Human Microbiome Journal 5–6 (2017) 11–13

Contents lists available at ScienceDirect

Human Microbiome Journal

journal homepage: www.elsevier.com/locate/humic

Gut microbiota diversity and T1DM onset: Preliminary data of a case-control study

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ARTICLE INFO

Keywords:
Type 1 diabetes mellitus
Microbiota
Children
Methanobrevibacter smithii
qRT-PCR

ABSTRACT

Type-1 diabetes incidence is increasing during the last decades. Recently, a role of microbiota alteration is proposed as pre-diabetic and diabetic risk factor. A bicentric case-control study is in progress in Northern Italy. Here preliminary results are shown. The microbiome clusterization showed a division between cases and controls even if fingerprint profiles are heterogenic. *Methanobrevibacter smithii* is highly present only in few patients. The diversity index and the microorganism sequenced in cases and controls, seems to be quite dissimilar. The conclusive results could show a significant predictive value for the bio-indicators evaluated.

Introduction

The global prevalence of diabetes has nearly doubled, during the last 35 years, reaching the 8.5% in the adult population [1]. Recently, evidences are produced on the interconnection among microbiome, metabolome and T1DM progression [2–4]. The aim of this work is to evaluate microbiome composition and specific quantitative difference between T1DM cases at the onset and controls.

Materials and methods

This case-control study (ClinicalTrials.gov ID: G12114000080001) included T1DM and healthy children. The T1DM cases were integrated into the study at the disease onset, with hyperglycemia – with/without ketoacidosis – polyuria/polydipsia symptoms, high value of glycated haemoglobin (> 42 mmol/mol) and T1DM-specific auto-antibodies positivity. Healthy children were included if comparable to cases in terms of age, gender and ethnicity. Inclusion criteria were: age (5–14 years), normal weight and residence in Piedmont (north-west Italy). Exclusion criteria were: celiac, chronic diseases diagnosis, eating disorders, overweight/obesity, active infections, use of antibiotics/probiotics/any other medical treatment that influences intestinal microbiota during the 3 months before the study start [5]. During the last control before the hospital discharge, the study participation was proposed to the children and his guardians. A kit for stool collection was delivered following validated procedure [6]. In the lab, the samples were homogenised and 2 g aliquots were stored at −80 °C. DNA extraction was performed using the PowerFecal DNA Isolation Kit (MoBio Laboratories Inc.). Fluorimetric quantification of DNA samples was performed using a Qubit™ fluorometer (Life Technology Ltd.). Within the first six months of project, 26 children aged between 5 and 13 years are included, 13 at the T1DM onset and 13 healthy as control. Extracted DNA concentrations ranged between 22 and 155 ng/μL (mean 30.4 ng/μL). Samples were stored at −20 °C until biomolecular analysis.

PCR-DGGE

PCR products for DGGE were obtained by amplifying total bacterial 16S rRNA genes. Primer pairs were 357F-GC and 518R [7]. All PCR reactions were performed with the T100 Biorad Thermocycler and the Master Mix (166-5009, Bio-Rad) was used (final volume 25 μl). DGGE was performed as previously described [8] using a DCode System (Bio-Rad), with gradient 30–50%. Electrophoresis ran at 200 V for 5 h at 60 °C in 1 × TAE buffer. Gels were stained for 30 min with SYBR® Green I (Sigma-Aldrich) and visualized under Gel-Doc XR (Bio-Rad). DGGE bands were excised, incubated one night at −20 °C, washed, and crushed in sterilized water. Supernatant (1 μl) was used as template and PCR was performed as above, except for BSA and the employment of

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https://doi.org/10.1016/j.humic.2017.11.002
Received 7 August 2017; Received in revised form 31 October 2017; Accepted 9 November 2017
Available online 11 November 2017

Table 1
(A) Summary of the population characteristics, comparing case and controls: number of children involved, sex, age and anthropometrics as mean and standard deviation (*T*-test for each independent variables is not significant p > .05, for sex Chi-square not significant). (B) Quantification by qRT-PCR of the specific bio-indicators into the stool samples collected (as mean and standard deviation), comparing cases and controls (*T*-test for each independent variables).

		Healthy controls	T1DM onset patients	p Value	
A	Subjects (number)	13	13	–	
	Male/Female	9/4	8/5	ns	
	Age (years)	8.00 ± 1.83	8.68 ± 1.75	.345	
	Height (cm)	132.6 ± 15.58	140.31 ± 12.49	.177	
	Weight (kg)	30.92 ± 12.68	35.95 ± 11.37	.298	
	BMI (kg/m^2)	16.89 ± 3.00	17.68 ± 3.21	.525	
B	*Methanobrevibacter smithii* (Log copies 16SrDNA/g stool)	4.516 ± 0.00	5.059 ± 1.040	.083	.012
	Methanobrevibacter smithii (Log copies niHf/g stool)	4.516 ± 0.00	5.086 ± 1.087	.084	
	Akkermansia muciniphila (Log copies gene target/g stool)	4.132 ± 1.504	4.056 ± 1.380	.895	
	Total bacteria (Log copies gene target/g stool)	7.588 ± 0.379	7.756 ± 0.433	.305	
	Simpson's diversity index	0.0669 ± 0.0149	0.0820 ± 0.0295	.115	
	Shannon index	2.87 ± 0.19	2.77 ± 0.30.	.317	

modified linker-PCR bacterial primers [9]. PCR products were sequenced (Genechron – Ylichron srl) and searched for sequence similarities in the NCBI database using BLASTn analysis.

qRT-PCR

The stool samples were quantified for total bacteria (TotBact) [10], *Methanobrevibacter smithii* (16S rDNA [10] and functional *nifH* gene [11]) and *Akkermansia muciniphila* [12]. RT-qPCR was performed with C1000 Thermal Touch (Bio-Rad). 2 µl of 1:10 extracted DNA was added to a reaction mixture (1:100 only for TotBact) consisting of IQ™ Multiplex PowerMix (Bio-Rad), molecular probe (10 µM), primers (10 µM final concentration, Thermo Fisher Scientific) and ultrapure water in a 20 µl final volume. The reaction conditions were 95 °C-3 min (1X), 95 °C-10 sec, 59 °C-15 sec, 72 °C-15 sec (39X), 72 °C-1 min. Genomic DNA of microorganism target (ATCC®) were used as standards. To confirm the amplification of each target, gel electrophoresis was performed on 2% agarose gels.

Statistical analysis

The DGGE gel analysis was performed with Bionumerics 7.2. The hierarchical classification was performed by UPGMA system (1% tolerance and optimization level) and Pearson correlation. Statistical analysis was performed using the SPSS Package 24.0. We applied log transformation of non-normally distributed data, Spearman rank-order correlation coefficient and *T*-test for independent variables.

Results and discussion

No difference could be observed for age and gender between case and control groups (Table 1A). The average of Shannon's diversity index was 2.82 ± 0.25, 4% lower in the patients and the Simpson's index showed a 27% less diversity in patients (both *T*-test p > .05). The hierarchical phylogenetic clustering showed a main division in two clusters with a similarity < 14%. 10/13 patients were included in one of this cluster while the other cluster include all the controls and showed a higher similarity (> 50%) (Graphical Abstract). Gut microbiota of all children was predominately composed by Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria, consistent with previous studies [5,13,14]. The major difference between case and control groups was found in the prevalence of bacteria at genus-division level. In the children with diabetes, we observed – by sequencing – an increase in the presence of two members of Bacteroidetes: *Bacteroides clarus* and *Alistipes obesi* and of *Bifidobacterium longum*.

Alistipes obesi is an obligate anaerobic rod, with optimal growth observed at 37 °C. It is different respect the other *Alistipes* sp. for its taxono-genomics profile moreover it is the first Alistipes isolated from the gut of an obese patient [15]. In recent published article is observed a growth of *Alistipes* sp. both in obese [16] and in T2DM patients [17].

Other remarkable results, in the group of patients, were the decrease of the presence of *Bacteroides vulgatus*, *oleiciplenus*, *coprophilus* and *dorei* as the decrease of some Firmicutes (i.e. *Eubacterium* and *Faecalibacterium prausnitzii*) and *Fusicatenibacter saccharivorans*.

A. muciniphila level significantly correlated with Shannon index (Spearman's rho = 0.415 p < .05).

The Table 1B shows the quantification of specific indicators in cases and controls. *Methanobrevibacter smithii* is under the method LOQ in all the samples, excluding 3 patients in which it is present at level of 10^7 gene copies/g stool. All the 3 patients are comprised into the same cluster (TDM1 cluster). There was no significant difference between the groups, except for *M. smithii* (Table 1B). Such methanogen species is increasing during the time starting from the onset in T2DM patient cohort [18] and in obese cohort. *M. smithii* seems to be able to potentiate the process of adipose tissue build-up and thus the obesity status [19]. Moreover, it is suggested a significant correlation between breath out methane and autonomic neuropathy exacerbation in T1DM [20]. This evidence can also suggest an implication of *M. smithii* – as major human gut methanogen – in such disease.

Conclusion

These are only preliminary data on the first part of the cases and controls recruitment. The data are yet lack in numbers and no NSG technique were yet applied, however it seems clear that some bio-indicators could be relevant at the end of the study. In particular, *M. smithii*, generally very low in children, as well Simpson's diversity index could become significant T1DM bio-indicators. Such research data will provide a tool to the T1DM early diagnosis and prevention.

Conflict of interest

None.

Acknowledgements

The authors wish to thank the Italian Ministry of Health for funding (RF-2011-02350617) and make a special acknowledgement to the participant children and their families.

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