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Evaluation of Gut Microbiota in Parkinson's Disease using Gas Chromatography with Mass Spectrometric Detection

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Abstract—The paper presents preliminary results of a comparative study assessing the gut microbiota in patients with Parkinson's disease and the control group using the gas chromatography with mass spectrometric detection. Sixteen patients with stage 3 Parkinson's disease and 94 age-matched persons without Parkinson's disease were examined. It was revealed that the total number of microbial markers in parietal intestinal microbiota in patients with Parkinson's disease was increased by 43% compared with the control group. This increase is due to a 2-fold increase in the number of conditional-pathogenic flora, and at the same time there was a 2-fold decrease in the number of microbial markers of useful microflora. The obtained results may be regarded as preliminary and need to be assessed in a large cohort of patients with Parkinson's disease. It is also necessary to assess the relationship between immune status and changes in microbiota, and to develop methods of correction of the revealed changes. Analysis of the efficiency of restoration of qualitative and quantitative composition of microbiota should be carried out using methods for the assessment of bioequivalence levodopa dose.

Keywords: Parkinson's disease, gut microbiota, gas chromatography-mass spectrometry

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Autonomic disorders are an integral part of the clinical presentation in most patients with Parkinson's disease (PD) [1–3]. Up to 80% of patients with PD have gastrointestinal problems [4], including intestinal dysmotility and constipation. The pathophysiology of constipation in PD is due to degeneration of the enteric nervous system caused by deposition of the pathological isoform of the α-synuclein protein [5]. This is accompanied by local inflammation, oxidative stress and intestinal permeability. The described changes long precede the motor disorders in PD, which leads to the hypothesis that the pathophysiological process of synucleinopathy begins in the gastrointestinal tract [6].

Constipation is not the only gastrointestinal problem that develops in PD. Increasingly, researchers are focusing on the gut microflora/microbiota composition in this disease. There is a reason why the gut microbiome (GMB) is called the 'forgotten organ'. It includes up to 100 trillion bacteria, which is ten times more than the number of cells in the human body. The whole genome of GMB is about 3 million genes, which is 150 times larger than the human genome. One-third of the GMB is the same in most people, while two-thirds are unique in each person [7]. Up to 50–60% of the GMB composition has not been fully studied [8]. There are hypotheses about the possible impact of such a large population of bacteria and their genome on human behaviour and physiology; in particular, a theory of a bidirectional gut microbiota-brain axis [9].

The interaction between the so-called small intestinal bacterial overgrowth (SIBO) syndrome, which is increased colonization of the small intestine by microflora, and PD is currently being intensively studied. This syndrome is detected in a quarter of patients with PD, which significantly exceeds its prevalence in the general population. Recent studies have shown that the stool of patients with PD contains a significantly reduced number of bacteria from the *Prevotellaceae* family compared to the control group, as well as increased levels of E. coli endotoxin [10]. There is a statistically significant decrease in the level of bacteria of the genus *Blautia*, *Coprococcus* and *Roseburia* in the stool of patients with PD compared to the control group. In addition, a decrease in the level of *Faecalibacterium* bacteria in patients with PD is combined with an increase in the level of *Ralstonia* in the intestinal mucosa [10].

At the genetic level, the GMB of patients with PD shows a significant dysregulation of genes involved in lipopolysaccharide synthesis and secretion [11]. The severity of the SIBO syndrome correlates with the

severity of motor disorders in patients with PD, and its correction leads to a decrease in the severity of motor fluctuations [12]. Moreover, there was a positive correlation between the number of enterobacteria and the severity of postural instability and ambulatory disturbances in patients with PD [13]. These data suggest that dopamine synthesis in the brain is influenced by dopamine-producing enzymes, whose activity is controlled by the GMB [14]. Considering that *Bacillus* bacteria, which are part of the GMB, are able to synthesize dopamine [15], we can assume that approximately half of the dopamine level in the body is contained in the GMB [16].

The influence of the GMB on the central nervous system can be seen in gnotobiotes, as well as when taking oral antibiotics and probiotics [17]. Gnotobiotic animals (gnotobiotes) are those obtained after a hysterectomy so that the mother's microbiota is not transferred to the animal during its passage through the birth canal. Gnotobiotes are raised under special conditions and are completely free of microflora, or are carriers of only certain types of microorganisms. The level of catecholamines (dopamine, noradrenaline and serotonin) measured in different parts of the brain (frontal cortex, striatum and hippocampus) differs significantly between gnotobiotic mice and mice from a control group. In sterile mice, the level of catecholamine metabolism in the striatum [18] and the overall level of dopamine in the brain [19] is significantly higher compared to the control group. These results explain the increased motor activity of sterile mice compared to the control group [18]. The synthesis of enzymes involved in the conversion of tyrosine to dopamine (tyrosine hydroxylase, DOPA decarboxylase) is influenced by the gut-brain axis [20]. There is thus a high probability that the level of dopamine in the brain stem is controlled by symbiotic bacteria. Moreover, the level of cerebral tyrosine is controlled specifically by GMB, because there is a significant decrease in sterile mice compared to the control group [19].

In gnotobiotic mice, there was increased expression of the dopamine receptor D1 gene in the hippocampus and a decrease in the striatum, in contrast to the control group. Expression of the nerve growth factor gene (*NGFI-A*), which plays an important role in neuroplasticity, is significantly reduced in the prefrontal cortex and striatum of sterile mice, which reflects decreased striatal synaptic plasticity. Increased expression of proteins involved in synaptogenesis (synaptophysin and PSD-95) was found in the striatum of sterile mice. At the same time, there were no differences in the frontal cortex and hippocampus in sterile mice and the control group. An additional study [18] showed a difference between sterile mice and the control group in the striatal expression of another 23 genes. These results confirm the influence of GMB on the striatum, which probably plays an important role in the pathogenesis of PD.

Among antibiotics, special attention should be paid to minocycline, a semisynthetic tetracycline antibiotic which, in addition to its effect on GMB, leads to a decrease in the activity of caspase-1 (interleukin-1β converting enzyme) and inducible nitric oxide synthase, which play an important role in apoptosis. Minocycline inhibits neurodegeneration of dopaminergic nigrostriatal neurons and depletion of dopamine in the striatum and nucleus accumbens in induced 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine Parkinsonism in mice [21]. *In vitro* minocycline has a neuroprotective effect on tyrosine hydroxylase-immunoreactive neurons in the rotenone model of PD [22]. It was found in experiments with fruit flies that minocycline has anti-inflammatory and antioxidant activity, with a potential dopaminergic effect [23]. Minocycline has shown itself as a potential drug for PD in phase II clinical trials, with phase III currently being planned [24]. It has the ability to restore the physiological GMB by reducing the *Firmicutes/Bacteroidetes* ratio [25]. Considering this fact and the discovered neuroprotective properties of minocycline in PD, we can assume that symbiotic gut bacteria play an important role in the pathogenesis of PD. Ampicillin also has a certain neuroprotective effect in PD. Ampicillin therapy prevented motor and behavioural disorders in mice injected with a group A streptococcus antigen, leading to dysfunction of the dopaminergic system [26]. The administration of ampicillin contributed to an increase in the level of tyrosine hydroxylase, D1- and D2-receptors in the striatum, without reducing the group A streptococcus antigen level. Since PD involves a pronounced dysfunction of the central dopaminergic system, we can hypothesize that ampicillin acts indirectly by affecting the GMB and the gut-brain axis.

In theory, the use of probiotics in correct doses can have a positive effect on the small intestine bacterial overgrowth syndrome and, presumably, on the central nervous system. The ability of the probiotic bacteria *Bacillus* sp. *JPJ* to convert L-tyrosine to levodopa (99.4% conversion) *in vitro* was demonstrated in 2011 [27]. Lactobacteria and bifidobacteria can produce antioxidants, vitamins and biologically active substances [28] and, therefore, can limit excess numbers of free radicals, leading to a decrease in neurodegeneration. A pilot study was conducted, which showed that regular consumption of fermented milk containing *Lactobacillus casei Shirota* reduces constipation severity in patients with PD [29].

The effect of chronic intestinal infection on the course of PD was studied most fully in the *Helicobacter pylori* model, which is often present in patients with PD. Carrying this bacterium leads to a decrease in levodopa absorption and increased motor fluctuations [30], and *H. pylori* infection leads to a decrease in dopamine levels in the motor cortical areas in mice [31]. *H. pylori* colonization is probably not directly involved in the pathogenesis of PD but leads to sys-

temic inflammation and an autoimmune response [32]. Eradication of the bacterium, in turn, reduces the severity of cachexia [33], increases levodopa absorption and reduces the degree of disability in patients with PD [34].

Thus, there are strong reasons to believe that qualitative or quantitative changes in the GMB can play a significant role in the pathogenesis of PD.

The evaluation of GMB changes in PD can help to solve practical problems such as:

—a final description of the hallmarks of GMB;

—assessing the relationship between the severity of GMB dysfunction and chronic inflammation in PD;

—an objective assessment of the effect of an altered GMB on levodopa absorption and, as a result, on the severity of motor disorders and motor fluctuations;

The methods for determining the microecological status as well as diagnosing infections, which are currently used in clinical practice, have certain limitations and disadvantages. For example, a significant disadvantage of the classic bacterial culture, in addition to the high cost and duration (7–10 days), is the inability to assess the role of uncultivated microorganisms in the infectious and inflammatory process, which is primarily associated with anaerobic bacteria. The immunoserological method, used as an adjunct to the classic method, is indirect since it does not detect the pathogen but the immune response to it, which may have individual variations. The well-known molecular biological methods, despite their advantages (direct identification of the pathogen, high specificity and sensitivity, universality, speed, the possibility of diagnosing chronic and latent infections), also have serious disadvantages such as frequent false positive results and the inability to adequately quantify the result [38].

Based on the above, there is an obvious need for a reliable, quantitative, rapid diagnostic test for dysbacteriosis and to identify the infectious agents. In our opinion, this could be chemodifferentiation of microorganisms using gas chromatography combined with mass spectrometry (GC-MS), based on the quantitative measurement of bacterial markers (fatty acids, aldehydes, alcohols and sterols).

GC-MS provides unique information about the composition of specific chemical monomer components of microbial cells entering the blood plasma and characteristic of certain taxa. These components (markers) can be isolated from the other chemical components of the total biomass of biological objects and used to detect microorganisms of the relevant genus or species. The analysis involves the direct extraction of higher fatty acids from the study sample using a chemical procedure, their separation on a chromatograph in a high-resolution capillary column and dynamic analysis of the composition on a mass spectrometer. Since the chromatograph is combined with the mass spectrometer in a single device, and is equipped with a computer with the appropriate programs for automatic analysis and data processing, the analysis itself takes 30 minutes, and the whole process, including time for preparing samples and calculating data, is no more than 3 hours. The result is a quantitative measurement of the microorganism composition present in biological fluids and tissues.

The fatty acid composition of most clinically significant microorganisms has been well studied to date, with proven reproducibility and genus and species specificity.

The proposed technology allows these compounds to not only be monitored in the samples but also the number of microorganisms of a certain taxon in the sample to be calculated. This is the fundamental difference of this method and what gives it a new qualitative property – the ability to separate the entire pool of microbial markers to assess the contribution of each of the hundreds of species of microorganisms that are present, for example, in faeces. In 2010, the GC-MS method was approved by Roszdravnadzor for use as a new medical technology in 'Assessment of the human microecological status by chromatography-mass spectrometry'. The homeostatic features of serum microbial markers, identified through systematic research, and the correspondence between the homeostatic profile and the GMB composition in healthy people has provided a unique opportunity to monitor the state of the GMB using blood tests.

The aim of the study was to assess the qualitative and quantitative composition of mural GMB in patients with PD and to compare it to the GMB composition in the control group using GC-MS.

MATERIALS AND METHODS

The pilot study included 16 patients (7 men and 9 women aged 58–67 years) with stage 3 PD on the Hoehn and Yahr functional scale. The comparison group included 94 patients (25 men and 69 women aged 55–65 years) with different physical conditions (selected by continuous sampling), who underwent inpatient examination and treatment at the Federal State Funded Institution, Nikiforov All-Russian Centre for Emergency and Radiation Medicine, and had no neurological diseases.

The quantitative composition of mural GMB was determined using the Agilent 7890 gas chromatograph (Agilent Technologies, USA) with mass selective and flame ionization detectors.

The taken blood samples in a volume of 40 μl were dried with the addition of an equal amount of methanol and subjected to acid methanolysis in 1 M HCl in methanol. Methanolysis was conducted in 0.4 ml of reagent per 10–15 mg of dry residue for 1 hour at 80°C. The release of fatty acids and aldehydes from the complex lipids of microorganisms and other liquid cells, in the form of methyl esters and dimethyl acetals, occurred at this stage. These components were

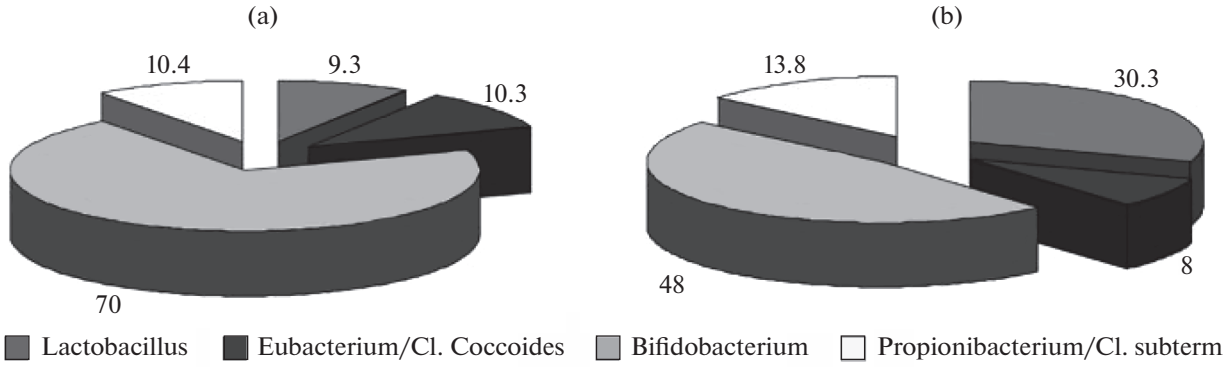

Fig. 1. Proportion of particular microorganisms in the structure of intestinal microbiota.

extracted with hexane (400 μL) for 5 min, then the hexane extract was dried, and the dry residue was treated with 20 μl of N,O-bis (trimethylsilyl)-trifluoroacetamide for 15 min at 80°C to obtain trimethylsilyl esters of oxyacids and sterols. Eighty microlitres of hexane were added to the ether reaction mixture.

To perform the analysis, 2 μL of the ether mixture were added to the GC-MS injector by means of an automatic sampling system (autosampler), which ensures the reproducibility of the retention time of chromatographic peaks and increases the accuracy of automatic data processing.

Sample chromatography was performed on a methyl silicone bonded phase capillary column HP-5ms, 25 m long and an internal diameter of 0.25 mm, with helium as the carrier gas. Programmed mode analysis, heating rate of the column thermostat—7°C/min in the 135–320°C range. Holding time at the initial temperature—1.5 min; evaporator temperature —250°C, interface temperature 250–300°C. Intervals and ions were selected in such a way as to selectively detect the markers of the species of microorganisms being identified.

The combined statistical indicators of mural GMB were calculated based on the principles set out in article [39]: total number of cells, number of markers of beneficial and opportunistic microflora, anaerobes, aerobes and their ratio.

Statistical processing of the results (median, quartiles and 50% confidence interval) was carried out using the Statistica 6.0 software package.

RESULTS AND DISCUSSION

The total number of microbial markers was 43% higher in the mural intestinal layer of patients with PD as compared to the control group (Table 1). The quantitative and qualitative composition of the GMB was different. Patients with PD had more microbial markers of opportunistic microflora than the control group: *Staphylococcus intermedius*—by 61%, *Eubacterium lentum* (group A)—5.8 times, *Clostridium hystolyticum*—2.8 times, *Peptostreptococ. anaerobius* – 3.6 times, *Ruminicoccus*—3.8 times, *Nocardia* and *Nocardia asteroides*—2 times, *Clostridium propionicum* and the *Enterobacteriaceae* family (*E. coli*, etc.), as well as microbial markers of Saccharomyces (sitosterol)—1.7 times, Saccharomyces (campesterol) and microbial markers of Herpes—2.8 times. The number of microbial markers of *Propionibacterium* and several other bacteria (*Streptococcus*, *C. difficile*, *Propionibacterium jensenii* and *Propionibacterium acnes*) was reduced by half.

The number of microbial markers of beneficial microflora in patients with PD was lower than in the control group: *Eubacterium/Clostridium coccoides*—by 6.3 times, *Bifidobacterium*—2.5 times, *Propionibacterium/Clostridium subterminale* —1.5 times, *Lactobacillus* – 24% (Fig. 1).

Thus, in patients with PD, the ratio of beneficial to opportunistic microflora was almost 4 times lower than in the control group (Table 2). The number of aerobic bacteria was double the number of anaerobes, as indicated by a decrease in the ratio of anaerobes to aerobes by 1.8 times.

The total number of microbial markers was thus 43% higher in the mural intestinal layer of patients with PD as compared to the control group. This increase is due to a twofold increase in the number of opportunistic microflora markers alongside a twofold decrease in the number of beneficial microflora markers.

For study reliability, the control group included older patients, whose GMB composition was significantly different from the conventional 'norm'. A comparison with the values in healthy younger people [38] will be incorrect in this case, because the differences between the groups will be much higher.

The obtained results are intermediate, and an assessment of GMB in a larger group of patients with PD is necessary to clarify them. In the future, once reliable and validated data is obtained, the focus should be developing ways to correct dysbiosis using antibiotics and metabiotics, and to assess the resulting

Table 1. Statistical indicators of near-wall intestinal microbiota in the control group and patients with Parkinson's disease (number of cells/g × 10^5)

Groups and taxons of microorganisms	Control (n = 94)		Parkinson disease (n = 16)	
	mediana	50% DI	mediana	50% DI
Gram-positive coccuses, aerobic or facultative				
Streptococcus (*oral forms*)	2670	2075–3655	–	–
Staphylococcus intermedius	1110	715–1630	1786*	1540–3482
Streptococcus mutans	270	200–350	292	279–344
Anaerobi				
Eubacterium lentum (*грунна A/group A*)	224	130–430	1300*	1238–1709
Eubacterium/Cl. coccoides	5130	2760–9300	817*	662–962
Clostridium hystolyticum	536	270–1070	1520*	1055–2010
Clostridium ramosum	5084	3840–7050	4348	3774–5292
Cl. difficile	130	86–200	–	–
Clostridium propionicum	–	–	11711	11119–13138
Propionibacterium	10	0.1–40	5*	2–31
Propionibacterium/Cl. subterm.	1350	730–2130	911*	897–1809
Propionibacterium jensenii	310	100–850	–	–
Propionibacterium acnes	44	0.1–135	–	–
Peptostreptococ. anaerobius 1, 2	131	0.1–230	475*	56–1498
Lactobacillus	8100	5700–11860	6167*	5550–7432
Bifidobacterium	2330	1400–050	914*	714–1621
Actinobacteria	35	25–40	36	25–52
Actinomyces viscosus	790	510–1190	883	464–1168
Ruminicoccus	1110	710–1340	4208*	3633–4295
Gram-positive bacilli, aerobic or facultative				
Nocardia, 14:1*d*11	3500	2580–4675	6436*	5797–7003
Nocardia asteroides	1090	546–1600	2237*	2095–2781
Rhodococcus	203	130–265	181	155–203
Corineform CDC-group XX	400	270–523	333	258–630
Gram-negative bacilli, aerobic or facultative				
Family Enterobacteriaceae (*E. coli and others*)	0	0–34	158*	124–207
Fungi, viruses, etc.				
Micro-fungi, cytosterol	19	9–40	33*	17–41
Micro-fungi, campesterol	–	–	1008	852–1219
Candida	290	100–450	351	297–528
Streptomyces	266	177–407	194	139–529
Herpes	1380	340–3240	3932*	2881–3932
@Цитомегаловирус	–	–	3539	3108–4333
Pseudonocardia	35	20–50	30	27–42
Whole number of cells	36591	28876–38540	52196*	50525–68404

here and in the Table 2 * – $p < 0.05$ in comparison with control group (Mann–Whitney test).

Table 2. Combined statistical parameters of near-wall intestinal microbiota in the groups under comparison (number of cells/g × 10^5)

Parameters of microflora	Control (n = 94)		Parkinson disease (n = 16)	
	mediana	50% DI	mediana	50% DI
Markers of useful microflora	16910	13500–26800	8667*	7963–11873
Markers of opportunistic microflora	17691	15300–24100	36431*	34295–46910
Markers of useful microflora/markers of opportunistic microflora	0.96	0.79–1.43	0.25*	0.22–0.27
Anaerobi	25358	19800–34200	26534	36028–36494
Aerobi	9243	630–17000	17891*	16484–20606
Anaerobi/aerobi	2.74	2.51–3.08	1.56*	1.40–1.67
Total sum	36591	36800–47800	52196*	50525–68404

changes in immune status and motor fluctuations over time. It is obvious that to reflect the changes in levodopa absorption secondary to GMB correction, assessing only the patient's subjective opinion (Hauser's diaries) is insufficient. We are currently implementing methods for assessing the bioequivalence of levodopa doses in the plasma, which will objectively assess the effect of GMB correction on levodopa pharmacodynamics and pharmacokinetics.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare there is no conflict of interest.

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