glomerular filtration rate (eGFR) < 30 mL/min), and previous surgery within the gastrointestinal tract. The study was approved by the local ethics committee and written consent was obtained from all patients prior to participation.

Demographic information, current treatment and diabetic micro- and macroangiopathy complications were retrieved from all patients. In particular, the following cardiovascular complications were considered: (a) Coronary heart disease, defined as history of myocardial infarction, procedure of percutaneous transluminal coronary angioplasty or coronary artery bypass graft, coronary vessel disease confirmed by coronary angiography; (b) Cerebrovascular vessel disease, defined as history of ischemic stroke, transient ischemic attack (TIA) or stenosis of a. carotis > 50% (c) Peripheral artery disease, defined as history of bypass operation, procedure of percutaneous transluminal angioplasty (PTA), amputation or ankle brachial index < 0.8.

The following laboratory parameters were taken into account: Blood count, HbA1c, glucose, lipids, creatinine, hepatic function parameters, hsCRP, ferritin, insulin, gliadin antibodies (IgA, IgG). The eGFR was calculated according to the CKD-EPI formula [13]. All laboratory parameters were measured at fasting state. Fresh stool samples from the investigated patients were also collected and immediately stored at -70 °C. Subsequent analyses of the intestinal microbiome were performed at the Center for Metagenomics in Tübingen, Germany.

The intestinal microbiome was analyzed using 16S rRNA sequencing. For 16S analysis, DNA was isolated from frozen stool samples using the MetaHIT Method [14]. DNA quantity and integrity were assessed using Nanodrop and agarose gel electrophoresis. On average DNA isolation resulted in a DNA concentration of 190.7 ng/µl. Agarose gel electrophoresis revealed defined bands > 10 kbp for all DNA samples.

For sequencing, the variable regions V3-4 of the bacterial 16S rRNA gene were amplified, using primers targeting the primer binding sites of the primers S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') [15]. Library preparation was performed using the Illumina Nextera XT Index Kit according to Illumina's recommendations for 16S metagenomics. Library purification, quantification, and quality control were performed using magnetic beads (AMPure XP), Qubit and electrophoresis (Agilent 2100 Bioanalyzer), respectively. Paired-end sequencing was carried out on an Illumina MiSeq sequencing system using the MiSeq Reagent Kit v2 (500 cycle, 2 × 250 bp). Raw sequencing reads were analyzed according to the CeMeT standard pipeline. Forward and reverse reads were quality trimmed using PRINSEQ [16] and merged with fastq-join [17]. An in-house developed filtering script was used to remove merged sequences shorter than 100 bp. The remaining high quality sequences were aligned to NCBI's Bacterial 16S rRNA database (downloaded January 2016) with the alignment tool MALT [18]. Taxonomic classification was performed within MALT using the implemented LCA (Lowest Common Ancestor) algorithm.

Sequencing statistics

Bacterial 16S rRNA gene sequencing of 60 DNA samples resulted in 15.7.6 million raw reads with an average number of 262,197 raw reads per sample. 80.6% of the sequenced bases had an Illumina Q score above 30 (> Q30 = 80.6%).

Biometric evaluation

Means, standard deviations (SDs) and relative fre-

Table 1: Demographic and laboratory characteristics of the investigated collective of diabetic patients with and without cardiovascular complications (CVCs).

Characteristic	Unit	Patients without CVCs (n = 32)	Patients with CVCs (n = 28)	p*
		Mean ± SD	Mean ± SD	
Female proportion	%	59.4	35.7	0.07
Age	Years	66.8 ± 6.3	68.2 ± 5.9	0.38
Diabetes duration	Years	12.9 ± 5.7	13.1 ± 6.5	0.90
BMI	kg/m²	32.1 ± 5.7	32.8 ± 6.2	0.65
hsCRP	mg/L	3.0 ± 3.04	4.68 ± 3.8	0.07
HbA1c	%	7.34 ± 0.97	7.62 ± 0.92	0.26
HbA1c	Mmol	56.6 ± 10.7	59.8 ± 9.9	0.24
Triglycerides	mg/dL	172.7 ± 71.3	168.3 ± 89.1	0.83
Cholesterol	mg/dL	189.9 ± 40.2	167.1 ± 40.3	**0.03**
HDL cholesterol	mg/dL	51.6 ± 10.8	44.2 ± 13.7	**0.02**
LDL cholesterol	mg/dL	119.1 ± 35.9	101.1 ± 34.2	0.05
eGFR	ml/min	82.6 ± 18.1	72.9 ± 19.4	**0.04**
Haemoglobin	g/dL	14.2 ± 1.2	14.1 ± 1.3	0.76
Hypertension	%	81	96	0.10
BP systolic	mmHg	140.3 ± 12	146.4 ± 16.1	0.06
BP diastolic	mmHg	78.4 ± 9.4	79 ± 14.2	0.74

*bold denotes $p < 0.05$; BP: Blood Pressure.

Table 2: Medication used in the investigated collective of diabetic patients with and without cardiovascular complications (CVCs).

Medication	Patients without CVCs (n = 32)		Patients with CVCs (n = 28)		p*
	n	%	n	%	
Metformin	24	75	19	67.9	0.05
DPP4 Inhibitors or Incretine mimetica	12	37.5	9	32.1	0.498
Sulfonylureas	4	12.5	6	21.4	0.49
Insulin	14	43.8	19	67.8	0.07
Statins	14	43.8	23	82.1	**0.01**
Diuretics	16	50	18	64.3	0.27
Beta Blockers	15	46.9	20	71.4	0.05
Calcium antagonists	12	37.5	9	32.1	0.49
ACE-Inhibitors or AT1-Blockers	26	81.3	26	92.8	0.26

*bold denotes $p < 0.05$.

quencies were used to summarize demographic characteristics, routine lab results and bacterial type abundances in type 2 diabetes patients with and without CVCs. Mean differences between the two groups of patients were assessed by t-tests. In addition, univariate logistic regression was used to identify bacterial types potentially associated to the prevalence of CVCs and to calculate odd 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).

Results

Twenty-eight patients with an average type 2 diabetes duration of 13 years presented with cardiovascular complications (CVCs), namely: Coronary heart disease n = 16 (57%), peripheral arterial disease n = 8 (29%) and cerebrovascular disease n = 4 (14%). Main demographic characteristics and laboratory results from patients with and without CVCs are summarized in Table 1. No statistical differences between patients with and without CVCs were found regarding demographic and lab results. Due to the higher frequency of statin therapy in patients with CVCs (82% compared to 43% in patients without CVCs; Table 2), their cholesterol values were lower (167 compared to 190 mg/dL). Kidney function was somewhat poorer in patients with CVCs, while the remaining laboratory values showed no significant differences. The type of antidiabetic therapy did not differ between both groups (Table 2). Most patients were taking metformin, about one third was treated with an incretine based therapy, insulin was given in a higher percentage in both patient groups. The prevalence of hypertension and the blood pressure values were somewhat higher in patients with than in patients without CVCs, but the differences were not significant (Table 1). Antihypertensive therapy was similar in both groups (Table 2). Exploratory survey of the patients' nutritional habits revealed that no study participant was on a vegetarian or vegan diet. No patient had been diagnosed as suffering from gliadin intolerance.

With the exception of *Verrucomicrobia*, with an average abundance of 1.25% in patients without and 1.01% in patients with CVCs ($p = 0.02$), the microbiome composition displayed no difference (Table 3). A potentially relevant but statistically only marginally significant finding was the detection of bacteria of the *Synergistetes* phylum in about one half of the patients - 54% with and 37.5% without CVCs. The abundance of these bacteria

Table 3: Abundance of bacteria (in %) at the phylum level in diabetic patients with and without cardiovascular complications (CVCs).

Phylum	Patients without CVCs	Patients with CVCs	p*
	Mean ± SD	Mean ± SD	
Firmicutes	75.46 ± 12.6	75.76 ± 12.8	0.93
Bacteroidetes	15.42 ± 11.2	15.23 ± 9.7	0.94
Ratio	4.90 ± 1.12	4.97 ± 1.32	0.90
Verrucomicrobia	1.35 ± 2.1↑	1.01 ± 2.1	**0.02**
Proteobacteria	1.30 ± 1.8	1.23 ± 1.8	0.88
Actinobacteria	1.32 ± 1.6	1.18 ± 1.0	0.69
Synergistetes	0.01 ± 0.03	0.06 ± 0.14	0.05

*bold denotes p < 0.05; The arrow represents average differences; SD: Standard Deviation.

Table 4: Abundance of bacteria (in %) at different classification levels in diabetic patients with and without cardiovascular complications (CVCs, significant findings only).

Level	Name	Patients without CVCs	Patients with CVCs	p*
		Mean ± SD	Mean ± SD	
Phylum	-			
Class	-			
Order	*Coriobacteriales*	0.13 ± 0.11	0.29 ± 0.25 ↑	**0.001**
Family	*Coriobacteriaceae*	0.12 ± 0.11	0.26 ± 0.25 ↑	**0.005**
Genus	*Collinsella*	0.11 ± 0.11	0.25 ± 0.24 ↑	**0.004**
Species	*Collinsella aerofaciens*	0.10 ± 0.11	0.23 ± 0.23 ↑	**0.009**

*bold denotes p < 0.05; The arrows represent average differences; SD: Standard Deviation.

Table 5: Results from logistic regression analyses on the relationship between the bacteria abundance at different classification levels and the prevalence of cardiovascular complications.

Level	Name	p*	OR	95%	CI
Phylum	*Actinobacteria*	0.67	0.918	0.617	1.367
Phylum	*Bacteroidetes*	0.94	0.998	0.951	1.048
Phylum	*Firmicutes*	0.93	1.002	0.962	1.043
Phylum	*Proteobacteria*	0.88	0.978	0.731	1.309
Phylum	*Verrucomicrobia*	0.52	0.918	0.708	1.191
	ratio_Firm_Bact	0.71	1.003	0.989	1.017
Order	*Coriobacteriales*	**0.004**	442.8	6.884	> 999.999
Family	*Coriobacteriaceae*	**0.01**	200.2	3.486	> 999.999

*bold denotes p < 0.05; OR: Odds Ratio; CI: Confidence Interval.

Table 6: Estimated odds ratios for cardiovascular complications according to the quartiles of *Coriobacteriales* abundance.

***Coriobacteriales* abundance**	**Without complications**	**With complications**	**OR**	**95%**	**CI**
< 0.08	12	5	Ref.		
0.08-0.13	9	5	1.33	0.29	6.04
0.14-0.29	8	6	1.80	0.41	7.96
> 0.30	3	12	9.60	1.86	49.5

was higher in patients with (0.06%) than in patients without CVCs (0.01%, p = 0.05). *Synergistetes* are bacteria of relevance in oral inflammations (gingivitis etc.).

From the order level downwards, the mean proportion of *Coriobacteriales* in patients with CVCs was significantly higher than in patients without CVCs at all subordinate classification levels (Table 4). The identified species was *Collinsella aerofaciens* with an average abundance of 0.10% in patients without and 0.23% in patients with CVCs. Logistic regression analysis revealed that each increase in the abundance of *Coriobacteriales* by 0.01% is associated with an odds ratio for CVCs equal to 4.4 (p = 0.004, Table 5).

Table 6 shows the odds ratios for CVCs according to the quartiles of *Coriobacteriales* abundance. The odds ratio for CVCs among patients in the highest quartile (*Coriobacteriales* abundance > 0.30%) was 9.60 (95% CI 1.86 to 49.5) compared to patients in the lowest quartile (abundance < 0.08%). At the family level, logistic regression analysis revealed a 2-fold increased risk of CVCs for every 0.01% increase in *Coriobacteriaceae* abundance (p = 0.01).

Collinsella belongs to the trimethylamine (TMA)-forming bacteria, which are known to exert atherogenic effects. There are further bacteria described in the literature that are also associated with arteriosclerotic vessel wall lesions, a fact that led us to investigate the abundance of these types of bacteria in type 2 diabetes patients with and without CVCs. Patients with CVCs presented with higher average proportions of *Collinsel-*

Table 7: 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*).

Bacteria name (Bibliography)	Unit	Patients without CVCs	Patients with CVCs	p*
		Mean ± SD	Mean ± SD	
Proteobacteria [7]	%	1.3 ± 1.75↑	1.23 ± 1.82	0.88
Desulfovibrionaceae [20,22]	%	0.19 ± 0.33↑	0.13 ± 0.16	0.52
Enterobacteriaceae [20,23]	%	0.82 ± 1.67	0.86 ± 1.78↑	0.58
Veillonellaceae [9]	%	0.64 ± 1.06	1.17 ± 2.52↑	0.28
Prevotellaceae [10]	%	1.11 ± 3.83	1.54 ± 3.61↑	0.66
Oscillibacter [20]	%	0.53 ± 0.74	0.53 ± 0.49	
Collinsella [19]	%	0.11 ± 0.11	0.25 ± 0.24↑	**0.04**
Streptococcus [9]	%	0.41 ± 0.71↑	0.28 ± 0.38	0.26
Lactobacillus [21]	%	0.14 ± 0.52	0.52 ± 1.28↑	0.15
Grand average	%	0.58 ± 0.43	0.72 ± 0.50↑	0.25

*bold denotes $p < 0.05$; The arrows represent average differences even if statistically not significant; SD: Standard Deviation.

Table 8: Abundance of butyrate-producing bacteria (in %) among diabetes patients with and without cardiovascular complications (CVCs).

Bacteria name	Unit	Patients without CVCs	Patients with CVCs	p*
		Mean ± SD	Mean ± SD	
Roseburia	%	0.03 ± 0.05↑	0.02 ± 0.02	0.32
Faecalibacterium	%	4.88 ± 4.25↑	3.57 ± 3.13	0.18
Eubacterium	%	3.96 ± 3.16↑	3.52 ± 3.24	0.59
Akkermansia	%	1.35 ± 2.04↑	1.01 ± 2.1	0.52
Bifidobacterium	%	0.74 ± 0.87↑	0.56 ± 0.57	0.35
Ruminococcus	%	6.09 ± 4.44	6.99 ± 4.59↑	0.44
Butyrivibrio	%	0.05 ± 0.19↑	0.02 ± 0.06	0.42
Coprococcus	%	0.85 ± 1.39↑	0.76 ± 1.27	0.79
Grand average	%	2.24 ± 2.37↑	2.06 ± 2.45	0.77

*p: Probability value; The arrows represent average differences even if statistically not significant; SD: Standard Deviation.

la and a higher but not statistically significant different average proportions in four further species. Patients without CVCs showed three bacteria species with higher abundances, but mean differences did not reach statistical significance (Table 7).

Last, we separately investigated the abundance of butyrate-forming bacteria, which are essential for the metabolism of the intestinal epithelium. As shown in Table 8, patients without complications exhibited higher average abundances of seven of the eight bacterial species under investigation; mean differences, however, did not reach statistical significance.

Discussion

This investigation shows that type 2 diabetic patients with cardiovascular complications (a) Present a higher prevalence of bacteria associated with atherogenic complications, significantly for *Collinsella aerofaciens*, (b) Show a lower prevalence of bacteria of the phylum *Verrucomicrobia*, and (c) Also show a lower prevalence of butyrate-producing bacteria.

The results of various studies conducted in humans with arteriosclerotic complications suggest that the composition of the microbiome has a major effect on the emergence of CVCs. The blood of patients with CVCs, for example, exhibits bacterial DNA, while that of patients with type 2 diabetes shows elevated concentrations of constituents of the walls of gram-negative bacteria (Lipopolysaccharide [LPS]) [5-9]. Investigations of arteriosclerotic plaques also yielded the DNA of bacteria, the taxa of which could also be detected in the oral cavity or the intestine [8,9]. It is assumed that the presence of bacteria in arteriosclerotic foci is capable of resulting in plaque instability and thus in a manifest cardiovascular complication. This assumption is given further weight by Karlsson, et al. in a study in patients with symptomatic stenosis of the carotid artery [19]. Compared with results obtained in asymptomatic patients, in the stools of the patients with symptoms the authors found a lower occurrence of butyrate-producing bacteria and a higher prevalence of bacteria that produce pro-inflammatory substances. Other work groups have also been able to demonstrate characteristic changes in the composition of the intestinal microbiome in patients with cerebrovascular events, compared with non-affected controls [20,21].

Our findings made in diabetes patients with predominantly cardiac or peripheral-arterial manifestations are in agreement with the named reports regarding cerebrovascular complications. We found a significantly positive correlation between the prevalence of *Collinsella* and the occurrence of CVCs. At the species level *Collinsella aerofaciens* was found, a bacterium that was also described by Karlsson, et al. [19]. In our study, other bacteria that have also been reported in the li-

terature in connection with cardiovascular events were also relatively more frequently detected in the stools of cardiovascular patients, albeit without achieving statistical significance (Table 5).

Collinsella belongs to TMA- producing bacteria which are known to produce atherogenic effects after transformation into TMA-Oxid in the liver [10]. Determination of serum TMA- or TMA-O -concentrations were not done in our study so that the impact of our finding for development of CVCs has to be clarified in further studies. Nevertheless, *Collinsella* seems not to be a bacteria that is specific for diabetic patients with CVCs. In Karlsson´s study with high presence of *Collinsella,* diabetes morbidity lay at 25% (3 of 12 patients investigated). In the other studies mentioned above but without significant abundance of *Collinsella*, diabetes morbidity was higher, i.e. 27-50% [9,19,20]. The reasons for the different results regarding *Collinsella* abundance may be due to the known fact that the composition of IMB is influenced by multiple factors such as 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 patients investigated regarding their age, metabolic disposition or therapy. This may be a reason for different microbiome signature in patients with CVCs.

One potential cause under discussion for the passage of bacteria or their constituents into the organism as an important component regarding development of CVCS is the raised permeability of the intestinal barrier [5,6,24]. The functionability of this barrier is also influenced by the microbiome. Bacteria of the phylum *Verrucomicrobia* i.e. *Akkermansia species* fulfill important tasks in the metabolism of the mucolemma of the intestine as one layer of the barrier [24]. Recent studies have shown that metformin treatment is associated with higher abundance of *Akkermansia* [25,26]. In the present study, patients with and without CVCs were treated in similar frequency with metformin. Nevertheless, the occurrence of *Verrucomicrobia* was significantly less frequent in patients with CVCs, reasons are unclear as yet. Further, butyrate-producing bacteria play an essential role for the energy metabolism of the intestinal epithelium and are also preconditional for the integrity of the intestinal barrier. The prevalence of butyrate-producing bacteria in seven of the eight bacteria strains that were investigated was also slightly lower in patients with cardiovascular complications. These findings may support the assumption of a compromised intestinal barrier in patients with CVCs.

Furthermore, the microbiome can also promote the emergence of vascular complications by forming bioactive metabolic products. In this connection, the substance trimethylamine (TMA), which is produced from phosphatidylcholine and carnitine through bacterial metabolism, has been the subject of intensive research in recent years [10,11,27]. After its resorption from the intestine, this substance is transformed into TMA oxide (TMAO). TMAO concentrations are positively associated with the occurrence of CVCs, as has been shown in several studies, some of which were prospective ones [5]. The underlying molecular mechanism of the atherogenic effect of TMAO has, however, not yet been fully clarified. In our study, the majority of TMA producing bacteria that were investigated was found in patients with CVCs, however, difference was significant only for *Collinsella.*

The main limitations of study are the relatively low number of investigated patients and the observational study design. Details are lacking for the nutritional habits of the subjects, in particular regarding the uptake of gliadin-containing foods and fats, which exert an influence on the function of the gut barrier [28] or foot products containing L-carnitine or phosphatidylcholine which are used for TMA formation [10,11,27]. In addition, factors such as alcohol consumption, smoking, and the use of pre- and probiotics may also have an impact on the composition of the intestinal microbiome; such factors should be taken into consideration in future prospective investigations. We are separately investigating the effect of antidiabetic therapy on the intestinal microbiome. In the present study, however, we found no statistically significant differences between patients with and without CVCs in terms of used antidiabetic drugs.

In summary, our study shows that patients with a long history of type 2 diabetes presenting a CVC have a less favorable composition of their intestinal microbiome than do patients without CVCs: (a) A significantly lower prevalence of members of the phylum *Verrucomicrobia* and in tendency a lower prevalence of butyrate-producing bacteria, both of which are essential for maintaining the integrity of the function of the intestinal barrier, and (b) A significantly higher prevalence of *Collinsella,* a bacterial species that is among the producers of atherogenic metabolic products. Our results thus underscore the options that in future will enable medical science to influence the intestinal microbiome (pharmaceutically, using pro- or prebiotic agents, or by faecal transplants) to reduce the cardiovascular risk in patients with diabetes.

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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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