GUT MICROBIOTA ALTERATIONS AND THEIR ASSOCIATION WITH IL6, IL8 AND TNFα LEVELS IN PATIENTS WITH EXTERNAL GENITAL ENDOMETRIOSIS

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Today, the association of gut microbiota with external genital endometriosis (EGE) is of special scientific interest. The study was aimed to assess alterations of the gut microbiota taxonomic composition and explore their correlations with plasma levels of IL6, IL8 and TNFα at the species level in patients with EGE. The cross-sectional comparative study involved 50 patients with EGE (index group) and 50 healthy women (control group). The changes in the gut microbiota taxonomic composition and plasma levels of IL6, IL8 and TNFα were assessed. A significant decrease in the abundance of such species, as *Coprococcus catu* ($p = 0.009$), *Turicibacter sanguinis* ($p = 0.008$) and *Ruminococcus gnavus* ($p < 0.001$), along with the increase in the abundance of *Eubacterium ramulus* ($p = 0.040$), *Bacterioides dorei* ($p = 0.001$), *Prevotella divia* ($p = 0.008$) and *Shigella flexneri* ($p < 0.001$) were found in the gut microbiota taxonomic composition in patients with EGE. Significant correlations between the IL6 levels and the abundance of Turicibacter sanguinis ($r = –0.92$; $p = 0.001$), IL8 levels and the abundance of *Shigella flexneri* ($r = 0.72$; $p < 0.001$), TNFα levels and the abundance of *Prevotella divia* ($r = 0.77$; $p = 0.001$) were revealed. The findings add to the available literature data on the features of gut microbiota alterations and their association with some inflammation biomarkers in individuals with EGE, which can justify further research in this area and probably open up new approaches to treatment of the disease.

Keywords: external genital endometriosis, gut microbiota, IL6,IL8, TNFα.

Author contribution: Gumenyuk LN — study concept and design; Zemlyanaya IA, Rami A, Seroshtanov NA — data acquisition, analysis, and interpretation; Badula ES, Ismailov AR — statistical data processing; Kokareva SS, Cheremisova AA, Kupreichyuk YuR — manuscript writing.

Compliance with ethical standards: the study was approved by the Ethics Committee of the SI Georgievsky Medical Academy, VI Vernadsky Crimean Federal University (protocol № 10 of 14 November 2021), planned and conducted in accordance with the Declaration of Helsinki. The informed consent was obtained from all study participants.

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ИЗМЕНЕНИЯ МИКРОБИОТЫ КИШЕЧНИКА И ИХ СВЯЗЬ С ПОКАЗАТЕЛЯМИ IL6, IL8 И TNFα У ПАЦИЕНТОК С НАРУЖНЫМ ГЕНИТАЛЬНЫМ ЭНДОМЕТРИОЗОМ

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Ассоциация микробиоты кишечника и наружного генитального эндометриоза (НГЭ) на сегодняшний день представляет собой особый научный интерес. Целью исследования было оценить изменения таксономического состава микробиоты кишечника и изучить на уровне видов их взаимосвязь с показателями IL6, IL8 и TNFα в плазме крови у пациенток с НГЭ. В одномоментное сравнительное исследование было включено 50 пациенток с НГЭ (основная группа) и 50 здоровых женщин (контрольная группа). Оценивали изменения таксономического состава микробиоты кишечника и уровни IL6, IL8 и TNFα в плазме крови. У пациенток с НГЭ в таксономическом составе микробиоты кишечника обнаружены статистически значимое снижение представленности видов *Coprococcus catu* ($p = 0{,}009$), *Turicibacter sanguinis* ($p = 0{,}008$) и *Ruminococcus gnavus* ($p < 0{,}001$), повышение представленности видов *Eubacterium ramulus* ($p = 0{,}040$), *Bacterioides dorei* ($p = 0{,}001$), *Prevotella divia* ($p = 0{,}008$) и *Shigella flexneri* ($p < 0{,}001$). Выявлены статистически значимые корреляции показателя IL6 с представленностью *Turicibacter sanguinis* ($r = –0{,}92$; $p = 0{,}001$), IL8 и *Shigella flexneri* ($r = 0{,}72$; $p < 0{,}001$), TNFα с представленностью *Prevotella divia* ($r = 0{,}77$; $p = 0{,}001$). Полученные результаты дополняют имеющиеся литературные сведения о специфике изменений микробиоты кишечника и их сопряженности с некоторыми биомаркерами воспаления при НГЭ, что может стать обоснованием для продолжения исследований в этом направлении и, возможно, открывает новые подходы к лечению этого заболевания.

Ключевые слова: наружный генитальный эндометриоз, микробиота кишечника, IL6, IL8, TNFα

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Endometriosis is a significant issue of modern gynecology, it remains under active consideration over the decades. According to the aggregate data, more than 176 million women all over the world have endometriosis [1], and its prevalence rate grows steadily in recent years. It is important to note that endometriosis is associated with infertility in 50–80% of cases and chronic pelvic pain in 50% of cases [1, 2]. These conditions worsen the patients' mental and physical health, as well as their quality of life [3]. The difficulties in differential diagnosis of endometriosis often result in the diagnosis delay of 4–11 years, and 65% of women are misdiagnosed [3, 4], which results in the disease progression and grave consequences [5]. The today's pharmacological and surgical approaches to treatment of endometriosis are associated with the risk of severe side

effects and show insufficient efficiency. The relapse rate is still high: it reaches 15–21% [3]. That is why the search for new pathophysiological mechanisms underlying external genital endometriosis (EGE), as well as for safe and efficient methods for prevention and treatment of the disease, is still relevant.

EGE, characterized by proliferation of endometrial tissue outside of the uterine cavity, is conventionally considered as a chronic estrogen-dependent immune inflammatory disease that is limited to the pelvis [6]. However, today EGE is more and more often considered as a systemic inflammatory disorder often associated with heterogeneous multiple organ dysfunction [3, 7]. It is believed that aberrant cytokine production accompanied by the immune response dysregulation plays a vital part in pathophysiology of systemic inflammation associated with EGE. In this regard, pro-inflammatory interleukins (IL6, IL8) and tumor necrosis factor alpha (TNFα) are considered to be among the most important. Assessment of the cytokine profile in blood of patients with EGE made it possible to detect the elevated levels of IL6, IL8 and TNFα [8–10]. Furthermore, elevated plasma IL6 levels were associated with the pain severity [11], disease severity [12], and relapse rate [11] in patients with EGE. While plasma levels of IL8 were associated with the size of active lesions [13] and infertility [14], the levels of TNFα were associated with the severity of EGE clinical manifestations, disease activity and depth [15].

Current research suggests that gut microbiota is involved in EGE pathophysiology, which can be explained by its fundamental role in maintaining the immune homeostasis and direct association with the development of numerous inflammatory diseases [16]. The experiments involving the heterologous surgical injection murine model of endometriosis showed that gut microbiota affected the EGE course and progression [17, 18] via modulation of various immune system components [18]. Particularly, administration of normal murine fecal microbiota to mice with experimentally induced endometriosis and gut microbiota depletion was associated with the decline in the endometriotic lesion growth, while administration of fecal microbiota obtained from mice with endometriosis resulted in the disease progression. Furthermore, depletion of intestinal microbiota reduces the severity of inflammatory response associated with endometriosis [17] and modulates the abundance of immune cells in the peritoneum [18]. Finally, the papers provide strong evidence of changes in the intestinal microbiota profile in mice [18–20] and humans [18, 19]. At the same time, clinical data on the gut microbiota species composition in patients with EGE are fragmentary, contradictory and insufficient for unambiguous conclusions. Thus, among 16 studies, focused on assessing the relationship between EGE and microbiome, only six involved the analysis of gut microbiota, and only four involved assessment of human intestinal microbiome [23]. It is also important to note that, among the reviewed papers there are no studies focused on assessing gut microbiota alterations in patients with EGE of Slavic ethnic background. In particular, there is little information on the association between gut microbiota and inflammatory biomarkers in patients with EGE.

The study was aimed to assess alterations of the gut microbiota taxonomic composition and explore their correlations with plasma levels of IL6, IL8 and TNFα at the species level in patients with EGE.

METHODS

The cross-sectional comparative study was performed in the Saint Luke Multidisciplinary Clinic (Simferopol, Republic of Crimea). The study involved 50 patients aged 18–45 with the confirmed diagnosis of stage I–IV EGE admitted to the Gynecology Department (index group) and 50 age-matched healthy women who underwent preventive medical examination (control group). All EGE patients and healthy women submitted the informed consent to study participation.

Inclusion criteria for the index group: age 18–45 years; the diagnosis of EGE verified by laparoscopy and histological assessment.

Non-inclusion criteria for the index group: age < 18 of > 45 years; body mass index >24.9 kg/m^2; pregnancy and lactation; type I or II diabetes mellitus, concomitant chronic systemic and somatic disorders; history of mental and behavioral disorders; verified functional and inflammatory disorders of the gastrointestinal tract, hepatobiliary system; history of inflammatory disorders within a month before the study; history of stool problems (constipation/diarrhea) within a month before the study; taking hormonal oral birth control or anti-inflammatory drugs, antibiotics, probiotics, prebiotics, antiviral drugs, symbiotics or acid–suppression medications within three months before inclusion in the study; taking medications affecting the stool passage within eight weeks before inclusion in the study; refusal to participate in research.

Inclusion criteria for the control group: age 18–45 years; body mass index < 24.9 kg/m^2; no somatic disorders or allergy; no infectious or acute disorders within two months before inclusion in the study; no history of mental and behavioral disorders; no stool problems (constipation/diarrhea) within a month before inclusion in the study; taking no hormonal oral birth control or anti-inflammatory drugs, antibiotics, probiotics, prebiotics, antiviral drugs, symbiotics or acid–suppression medications within three months before inclusion in the study; taking no medications affecting the stool passage within eight weeks before inclusion in the study.

Non-inclusion criteria for the control group: body temperature above 36.9 °C.

The characteristics of patients with EGE and controls are provided in Table 1. The groups were matched for age (p = 0.94; χ^2) and body mass index (p = 0.052; χ^2). A total of 36 patients (70.0%) had stage III–IV EGE.

The diagnosis of endometriosis was verified during surgery in accordance with the criteria of the American Society for Reproductive Medicine (ASRM) classification.

To analyze the taxonomic composition of the gut microbiota of patients with EGE and healthy women, fecal samples were collected in the morning (8 a.m. to 10 a.m.), and in EGE patients sampling was performed on the day of hospital admission. The samples were frozen and stored in disposable plastic containers at a temperature of –80 °C prior to metagenomic analysis. Isolation of total DNA was performed by phenol-based

Table 1. Characteristics of patients with external genital endometriosis and healthy women

Parameter	EGE patients (n = 50)	Control group (n = 50)
Average age, years, median [25%; 75%]	37,0 [32,0; 44,0]	37,7 [32,7; 43,2]
Body mass index, kg/m^2, median [25%; 75%]	23,0 [21,0; 24,3]	22,06 [20,8; 24,1]
Stage I–II EGE, n (%)	14 (28,0%)	–
Stage III–IV EGE, n (%)	36 (70,0%)	–

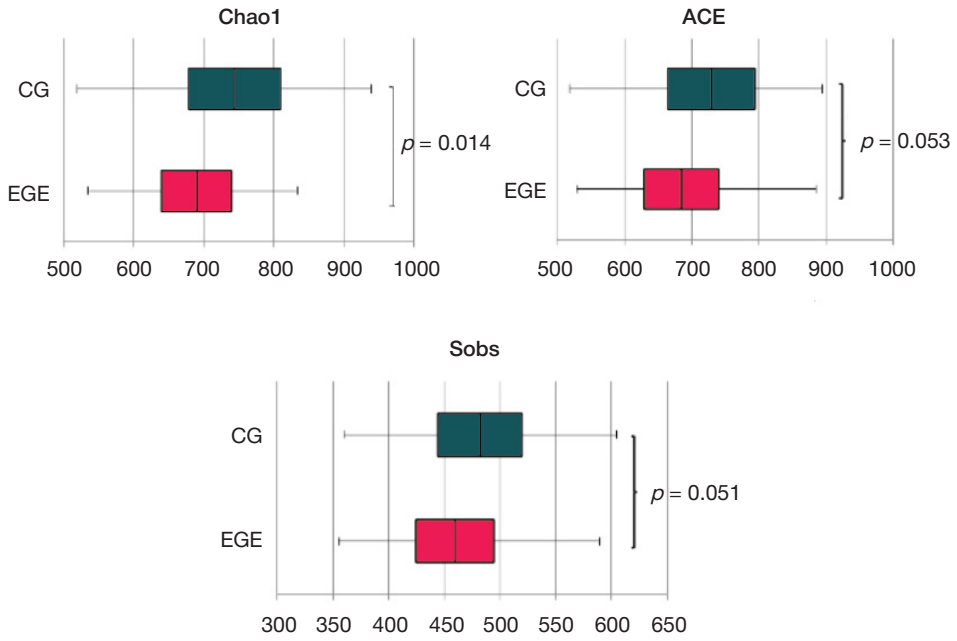

Fig. 1. Phylogenetic composition of gut microbiota in patients with external genital endometriosis (EGE) and healthy women. CG — control group

extraction; the DNA nucleotide sequence was determined by shotgun sequencing using the SOLiD5500 Wildfire high-throughput sequencing system (AppliedBiosystems; USA) [24].

The reads were filtered based on their quality, and the taxonomic classification was performed using the QIIME ver. 1.9.1 software [25]. Taxonomic assignment of the reads was based on the data taken from two taxonomic databases: during the first phase the reference set of bacterial operational taxonomic units (OTUs) was selected based on matching the acquired reads of 16S rRNA genes with the GreenGenes database, ver. 13.5 [26]. During the second phase taxonomic assignment of these OTUs was performed using the RDP algorithm based on the specialized HITdb human intestinal microbiota database [27].

The qualitative and quantitative assessment of gut microbiota composition was performed by identification of microbial species, genera, and phyla; the microbial community α-diversity was assessed by calculating the Chao1 index, the number of taxa observed (Sobs), and the indicator of species richness (ACE) using the Mothur v.1.22.0 software (http://www.mothur.org).

Blood samples of EGE patients and healthy volunteers to be used for immunosorbent assay were collected by venipuncture in the morning in a fasting state at rest (for at least 15 min). Plasma levels of IL6, IL8 and TNFα were assessed by enzyme-linked immunosorbent assay (ELISA) using the test system (Vector-Best; Novosibirsk, Russia). The tubes containing blood serum were frozen and stored at a temperature of –20 °C.

Statistical data processing was performed using the STATISTICA 8.0 software package (StatSoft.Inc.; USA). As for quantitative indicators, the distribution type was determined using the Kolmogorov–Smirnov test. Given that the majority of

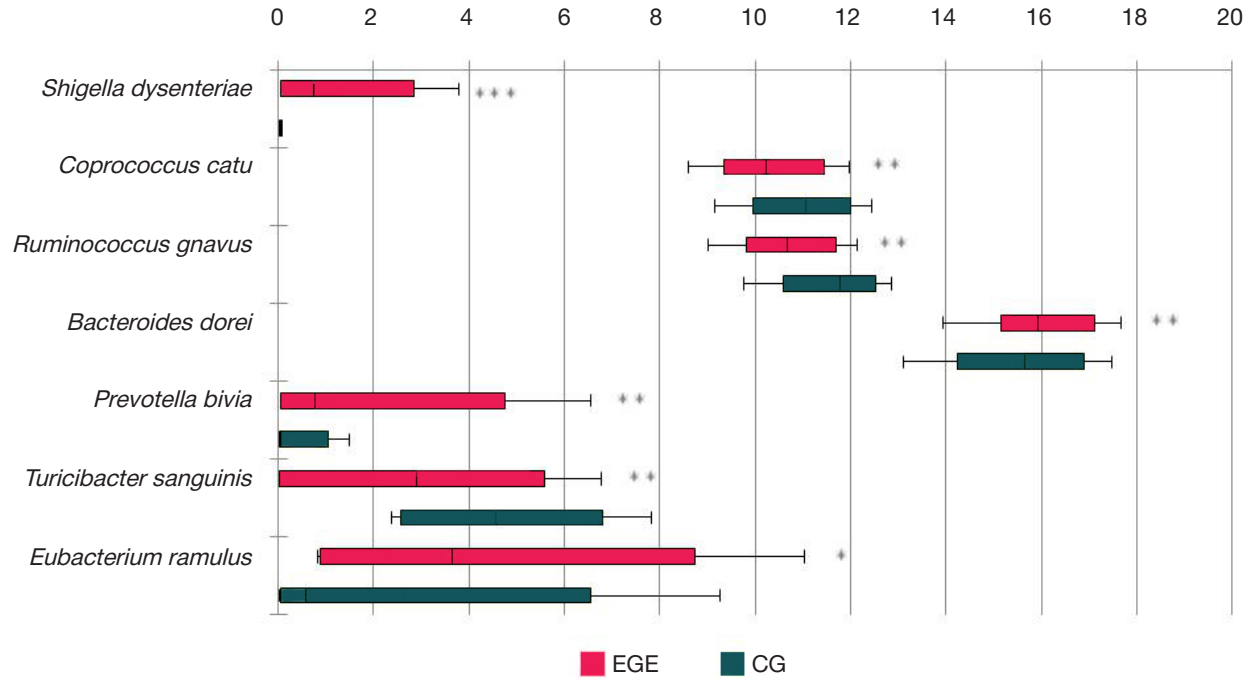

Fig. 2. Species composition of gut microbiota in patients with external genital endometriosis (EGE) and healthy women. CG — control group

Table 2. Comparative analysis of plasma IL6, IL8 and TNFα levels in patients with external genital endometriosis (EGE) and healthy women. CG — control group; *p* — significance of differences between the values of patients with EGE and the CG

Parameter	EGE patients (*n* = 50)	Control group (*n* = 50)	*P*
IL6, pg/mL, median [25%; 75%]	14,7 [8,1; 18,3]	3,8 [2,0; 6,6]	< 0,001
IL8, pg/mL, median [25%; 75%]	14,6 [9,6; 28,8]	2,2 [1,4; 6,8]	< 0,001
TNFα, pg/mL, median [25%; 75%]	17,9 [9,3; 26,5]	5,2 [2,8; 7,6]	< 0,001

quantitative indicators were not normally distributed, the median (Me) and interquartile range (25th percentile; 75th percentile) were calculated. As for qualitative traits, the percentage and absolute values were determined. The chi-squared test (χ^2) was used to compare qualitative traits, and quantitative traits were compared using the Mann–Whitney U test. Spearman's rank correlation was applied to assess correlations between the factors. The significance level for comparison of qualitative and quantitative traits, as well as for correlation analysis was set as $p < 0.05$.

RESULTS

Assessment of the gut microbiota taxonomic composition revealed a significant decrease in the bacterial community α-diversity (Chao1 index $p = 0.014$) in patients with EGE compared to healthy women. Furthermore, patients with EGE had lower ACE and Sobs indices than healthy women, however there were no significant differences between groups ($p = 0.053$; $p = 0.051$, respectively) (Fig. 1).

Comparative analysis of the gut microbiota species composition in patients with EGE relative to healthy women revealed a significant decrease in the abundance of *Coprococcus catu* ($p = 0.009$), *Ruminococcus gnavus* ($p < 0.001$) and *Turicibacter sanguinis* ($p = 0.008$) along with the increased abundance of such bacterial species, as *Eubacterium ramulus* ($p = 0.040$), *Bacterioides dorei* ($p = 0.001$), *Prevotella divia* ($p = 0.008$), and *Shigella flexneri* ($p < 0.001$) (Fig. 2).

The IL6, IL8 and TNFα plasma levels of patients with EGE were significantly higher than that of healthy women (Table 2).

At the same time we revealed a strong negative correlation between the abundance of *Turicibacter sanguinis* and the IL6 levels ($r = –0.92$; $p = 0.001$); there was a strong significant positive correlation between the increase in abundance of *Shigella flexneri* bacteria and the levels of IL8 ($r = 0.72$; $p < 0.001$). Furthermore, a strong positive correlation between the TNFαlevels and the abundance of *Prevotella divia* ($r = 0.77$; $p = 0.001$) was reported.

DISCUSSION

Gut microbiota is associated with many inflammatory disorders, including EGE [16–19]. However, today there are just a few human studies on the issue, the results of which do not allow any consensus-based conclusions. Given the lack of knowledge of the issue, the primary objective of our study was to refine the gut microbiota taxonomic composition alterations in the group of patients with EGE. Our study has confirmed that gut microbiota composition of EGE patients is quite different from that of healthy women. The findings show that the lower bacterial α-diversity relative to healthy women is typical for patients with EGE, which is a common distinctive feature of chronic inflammatory disorders [28]. Our findings are consistent with the data of the earlier reported study [22], but do not confirm other data [21], according to which patients with EGE are characterized by the decrease in both α- and β-diversity. The results of our study have also shown that dysbiotic intestinal alterations in patients with EGE are characterized by the decrease in the abundance of bacteria having the potential for immunomodulation: *Coprococcus catu* and *Turicibacter sanguinis* species representatives that are known to produce short-chain fatty acids (SCFAs), i.e. endogenous signaling molecules essential for maintaining the host's immune homeostasis, and *Ruminococcus gnavus*. Moreover, the decrease in the levels of SCFAs results in the increased abundance of Gram-negative bacteria, and therefore lipopolysaccharide (LPS) levels [29]. There is evidence that feces of mice with endometriosis have low levels of SCFAs, specifically butyrate, while butyrate administration inhibits endometriotic cell growth *in vitro* and *in vivo* via inhibition of histone deacetylase activity and activation of expression of the Rap1GAP protein that inactivates the Rap1 intracellular signaling protein [19]. In addition, we have detected the increased abundance of *Eubacterium ramulus*, *Bacterioides dorei*, *Prevotella divia* and *Shigella flexneri*. Among these the presence of Shigella flexneri should be noted, since these bacteria have been earlier detected in the fecal samples of patients with stage III–IV EGE in the study [30]. It is suggested that this species plays a role of the trigger that initiates the immune alterations resulting in the development and progression of endometriosis [31]. Our findings are partially in line with the data of the number of other studies. For example, one of the studies has shown that the decrease in the abundance of *Coprococcus* along with the increase in the abundance of Bacterioides is typical for patients with EGE [21]. The other study has shown that patients with EGE are characterized by the increase in abundance of *Eubacterium* and *Bacterioides* [22]. The data obtained may be inconsistent due to the fact that, firstly, the studies involved patients of different ethnic groups, and secondly, in contrast to the listed above researchers, we did not enroll overweight patients with EGE (since the effects of this factor on gut microbiota alterations was proven) and the patients taking hormonal, birth control and anti-inflammatory drugs in order to avoid their effects on the study results.

As stated earlier, patients with EGE demonstrate a significant increase in plasma levels of IL6, IL8 and TNFα, the role of which in the disease development and progression to severe forms has been proven [8–10]. Our study has also revealed significantly higher levels of IL6, IL8 and TNFα compared to healthy women in patients with EGE. Meanwhile, intestinal dysbiosis, that is more and more often considered to be a factor of inflammation, autoimmune and immune-mediated disorders, can trigger the inflammatory immune response associated with elevation of pro-inflammatory cytokine levels at the whole-body level [32]. That is why the second objective of the study was to assess the association of gut microbiota composition at the species level with plasma levels of IL6, IL8 and TNFα in the group of patients with EGE. We have found that some intestinal microbial species of patients with EGE are associated with plasma levels of the studied cytokines, which can indicate the association of gut microbiota composition with EGE. In particular, a negative correlation between the elevated IL6 levels and the abundance of Turicibacter sanguinis bacteria has been revealed. We have found a probable explanation for

this correlation in the literature. As is well known, the Turicibacter bacteria are involved in production of metabolites having a protective effect on the intestinal epithelium and reproductive system, specifically such SCFAs, as acetic, valeric and butyric acids. The decrease in the levels of the latter leads to activation of histone deacetylase and the related NF-kB nuclear transcription factor, as well as to inhibition of the GPR41, GPR43 and GPR109A G protein-coupled receptors, thereby inducing expression of the genes responsible for synthesis of pro-inflammatory cytokines, including IL6 [33], and promoting the development of chronic inflammation [16]. The earlier reported [34] association of the IL8 levels with the abundance of bacteria of genus *Subdoligranulum* in patients with EGE has not been confirmed in our study. According to our findings, a positive correlation of the IL8 levels with the abundance of *Shigella* flexneri bacteria is typical for patients with EGE, which can be mediated by the ability of the latter to induce persistent NF-kB inhibitory kinase complex (IKK) activation and subsequent I-kB degradation via initiation of the pattern recognition receptors TLR4. This, in turn, promotes the release of NF-κB with subsequent translocation into the nucleus and triggering the IL8 transcription [35]. The literature reports such associations in patients with confirmed *Shigella* infection (*shigellosis*) that have been confirmed by strong positive correlations between the abundance of *Shigella* flexneri and the levels of IL8 in blood plasma [36]. As we have already stated, the contrast between our findings and the results of the study these are compared with may be due to the differences in design, specifically to the fact of selective enrollment of normal-weight EGE patients having no extragenital comorbidities in our study, while in the other study [34] these characteristics were not considered as exclusion criteria. Furthermore, the differences may result from the fact that we enrolled patients with stage I–IV EGE, while the study [34] involved patients with stage III–IV EGE. This fact could also affect the differences between the associations of IL8 with gut microbiota representatives in EGE patients and the associations reported in the literature. The small sample size (12 patients) used in the earlier reported study should be also noted [34]. Moreover, our study revealed a strong positive correlation between the TNFα blood levels and the abundance of *Prevotella* divia. We have found no reports of the research focused on studying this subject in patients with EGE. However, it has been previously shown that treatment of monocytic cell line with LPS from *Prevotella* results in simultaneous activation of three basic signaling pathways of mitogen-activated protein kinase (MAPK) (extracellular signaling kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2), and p38) with subsequent induction of the TNFα mRNA expression and TNFα secretion stimulation [37].

Our findings suggest that gut microbiota plays a vital part in EGE immunogenesis. Apparently, the causal relationships between gut microbiota and blood levels of pro-inflammatory cytokines in individuals with EGE require a more detailed study and further research in this area.

CONCLUSIONS

Significant alterations in the gut microbiota abundance and taxonomic composition have been found in patients with EGE. Furthermore, the significant correlations of some bacterial species with plasma levels of IL6, IL8 and TNFα we have revealed suggest the association of the gut mictobiota abundance and composition with the EGE immunopathogenesis. Further research is required to confirm the role of gut microbiota in the EGE pathophysiology. The targeted effects on gut microbiota may contribute to the efficiency of approaches to treatment of EGE.

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