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FERROPTOSIS IN CD4+ AND CD8+ T-CELLS IN THE SETTINGS OF HIV INFECTION

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ABSTRACT

Introduction: Elevation of intracellular iron concentration triggers ferroptosis. Understanding the regulation and pathophysiological mechanisms of this process in HIV infection may contribute to antiretroviral therapy (cART) monitoring.

Aim: To perform a correlation analysis of the intracellular labile-bound iron pool (LIP) in CD4+ and CD8+ T cells in association with CD4+, CD8+ T cells absolute count (AC) and CD4/CD8 index in HIV+ individuals on continuous cART with sustained viral suppression.

Material and methods: Peripheral blood samples (Li heparin, n=34) were collected in the course of the routine immune monitoring of HIV+ individuals at four time points during 24 months. Plasma HIV viral load (VL) was determined with the Abbott Real-Time HIV-1 test (sensitivity 40 copies/ml). AC and percentage of CD4+ and CD8+ T cells were determined by direct flow cytometry (Multitest, BD Trucount, FACS Canto II). The intracellular content of LIP in CD4 and CD8 T cells ($LIP_{CD4,}$ $LIP_{CD8)}$ was measured at the beginning of the study, using acetoxymethyl ester and subsequent incubation with a chelator (Deferiprone). LIP was quantified according to the mean fluorescence intensity (MFI) (FACSCanto II, Diva 6.1.2).

Results: In the settings of a higher LIP_{CD4} , high LIP_{CD8} correlated with increased CD8AC (Rho= 0.70, $p<0.05$) up to 11 (min. 6, max. 15) months after LIP measurement., and decreased CD4/CD8 ratio correlated inversely with LIP_{CD8} in all consecutive measurements (Rho= -0.71, $p<0.01$ for all), Importantly, high LIP_{CD8} correlated with a lower CD4AC (Rho= -0.65, $p<0.05$) up to five (min.1, max.8) months after LIP measurement.

Conclusion: The increased concentration of intracellular LIP in CD8 cells in HIV+cART individuals could indicate viral activity in the settings of undetectable HIV VL, directly associated with ongoing cell ferroptosis.

Keywords: HIV, ferroptosis, immune restoration

INTRODUCTION:

Ferroptosis was recently identified as a non-apoptotic, iron- and reactive oxygen species (ROS)-dependent form of lytic cell death characterized by mitochondrial dysfunction, and accumulation of lipid peroxides on biological membranes (1, 2, 3). It has been implicated in a variety of human diseases, including cancer cells' death, neurotoxicity, neurodegenerative diseases, acute renal failure, hepatic inflammation, heart ischemia/reperfusion injury, as well as in HIV, COVID-19, tuberculosis and other infections (3, 4). The role of ferroptosis in viral infections is just beginning to be elucidated.

Ferroptosis results from imbalanced production and degradation of intracellular lipid ROS. Optimal concentrations of ROS are critical for normal cell function and survival. Excessive and uncontrolled production as well as accumulation of ROS due to insufficient cellular antioxidant capacity can be detrimental to the cells (4). When glutathione, the main intracellular enzyme responsible for the reduction of ROS is lacking, cells become susceptible to lipid peroxidation (5, 6). The enzyme lipoxygenase catalyzes this process, utilizing iron as an essential cofactor (7, 8). Thus iron participates in the production of ROS and oxidative stress. Dysregulation of cellular labile iron levels can be triggered via higher accumulation of ferrous iron Fe^{2+} in the cytoplasm (labile iron pool, LIP), increased Heme oxygenase

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1 (HO-1), increased transferrin uptake, reduced expression of ferroportin and depletion of ferritin (4, 8, 9). The transferrine receptor (CD71) can transport exogenous iron ions into the cell by binding to iron storage ferritin, creating an intracellular labile iron pool, which induces the Fenton reaction, leading to the production of ROS (9).

Ferroptosis, oxidative stress, and mitochondrial disruptions are all closely associated and may cooperate to contribute to the residual inflammation and latent viral load in the settings of treated chronic HIV infection (8, 10).

The objective of the present study was to analyze labile bound iron (LIP) in CD4 (LIP_{CD4}) and CD8 T cells (LIP_{CD8}) of HIV+ patients on continuous cART with sustained viral suppression (SVS) in correlation with the routinely monitored immunological parameters CD4 T AC and CD4/CD8 ratio.

MATERIAL AND METHODS

Study design and participants

This study was approved by the Ethical Committee at the National Center of Infectious and Parasitic Diseases, Sofia (Approval number: 2019-026-01). The study was conducted during the follow-up of patients according to European ethical standards.

Peripheral blood samples (Li heparin, n=25) were obtained in the course of routine immune monitoring of HIV+ male individuals, registered at the Specialized Hospital for Active Treatment of Infectious and Parasitic Diseases, Sofia. Four samples per participant were analyzed in the course of 24 months at five-month (range 2-8 mo) intervals,

The inclusion criteria were: continuous cART for more than four years and SVS for at least two years.

Methods: Plasma HIV viral load (VL) was determined with reverse transcription polymerase chain reaction (Abbott Real-Time HIV-1) with the lowest limit of detection at 40 copies/ml.

The absolute count (AC) and percentage of CD4+ and CD8+ T cells were determined by direct flow cytometry (Multitest, BD Trucount, CD3/CD8/CD45/CD4/TRU Count, FACS Canto II) as described before (10).

The intracellular content of LIP was determined at the beginning of the study. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque Plus (Sigma-Aldrich) and incubated with calcein acetoxymethyl ester (CA-AM, Biolegend) followed or not by chelator (Deferiprone, Sigma Aldrich) using the method described by Prus et. al (11) and modified by Emilova et al (10). The difference between the mean fluorescence intensity (ΔMFI) of chelator-treated and untreated cells was used to evaluate the amount of LIP in CD4+ and CD8+T (FACSCanto II, Diva 6.1.2).

Statistical analysis: Between group differences were evaluated with nonparametric Mann–Whitney U test, and data are presented as median, min-max. Relationships between two variables were analyzed by Spearman's rank correlation coefficient. P values less than 0.05, at CI 0.95 were considered significant (SPSS Statistics v.23.0 and Graphpad Prism v.9.0).

RESULTS:

Patients' characteristics are given in Table 1. Despite sustained viral suppression in all participants, immunological responses to cART varied during the follow-up period. Baseline CD4AC and CD4/CD8 ratio ranged from 220 to 1032 cells/μl, and 0.26 to 2.01, respectively. The last measured values ranged from 292 to 1519 cells/μl, and 0.31 to 1.93 respectively. Notably, approximately 45 % of participants had a suboptimal CD4/CD8 ratio (<0.9). A wide range of LIP_{CD4} (median 625, min 83 – max 2935) was observed that was not associated with the age or the immune responses to therapy. Based on the median of LIP_{CD4} two groups were defined: Group A (n=14) with lower than median LIP and B (n=11) with higher than median LIP (320, range 83-594 vs. 1342, range 625-2935, p<0.0001) **(Table 1)**.

Interestingly, in group B a higer LIP_{CD8} was found as compared to group A (LIP_{CD8} 1121, range 389-2968 vs. 444, range 154-1045, p<0.01), indicating concomitant changes of LIP in the two T cell subsets **(Fig.1)**.

While CD4AC and CD4/CD8 did not differ significantly between the groups for the whole follow-up period, a slight decline of CD4 AC was observed at the second determination in group B **(Fig.2)**, as opposed to some increase of CD4 AC in group A **(Table 1, Fig.2).**

At high LIP_{CD4} values (group B), a strong inverse correlation was observed between CD4/CD8 and LIP_{CD8} at all studied points (Rho= -0.65, p<0.05 for all, **fig 3A**), which was associated with an increase of CD8AC (Rho= 0.70, p<0.05, **fig3B**) up to 11 (min. 6,

Table 1. Clinical and laboratory characteristics of study participants.
All data are represented as median, min-max.

HIV+ participants	Group A low LIP_{CD4}	Group B high LIP_{CD4}	Mann–Whitney U test
Number (n)	14	11	p>0.05
Age (years)	42 27-52	39 26-53	
Time after diagnosis of HIV infection (years)	8.5 6-24	7.0 5-13	
cART duration (years)	7.0 6-20	6.0 5-11	
Baseline* CD4 AC (cells/µl)	557 220-947	633 264-1032	
Second CD4 AC (cells/µl)	638 247-1145	506 232-981	
Third CD4 AC (cells/µl)	737 264-1007	581 312-1123	
Last CD4 AC (cells/µl)	724 292-1125	797 349-1519	
Baseline* CD4/CD8 (ratio)	0.78 0.26-2.01	0.54 0.30-0.99	
Second CD4/CD8 (ratio)	0.75 0.24-1.77	0.49 0.22-1.13	
Third CD4/CD8 (ratio)	0.81 0.30-1.95	0.55 0.39-1.27	
Last CD4/CD8 (ratio)	0.80 0.31-1.93	0.60 0.33-1.24	

*First measurement for the present study

max. 15) months after LIP measurement. In addition, the increase of LIP_{CD8} inversely correlated with CD4AC (Rho= -0.65, p<0.05) up to five (min.2, max.8) months after LIP measurement **(Fig.3C)**.

DISCUSSION:

Immune restoration in the settings of continuous cART has a variable course, and is most often incomplete (13, 14, 15). Our data shows that despite SVS, approximately 45% of the participants had a suboptimal CD4/CD8 ratio (<0.9) as a sign of insufficient immune restoration (14).

The reasons for this are numerous and for the most part are not elucidated. Residual viral reservoirs and low level HIV reactivation are a major concern, but no specific predictive markers or targeted therapy to enhance the recovery of CD4+ AC are available yet. People with an incomplete immune restoration are at higher risk of AIDS and non-AIDS-related morbidity and mortality (16).

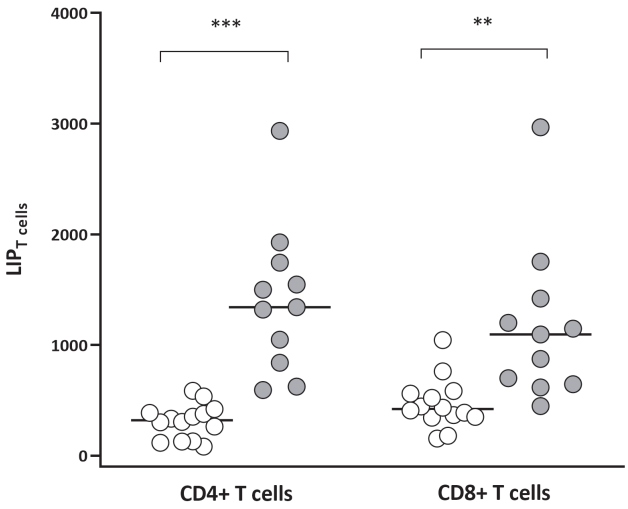

Figure 1. Comparison of MFILIP in CD4+ and CD8+ T cells between group A (open symbols) and B (gray symbols). The difference was statistically significant for CD4+T and CD8+ T cells, ($p<0.0001$ and $p<0.001$).

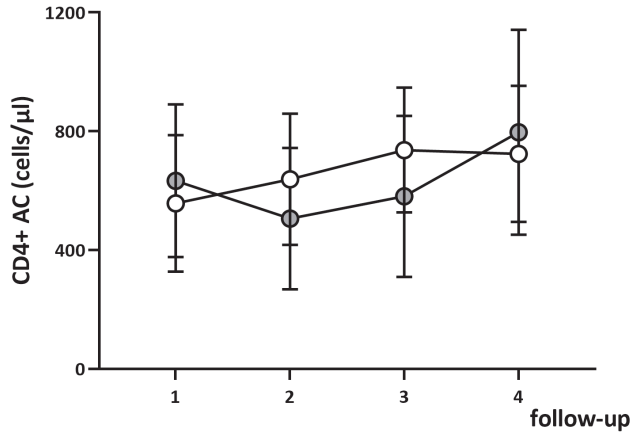

Figure 2. CD4 absolute count in group A (open symbols) and B (gray symbols) during the follow-up period ($p>0.05$).

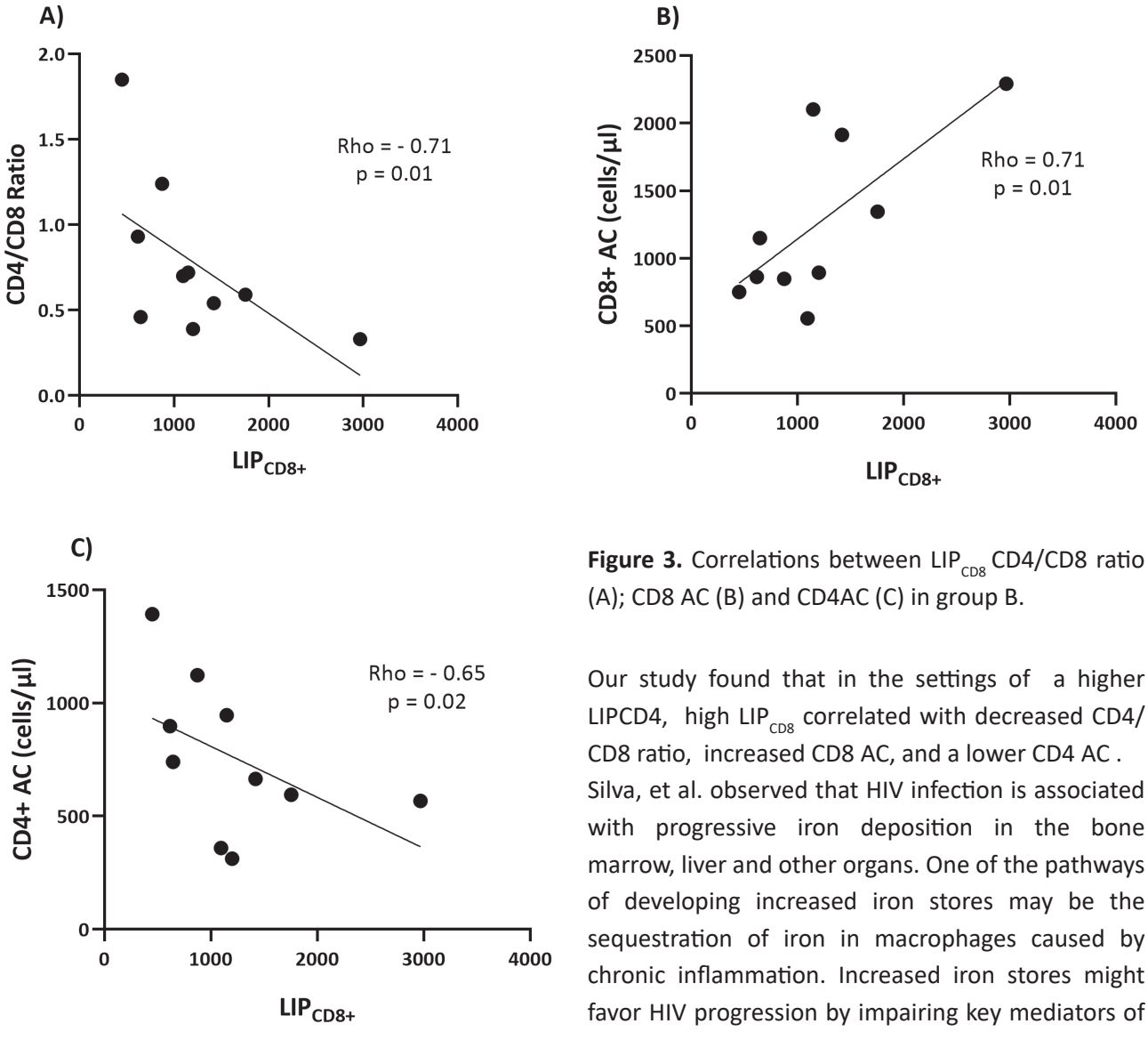

Figure 3. Correlations between LIP_{CD8} CD4/CD8 ratio (A); CD8 AC (B) and CD4AC (C) in group B.

Our study found that in the settings of a higher LIPCD4, high LIP_{CD8} correlated with decreased CD4/CD8 ratio, increased CD8 AC, and a lower CD4 AC . Silva, et al. observed that HIV infection is associated with progressive iron deposition in the bone marrow, liver and other organs. One of the pathways of developing increased iron stores may be the sequestration of iron in macrophages caused by chronic inflammation. Increased iron stores might favor HIV progression by impairing key mediators of

the host response. (12)

Our previous results clearly show that chronic HIV infection affects the regulation of iron turnover leading to increased LIP in the settings of SVS, independently of age, baseline CD4AC, CD4/CD8, HIV VL, and cART duration (12). It is well known that Fenton reaction depends on labile iron concentrations and leads to the formation of free radicals, and intracellular accumulation of ROS (4, 18, 19).

In addition, we have shown that, in HIV+cART+ individuals with SVS, the amount of ROS in CD4+ T cells correlated inversely with the CD4/CD8 ratio, suggesting that the low level of immune activation in patients with suboptimal ratio might reflect reactivation of latent HIV reservoirs (17, 20). The intracellular levels of ROS in monocytes from HIV+ individuals are associated with high viral load. The subsequent iron depletion in other cell compartments induces lysosomal ferritin degradation, iron loading of lysosomes, lysosomal ROS production, lysosomal and mitochondrial lipid membrane permeabilization and cell death with features reminiscent of ferroptosis (4).

In our hands, elevated labile iron levels were in strong inverse correlation with CD4 AC and CD4/CD8 ratio up to five months after the measurement. This observation is in line with recent studies on SARS-CoV-2, HCV, and HIV, suggesting a strong link between viral infection and ferroptosis (3, 10, 21, 22, 23, 24, 26). Viruses can trigger ferroptosis by disrupting different stages of cell metabolism, and affect host immune system through various pathways (10). Ferroptosis provides a favourable environment for viral survival, replication, and evasion of host immune response (10,22). Thus, targeting ferroptosis could be a promising approach for antiviral treatment (21, 27).

Our study is limited by the small number of the participants and the lack of established reference values for LIP. Therefore, we defined A and B subgroups somewhat artificially, based on the median LIP_{CD4}.

CONCLUSION:

In conclusion, chronic HIV infection affects iron metabolism and leads to increased LIP in the settings of SVS, and independently of age, baseline CD4AC, CD4/CD8, HIV VL and cART duration. Since elevated LIP promotes HIV replication, and is associated with T-cell dysfunction, exhaustion and ferroptosis, it may serve as a sensitive predictive marker for clinical follow-up. A larger prospective study is justified to assess the independent prognostic significance of LIP, and the undesirable effects of certain cART components on iron homeostasis.

Acknowledgements: The study is supported by the European Fund for regional development through Operational Program Science and Education for Smart Growth, Grant BG05M2OP001-1.002-0001-C04 "Fundamental, Translational and Clinical Investigations on Infections and Immunity" and the National Program for Prevention and Control of HIV and STI in Bulgaria 2021 – 2025

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MOLECULAR VIROLOGICAL ANALYSIS OF THE TRANSMISSION CLUSTERS AND RESISTANCE MUTATIONS OF HIV-1 SUBTYPE B IN BULGARIA (2012-2020)

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ABSTRACT

HIV-1 infection in Bulgaria is known for its high level of genetic diversity. Previous studies have indicated that subtype B is the most common strain in Bulgaria, particularly among men who have sex with men, who are at a high risk of transmission. The primary objective of this study was to identify any transmission clusters and transmission resistance in individuals newly diagnosed with HIV-1 who have not yet received antiretroviral therapy (ART).

To this end, we sequenced the HIV-1 pol gene in the samples from the study participants using either the Viroseq HIV-1 Genotyping Test (Abbott) and the Applied Biosystems 3130xl genetic analyzer or the TruGene DNA Sequencing System (Siemens Healthcare) and an OpenGene DNA sequencing system. We then subtyped the HIV-1 pol sequences, and further analyzed those that met the criteria for subtype B.

The study included a total of 595 HIV-1 subtype B sequences. Our analysis revealed that the majority of those diagnosed with HIV-1 subtype B were male and lived in Sofia region. The most common transmission mode was through sexual intercourse among men who have sex with men, followed by heterosexual transmission. We also observed the presence of multiple transmission clusters , and a low percentage of transmitted drug resistance mutations (TDRMs).

Overall, our study confirms that HIV-1 subtype B remains the most dominant strain in Bulgaria.

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INTRODUCTION

There is a wide variety of HIV-1 subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs) in Bulgaria, which can complicate the understanding of HIV-1 epidemiology [1,2]. Due to multiple introductions of various HIV-1 genotypes from abroad into Bulgaria and random founder events, numerous HIV-1 subtypes have spread unevenly into different transmission risk groups [3]. For instance, subtype B infections are mostly found in men who have sex with men (MSM), while CRF01_AE and CRF02_AG affect two geographically distinct subgroups of people who inject drugs (PWIDs). The greatest diversity of HIV-1 subtypes was identified in persons reporting heterosexual (HET) transmission [4,5,6,7,8].

It is crucial for individuals living with HIV to receive appropriate antiretroviral therapy (ART). ART helps to reduce morbidity, mortality, and viral transmission by modulating the amount of viral load in the body [9]. However, due to its high mutation rate, HIV-1 can easily develop drug resistance mutations, which can negatively impact the effectiveness of ART [10]. HIV drug resistance mutations (DRMs) can be transmitted and adversely affect HIV-1 therapy [11].

Therefore, before administration of therapy, a HIV drug resistance test is carried out to identify any transmission resistance mutations as recommended by the European and Bulgarian national guidelines. There is a list of 93 non-polymorphic HIV-1 DRMs in the surveillance DRM (SDRM), that require monitoring due to their ability to be transmitted to newly infected individuals; these are known as transmitted DRMs (TDRMs). These 93 mutations include 34 DRMs associated with nucleoside reverse transcriptase inhibitors (NRTIs) at 15 positions in the reverse transcriptase (RT) gene, 19 DRMs associated with non-nucleoside reverse transcriptase inhibitors (NNRTIs) at 10 positions in the RT gene, and 40 DRMs associated with protease inhibitors (PIs) at 18 positions in the protease gene (PR).

In our previous studies, we found a wide variety of different HIV-1 subtypes, CRFs, and URFs in Bulgaria [1,3,4,5,6]. The presence of multiple HIV-1 subtypes further complicates the understanding of HIV-1 epidemiology and the interpretation of DRM [1,2].

Our current study aims to determine the transmission clusters in HIV-1 subtype B between 2012 and 2020 in Bulgaria using newly developed bioinformatics tools. Our findings will help to focus intervention efforts more effectively to control the ongoing spread of the HIV-1 epidemic in Bulgaria.

MATERIALS AND METHODS

EDTA plasma samples (n=1053) were obtained from patients diagnosed with HIV in Bulgaria between 2012 and 2020. At the time of diagnosis, demographic and epidemiological data were collected and linked to an anonymous code [1]. Viral RNA was extracted from plasma samples according to the standard protocol of Abbott Viroseq HIV-1 Genotyping Test and/or QIAmp Viral RNA Mini Kit (Qiagen). The HIV-1 pol gene was sequenced utilizing Viroseq HIV-1 Genotyping Test (Abbott), Applied Biosystems 3130xl genetic analyzer, TruGene DNA Sequencing System (Siemens Healthcare), and OpenGene DNA sequencing system [7]. The sequences were subtyped using the automated subtype tool COMET v2.4 [12], the REGA HIV-1 subtyping tool v3.0 [13], and the jumping profile Hidden Markov Model (jpHMM) [14]. Only subtype B sequences underwent further manual analysis. The sequences were aligned using MAFFT v7 [15,16] and were manually edited using the AliView v1.23 program [17]. The phylogenetic tree was reconstructed with a set of Bulgarian subtype B sequences and reference sequences from the Los Alamos HIV Sequence Database using the maximum likelihood (ML) method. [18]. Fisher's exact test was used to compare categorical variables. Specifically, the summation method of small p-values was employed to evaluate the significance of the findings, with a statistically significant p-value being defined as less than 0.05.

RESULTS

Epidemiological analysis

A total of 595 ART-naïve patients diagnosed with HIV-1 subtype B were included in the study. Epidemiological and demographic data is presented inTable 1. The analysis revealed a significant prevalence of men infected with HIV-1 subtype B. Of all tested samples, 92.1% belonged to male patients, while only 7.9% belonged to female patients. The mean age at diagnosis was 34 years, with the youngest patient being 16 years old and the oldest 78 years old. Regarding the most probable location of acquiring the infection with HIV-1 subtype B virus, 83.4% of the sequences belonged to Bulgarian citizens who were infected within the country, 14.1% were Bulgarians most likely infected abroad, and 2.5% were foreigners who were diagnosed in Bulgaria.

Our study demonstrated a low prevalence of TDRMs in HIV-1 subtype B in Bulgaria, with only 5.2% of individuals infected with HIV-1 subtype B having any of the SDRMs of interest.

At the time of diagnosis, each individual was demanded to fill out a questionnaire assessing the risk factors and the probable route of HIV transmission. Approximately, while the MSM group was the most strongly represented, with 63.4% of infections, followed by 34.1% of heterosexual individuals, and 2.0%. of PWIDs with HIV-1 subtype B virus. Only 0.5% of the studied individuals belonged to the MSM+PWIDs subgroup.

Table 1. Characteristics of individuals infected with HIV-1 subtype B in Bulgaria.

HIV-1 subtype B	Total number	(%)
	595	100
Gender		
Women	47	7,9
Men	548	92,1
Likely country of infection		
Bulgarians infected in Bulgaria	496	83,4
Bulgarians infected abroad	84	14,1
Foreigners diagnosed in Bulgaria	15	2,5
Transmission category		
HET	203	34,1
MSM	377	63,4
PWIDs	12	2
MSM+PWIDs	3	0,5

Table 2. Statistical analysis of HIV-1 subtype B patients according to the country of infection and transmission category criteria.

HIV-1 subtype B	Bulgarians infected in Bulgaria n=496 (%)	Bulgarians infected abroad n=84 (%)	Foreigners diagnosed in Bulgaria n=15 (%)	p value Bulgarians infected in Bulgaria / Bulgarians infected abroad	p value Bulgarians infected in Bulgaria / Foreigners diagnosed in Bulgaria	p value Bulgarians infected abroad / Foreigners diagnosed in Bulgaria
HET	63 (12,7)	36 (42,9)	3 (20)	0,0001	0,4257	0,1504
MSM	319 (64,3)	47 (55,9)	12 (80)	0.1445	0.2778	0.0942
PWID	11 (2)	1 (1,2)	0 (0)	1	1	1
MSM +PWID	2 (0,4)	1 (1,19)	0 (0)	0.3751	0.0910	1

Statistical analysis

In this study, we categorized patients into three groups based on the country of their infection: Bulgarians infected in Bulgaria, Bulgarians infected abroad, and foreigners diagnosed in Bulgaria. We analyzed each patient's transmission category within these groups (Table 2). The percentage of HET Bulgarians infected with HIV-1 subtype B was significantly higher among those infected abroad than those infected in Bulgaria (42.9% compared to 12.7% p=0.0001). No significant statistical differences were found between the other groups.

Analysis of transmission categories

After conducting a summary analysis of transmission categories, we proceeded with analysis based on the year of diagnosis for patients with HIV-1 subtype B (Figure 1). Throughout the study period, the percentage of PWIDs and MSM+PWIDs remained constant and approached zero, with the exception of 2018, where we observed a rise in PWIDs from zero to 5.9%.

In contrast, HETs exhibited fluctuations during the studied period. In 2012, the percentage of diagnosed HETs was 39.4%, with a 1 percent increase in 2013 and a 1 percent decrease in 2014. The percentage of HETs diagnosed with HIV-1 subtype B was at its lowest in 2015, with 22.7%. However, the rates increased to 37.5% in the following year, decreased to 24.6% in 2018 and then rose again to 43.1% in 2020. In the MSM group, there were also some fluctuations in the rate of infections. At the beginning of the period, they accounted for 60.6%, and over the following two years, this percentage remained relatively consistent. In 2015, we observed a peak with 76% diagnoses, while the percentage of diagnosed MSM in 2016 returned to its original levels of 60.7%. In 2017 and 2018, an increase in the percentage of infected individuals was noted, with 68.6% in 2018. In the last two years of the study, there was a decrease in the percentage of diagnosed cases, reaching its lowest point in 2020, with 52.9%.

Demographic analysis

The demographic analysis presented in Table 2 revealed that the majority of patients (57.5%), were residents of Sofia region. Plovdiv and Varna districts represented 5.9% and 4.0% of the patients respectively, while the remaining 32.6% were

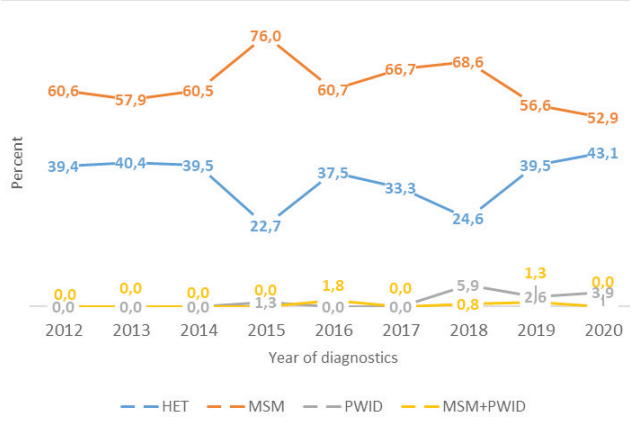

Figure 1. Percentage distribution of HIV-1 subtype B transmission categories in Bulgaria by year from 2012 to 2020.

Table 3. Regional distribution of individuals infected with HIV-1 subtype B in Bulgaria.

Region in the country	Total number of patients infected with HIV-1 subtype B	(%)
Sofia	342	57,5
Plovdiv	35	5,9
Varna	24	4,0
Other	194	32,6

dispersed across other regions in the country in an uneven manner.

Phylogenetical analysis

A phylogenetic analysis was conducted to reconstruct a tree of 595 HIV-1 subtype B sequences and 19 reference sequences [18]. Our objective was to identify clusters of sequences containing resistant mutations. Following the reconstruction of the phylogenetic tree (Figure. 2), all sequences with TDRMs were marked in red. Our analysis revealed multiple introductions of viruses with resistant mutations in clusters across the phylogenetic tree.

Of particular interest was a cluster containing 15 sequences, each of which harbored the TDRM L90M. This cluster comprised 11 sequences from patients living in Sofia, 2 from Kyustendil region, and 1 each from Plovdiv and Blagoevgrad regions. Thirteen patients self-identified as MSM, while two identified as HET. While most of the patients were infected in Bulgaria, the introduction of viruses from other European countries was also evident. Two of the patients believed to have contracted the virus in Spain, while other two - in Serbia and Germany. Additionally, we analyzed the presence of other sexually transmitted infections (STIs) from the information obtained during the diagnostic process and found that 46.7% of the sequences forming the cluster belonged to individuals with other STIs. The

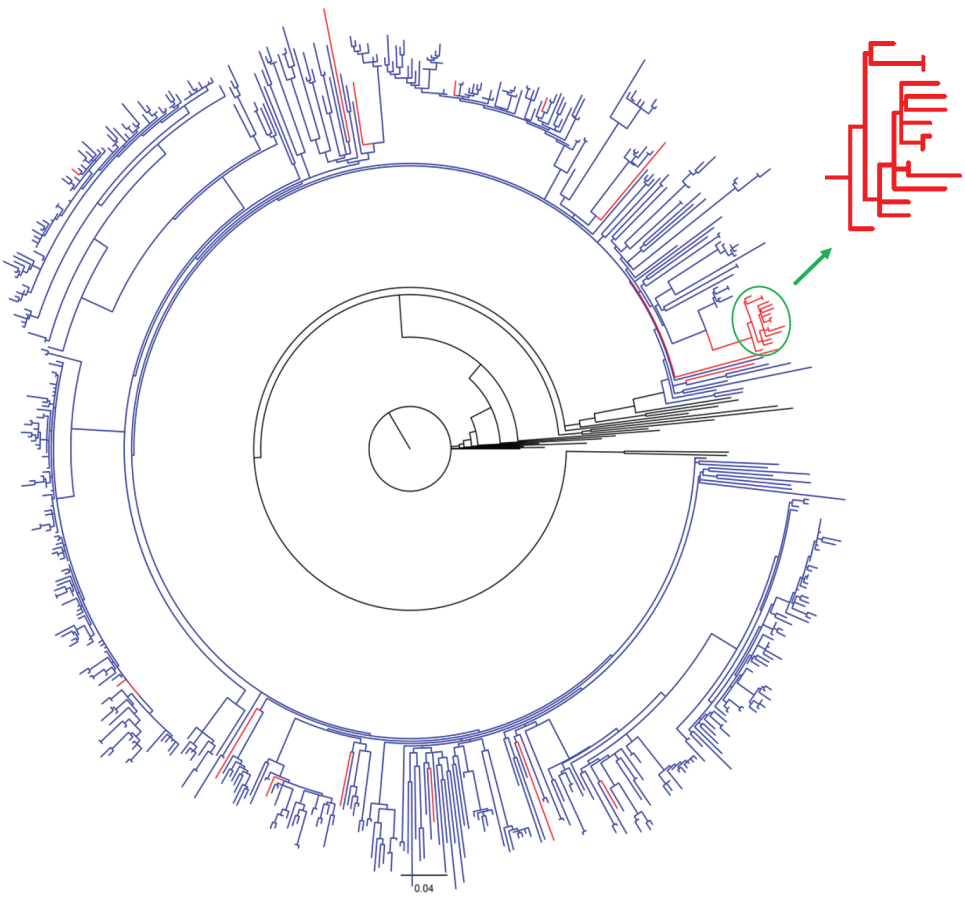

Figure 2. Phylogenetic tree of sequences from HIV-1 subtype B in Bulgaria. The phylogenetic tree was composed of 614 sequences, 595 were Bulgarian isolates and 19 - reference sequences. Reference sequences are colored in black, Bulgarian sequences are colored in blue and sequences containing resistance mutations are colored in red. The red cluster is composed of 15 resistance sequences.

most frequently reported STI was syphilis, followed by genital herpes, chlamydia, and hepatitis B.

DISCUSSION

In this study, we combined demographic, molecular and virological data from 595 individuals with HIV-1 subtype B diagnosed in Bulgaria between 2012 and 2020. We analyzed the phylogenetic clusters formed, transmitted drug resistance and routes of HIV-1 subtype B transmission.

Our analysis of the ART-naive transmission network of subtype B found that most phylogenetic clusters were composed primarily of sequences isolated from MSM with the potential to facilitate the accelerated spread of resistance mutations among these individuals.

Indeed, we identified a 15-member cluster of subtype B sequences from 13 MSM and 2 male HET. The presence of two male HET sequences in a cluster of 13 MSMs indicates possible bridges of transmission of HIV infection and/or inaccurately filled self-reporting forms. The analysis of the 15-member cluster showed 14 PI SDRMs, 4 NNRTI SDRMs and one NRTI SDRM, indicating transmission among these individuals with the potential for further spread among HET. The study found that 4 s out of 15 sequences in a cluster were from patients who acquired the infection abroad, meaning that those SDRMs could be introduced into Bulgaria from other countries and spread locally.

HIV infection was introduced in the MSM community in Bulgaria around 2010, much later, than in the HET group [6]. However, infection spread much more rapidly in the MSM group, making the study of the major prevalent subtype in this group important. Half of the cases with SDRM were detected in the capital city of Sofia, where the highest number of HIV-1 cases were registered during our survey period. However, SDRMs were also detected in persons from 15 other districts of the country, including remote locations in Bulgaria, suggesting that SDRMs are widespread in the country despite the low overall prevalence. SDRMs were also identified in 25.0% of drug-naive individuals who were co-infected with other STIs and in 3.3% of individuals engaged in sex work, suggesting additional potential mechanisms for onward spread of these SDRMs.

Genetic analysis confirmed the dominance of HIV-1 subtype B found in our previous study [6]. The study revealed that Subtype B was predominant among men and was unevenly distributed among persons with transmission risk behavior in Bulgaria. The majority of these sequences belonged to MSM. These results were expected, as the HIV-1 subtype B epidemic in Western Europe has similar characteristics [5,6]. The detection of clusters containing individuals from different transmission groups indicated the presence of transmission bridges between those groups.

O study has some limitations. Some individuals with subtype B were excluded from analysis due to receiving therapy in another country before their diagnosis in Bulgaria, and others - due to the lack of HIV-1 pol sequence. It is important to note that the self-reporting process also may have some limitations. At the beginning of the epidemic, the majority of men with HIV subtype B reported HET transmission, while subsequently, an increasing number of individuals infected with subtype B reported MSM behavior. This fact may be related to a heavier stigma in the early years of the epidemic.

CONCLUSION

HIV-1 subtype B is still the most common cause of HIV infections diagnosed in Bulgaria, although previous studies have shown that a variety of HIV subtypes are distributed throughout the country, including rare subtypes. The incidence of infected women differs significantly from that of infected men, which is a likely consequence of the introduction of HIV-1 subtype B in the vulnerable group of MSM, where it spreads rapidly. Transmission clusters involving vulnerable groups can serve as a springboard for the accelerated spread of resistant mutations to the general population.

The implementation of molecular virological surveillance specifically designed for vulnerable groups can be an effective measure for limiting the transmission of HIV within a community. By closely monitoring the molecular characteristics of the virus, it becomes possible to identify and target specific groups at a higher risk of contracting and spreading the disease. This approach can help the development of better targeted and effective prevention strategies, ultimately reducing the prevalence of HIV in the community.

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EPIDEMIOLOGICAL COMPARISON OF INFLUENZA, ARI AND COVID-19 PREVALENCE IN BULGARIA FOR THE PERIOD 2017-2022

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ABSTRACT

Introduction: The report presents a comparison of the incidence of respiratory infections - influenza, other acute respiratory infections and COVID-19 for the period 2017-2022. A comparative analysis of the epidemiological dynamics in different areas of the country is made and factors such as demography and vaccine coverage are also analyzed.

Materials and Methods: A comparative analysis of the prevalence of Influenza/ARI, and COVID-19 was made for the years of the studied period. Regional values were compared to the country total in order to rank regions according to the experienced disease incidence burden at a regional level. The percentile method was used to identify and filter the regions where Influenza/ARI waves appeared with the highest intensity in the country. To compare intensity of COVID-19 waves between the selected regions we used the maximal weekly incidence values per 100 000 population reached for the different periods of the pandemic. Information on the age structure of the population in the respective regions and the vaccine uptake was retrieved and compared.

Results: The regions of Blagoevgrad, Montana, Haskovo, Razgrad, Kardzhali, Sliven and Targovishte were filtered among all regions as the ones with the most intensive Influenza waves for the period 2017-2022. Blagoevgrad and Sliven were the regions with higher maximal COVID-19 incidence values before the Delta circulation and during the Omicron predominance. When the Delta variant was predominant, Blagoevgrad and Montana surpassed the incidence recorded for the country.

No data supporting the initial hypothesis that demographic structure at regional level determines intensity of spread of the two viral respiratory infections was found. However, the regions with higher COVID-19 vaccines coverage were found to have the lowest incidence levels of the infection.

Conclusion: Although COVID-19 and Influenza/ARI are both respiratory infections, they differ in their epidemiology and no specific pattern can be found. We recommend that anti-epidemic measures should be followed to limit incidence regardless of the circulating respiratory pathogen.

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INTRODUCTION

The report presents a comparison of the incidence of respiratory infections: influenza, other acute respiratory infections (ARI) and COVID-19 for the period 2017-2022. A comparative analysis of the epidemiological dynamics in different regions in Bulgaria is made.

The study focuses on three research questions: What was the prevalence of COVID-19 in regions with higher influenza circulation in the years preceding the pandemic? (1) Can a common pattern be found in the incidence of COVID-19 and influenza and ARI at a regional level? (2) Does specific regional demography together with the vaccine coverage for the specific pathogen determine the prevalence of the two infections? (3).

MATERIALS AND METHODS

Retrospective data from the Annual analyses of the incidence of acute infectious diseases in Bulgaria and the available Information System for data collection and analysis on Influenza and ARI incidence was used for the period 2017-2022. The Annual analyses are prepared at the National Centre of Infectious and Parasitic Diseases (NCIPD) and are accessible through the website of the Centre. To access data on Influenza and ARI referring to the years before 2022 through the Information System that provides evidence from

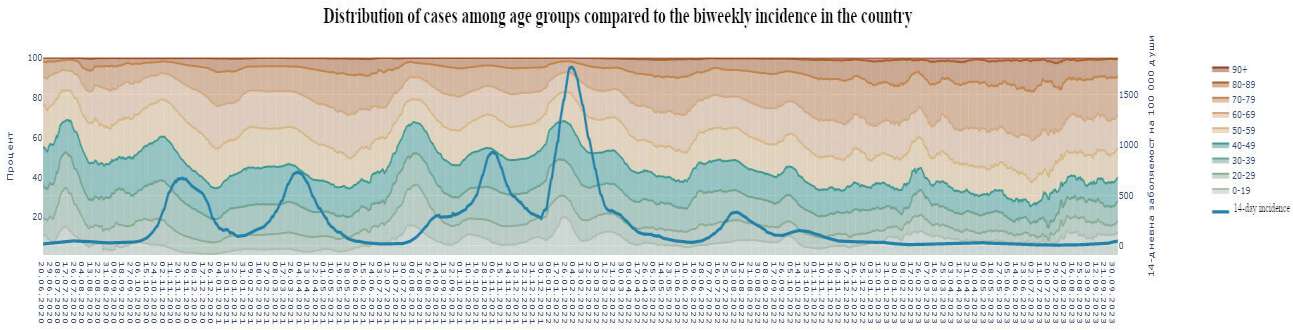

Figure 1. Distribution of cases among age groups compared to the biweekly incidence registered in Bulgaria

selected sentinel practices, a login available at the Department of Epidemiology at the NCIPD was used. For both studied infections, incidence was measured at a weekly basis. For Influenza/ARI it was counted per 10 000 population, while for COVID-19 the 100 000 population basis was used. For COVID-19, denominators have been updated with the last population enumeration data as of 31.12.2022. Sentinel data used for the Influenza/ARI surveillance is also updated with the latest population evidence on a yearly basis.

Influenza and ARI incidence were followed for the whole study period from 2017 onwards, while COVID-19 incidence was followed since the start of the local spread in the country in March 2020.

A comparative analysis of the prevalence of Influenza/ARI, and COVID-19 was made for the years of the studied period. Regional values were compared to the country total in order to rank regions according to the experienced disease incidence burden at a regional level. Incidence of the two respiratory infections was not compared in-between as their basic reproductive numbers differ significantly which naturally explains the more intensive spread observed during COVID-19 epidemic waves [1].

The percentile method was used to identify regions where Influenza/ARI waves appeared with the highest intensity in the country. The percentiles were calculated using data on Influenza/ARI spread for a 10 year period. This method was preferred as influenza epidemic intensity traditionally differs by region. For each region the threshold value of the 99th percentile marking "very high" levels of spread was identified. An additional indicator calculated through multiplication of the 99-th percentile value by 1.5 was introduced to facilitate the process of filtering the regions with the highest intensity of Influenza/ARI.

To compare intensity of COVID-19 waves between the regions we used the maximal weekly incidence values per 100 000 population reached.

To answer the third research question, the study used data on the distribution of the population within age groups for 2020. The information is used for the preparation of the Annual analyses. The shares of population in the age groups 0-14 and 30-64 years of age were compared across the regions with the highest intensity of Influenza/ARI to determine whether the different levels of prevalence of COVID-19 might be attributed to the demography of the specific region. The two age groups were selected according to available data showing that Influenza spread was more intensive in childhood population, while COVID-19 circulated more intensively among the working population (Figure 1).

During the Delta wave the tendency changed slightly and younger age groups were affected by COVID-19, as well. That is why we differentiated the periods for which the maximal COVID-19 incidence is taken according to the circulating variant in the specific period. For onset of the Delta variant we accepted 1st July 2021 when the number of infections started growing in Europe [2]. We considered infections registered until the end of November 2021 to be caused by Delta and from 1st December 2021 onwards we considered Omicron prevalence [3, 4].

Vaccination rates with the available vaccines against influenza and COVID-19 were compared at a regional level and to the country total. Data on immunizations against COVID-19 was obtained from the national COVID-19 information portal [5]. Data on immunizations against Influenza was retrieved from reports submitted by the Regional Health

Table 1. Maximal values of Influenza/ARI incidence measured per week at 10 000 within regional cities. Values higher than the 99-th percentile threshold are highlighted.

Област	Стойност 99-ти персентил 2013-2022	2017	2018	2019	2020	2021	2022
Благоевград	409.24	616.0194	507.896	410.5445	433.714	71.335	602.7077
Бургас	236.53	189.1439	226.8692	243.2285	263.275	166.0142	173.9923
Варна	199.74	159.2518	198.6914	248.887	200	194.3536	179.1976
Велико Търново	158.54	83.4188	99.2989	135.2137	223.2532	40.8401	100.6418
Видин	223.29	164.3348	290.9171	326.2348	235.816	138.8188	123.675
Враца	242.56	143.6234	351.2719	295.899	266.4821	224.9523	192.0749
Габрово	291.02	378.526	379.6296	239.2615	210.8491	253.019	291.3553
Добрич	179.09	151.7433	161.5169	265.9889	197.3685	104.4069	140.8281
Кърджали	164.84	164.8728	158.0031	486.3363	573.543	111.0608	101.9946
Кюстендил	190.14	275.4955	196.6568	206.3137	258.2391	65.1466	84.7242
Ловеч	279.05	238.1652	302.1239	115.7479	289.855	228.3511	272.8352
Монтана	340.15	658.8735	589.9021	315.6565	265.9975	122.5033	141.1452
Пазарджик	299.41	337.857	270.0027	397.2294	309.3842	174.4868	282.9913
Перник	229.17	208.5004	280.6638	277.0233	262.6181	142.5606	148.9432
Плевен	264.31	180.6322	223.3955	305.2966	309.5879	136.0544	168.9708
Пловдив	147.08	147.5549	164.4107	136.5395	183.2012	83.3212	61.8315
Разград	151.83	151.4683	319.0491	156.6997	131.2839	112.8748	144.6208
Русе	240.93	257.2369	246.3088	247.693	192.5254	181.9083	143.2792
Силистра	291.8	227.6867	297.4326	328.7415	327.3998	264.6869	277.5985
Сливен	209.75	151.2737	209.574	265.2429	316.3384	98.3213	118.7051
Смолян	207.82	209.0591	243.2046	261.0873	241.2676	155.4404	179.7754
София-град	205.86	204.1976	240.1729	276.8912	269.7993	131.58	114.529
София-област	259.31	268.6361	256.5431	259.9983	234.5416	77.2921	191.8976
Стара Загора	306.11	370.587	395.8901	310.3222	255.5136	97.1107	145.6658
Търговище	220.17	171.2423	138.6247	206.578	364.2294	122.3159	266.3768
Хасково	255.59	387.1146	199.3865	230.7259	364.6942	139.0922	332.3573
Шумен	222.39	221.3642	216.2161	326.8984	205.9202	205.9202	248.3912
Ямбол	321.62	392.9945	438.5334	377.5777	226.5466	84.2288	176.558
Общо	212.73	215.5172	230.8299	247.9181	242.6937	97.4997	126.6736

Inspectorates for the preparation of the Annual analyses of immunoprophylaxis.

RESULTS

Epidemic waves of the two infections:

Influenza/ARI thresholds in 18 of the country regions reached "very high" levels (set at the 99th percentile) and surpassed the average for the country: Blagoevgrad, Bourgas, Vidin, Vratsa, Gabrovo, Lovech, Montana, Pazardzhik, Pernik, Pleven, Russe, Silistra, Sofia-district, Stara Zagora, Targovishte, Haskovo, Shumen and Yambol. Respectively, 10 regions had a threshold scoring lower than the total for Bulgaria: Sofia-city, Smolyan, Sliven, Razgrad, Plovdiv, Kyustendil, Kardzhali, Dobrich, Veliko Tarnovo and Varna (Table 1). Considerably higher is the threshold value measured in Blagoevgrad compared to the rest of the values in the other regions in the country.

The retrospective analysis showed that in 2017 13 of the country regions registered very high incidence of Influenza/ARI. In 2018 their count was 16, for 2019 – 21, 2020 – 20, 2021 – 0 and 2022 – 5 (Table 1).

Further filtering with a new threshold set at 1.5 times the value of the 99th percentile showed that in 2017 the regions that surpassed it were Blagoevgrad (new threshold 613.86, maximum weekly incidence per 10 000 – 616.02), Montana (new threshold 510.23, max. 658.87) and Haskovo (new threshold 383.39, max. 387.11). In 2018 Montana (max. 589.9) and Razgrad (new threshold 227.75, max. 319.05) had incidence scoring above the newly introduced indicator for the respective region. In 2019 only Kardzhali (new threshold 247.26, max. 486.34) fulfilled this criterion. In 2020 there were three regions surpassing the new threshold incidence – Kardzhali (max. 573.54), Sliven (new threshold 314.63, max. 316.34) and Targovishte (new threshold 330.26, max. 364.23). In 2021 no region was affected by very high incidence of Influenza/ARI. In 2022 only the region of Blagoevgrad scored closely to the new threshold value (max. 602.71) but no region was filtered to surpass the newly introduced marker (Table 2).

After the filtering based on the Influenza prevalence,

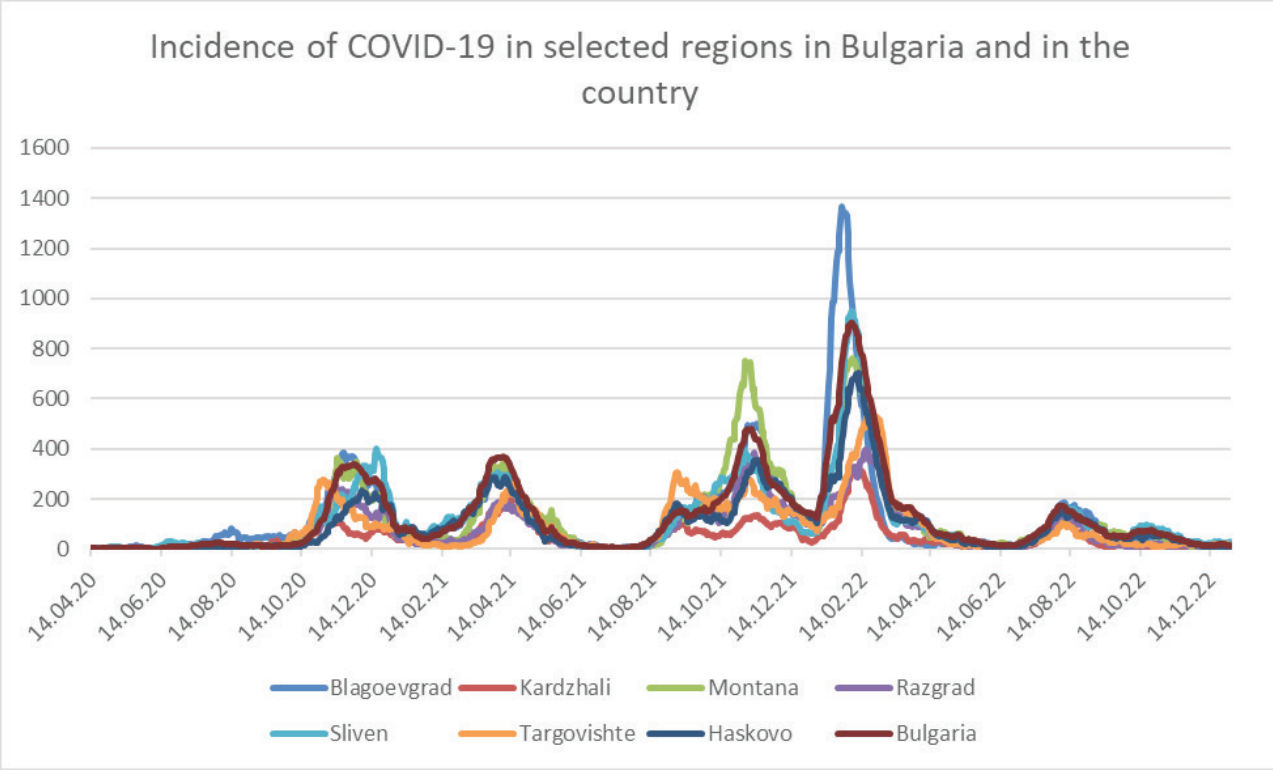

Figure 2. Incidence of COVID-19 in selected regions in Bulgaria and in the country in the period 2020-2022

the regions Blagoevgrad, Kardzhali, Montana, Razgrad, Sliven, Targovishte and Haskovo were selected for analysis of the peak values of COVID-19 incidence at a weekly level. Regarding the epidemic dynamics of COVID-19 in the selected regions, as shown in Fig. 2, no considerable deviations from the average for the country were observed in the period before the Delta variant started circulating. In Table 3 the maximal incidence values during the periods before Delta, during Delta circulation and during Omicron circulation could be seen.

The regions that registered higher maximal incidence values than the country maximum (369.49) before Delta took over were Blagoevgrad (384.22) and Sliven (402.46). During Delta circulation the national incidence value (481.62) was surpassed in the regions of Blagoevgrad (502.49) and Montana (749.60).

After the onset of the wave caused by the Omicron variant, only the regions of Blagoevgrad (1369.04) and Sliven (949.93) had higher maximal incidence values than the maximal for the country (900.35). The lowest values of the indicator were observed in Kardzhali (330.59) and Razgrad (398.05). The regions Montana, Targovishte and Haskovo can be categorized as regions with intermediate intensity of COVID-19 prevalence during the spread of Omicron according to the maximal values of the incidence there.

Demographics:

The regions Montana, Kardzhali and Razgrad presented with slightly lower share (less than 1%) of children population (0- 14 years old) as compared to the average for the country (14.44%). Sliven was the region with a considerably higher share of the 0-14 age group – 18.53%. The rest of the regions had a slightly higher share as compared to the country total – less than 0.3% difference (Table 4).

Regarding the share of actively working population (30-64 y.o.), in Blagoevgrad and Kardzhali it is higher than the country total (49.56%) and in Montana and Sliven it is lower. No deviation higher than 1% from the country total was found in the other analyzed regions.

Vaccine coverage:

Data on the vaccine coverage for COVID-19 in the selected regions is available in Table 5. All of the selected for this analysis regions but Kardzhali had lower percentage of fully immunized population as compared to the country average. Coverage with the COVID-19 vaccines was considerably higher than the uptake of the influenza vaccines in the period 2017-2022 (Table 6).

DISCUSSION

Table 2. Maximal values of Influenza/ARI incidence measured per week at 10 000 within regional cities. Values higher than 1.5 times the value of the 99-th percentile threshold are highlighted.

Област	Стойност 99-ти персентил 2013-2022	2017	2018	2019	2020	2021	2022	1.5 * прагова стойност
Благоевград	409.24	616.0194	507.896	410.5445	433.714	71.335	602.7077	613.86
Бургас	236.53	189.1439	226.8692	243.2285	263.275	166.0142	173.9923	354.795
Варна	199.74	159.2518	198.6914	248.887	200	194.3536	179.1976	299.61
Велико Търново	158.54	83.4188	99.2989	135.2137	223.2532	40.8401	100.6418	237.81
Видин	223.29	164.3348	290.9171	326.2348	235.816	138.8188	123.675	334.935
Враца	242.56	143.6234	351.2719	295.899	266.4821	224.9523	192.0749	363.84
Габрово	291.02	378.526	379.6296	239.2615	210.8491	253.019	291.3553	436.53
Добрич	179.09	151.7433	161.5169	265.9889	197.3685	104.4069	140.8281	268.635
Кърджали	164.84	164.8728	158.0031	486.3363	573.543	111.0608	101.9946	247.26
Кюстендил	190.14	275.4955	196.6568	206.3137	258.2391	65.1466	84.7242	285.21
Ловеч	279.05	238.1652	302.1239	115.7479	289.855	228.3511	272.8352	418.575
Монтана	340.15	658.8735	589.9021	315.6565	265.9975	122.5033	141.1452	510.225
Пазарджик	299.41	337.857	270.0027	397.2294	309.3842	174.4868	282.9913	449.115
Перник	229.17	208.5004	280.6638	277.0233	262.6181	142.5606	148.9432	343.755
Плевен	264.31	180.6322	223.3955	305.2966	309.5879	136.0544	168.9708	396.465
Пловдив	147.08	147.5549	164.4107	136.5395	183.2012	83.3212	61.8315	220.62
Разград	151.83	151.4683	319.0491	156.6997	131.2839	112.8748	144.6208	227.745
Русе	240.93	257.2369	246.3088	247.693	192.5254	181.9083	143.2792	361.395
Силистра	291.8	227.6867	297.4326	328.7415	327.3998	264.6869	277.5985	437.7
Сливен	209.75	151.2737	209.574	265.2429	316.3384	98.3213	118.7051	314.625
Смолян	207.82	209.0591	243.2046	261.0873	241.2676	155.4404	179.7754	311.73
София-град	205.86	204.1976	240.1729	276.8912	269.7993	131.58	114.529	308.79
София-област	259.31	268.6361	256.5431	259.9983	234.5416	77.2921	191.8976	388.965
Стара Загора	306.11	370.587	395.8901	310.3222	255.5136	97.1107	145.6658	459.165
Търговище	220.17	171.2423	138.6247	206.578	364.2294	122.3159	266.3768	330.255
Хасково	255.59	387.1146	199.3865	230.7259	364.6942	139.0922	332.3573	383.385
Шумен	222.39	221.3642	216.2161	326.8984	205.9202	205.9202	248.3912	333.585
Ямбол	321.62	392.9945	438.5334	377.5777	226.5466	84.2288	176.558	482.43
Общо	212.73	215.5172	230.8299	247.9181	242.6937	97.4997	126.6736	319.095

All 7 selected regions in the country demonstrated a much higher prevalence of Influenza and ARI in at least one of the 3 years before the pandemic than the usually observed. The maximal Influenza/ARI incidence values recorded there surpassed the threshold of 1.5 times the value of the 99th percentile.

Blagoevgrad and Sliven are the regions with higher maximal COVID-19 incidence values before Delta started circulating. This is the period when according to the evidence (Fig. 1) the actively working were the most affected by the coronavirus circulation. While it is true that Blagoevgrad has a higher share of working population as compared to the average for the country, Sliven is the region where the age group 30-64 is estimated to be less represented as compared to the average for the country.

During Delta when the number of infections among the younger population increased, the regions of Blagoevgrad and Montana surpassed the incidence recorded for the country. The region of Montana which has the lowest vaccination rate of the selected

Table 3. COVID-19 maximal weekly incidence per 100 000 recorded in selected regions in the period until Delta predominance, Delta predominance and the onset of the Omicron wave

Region	Max weekly COVID-19 incidence – before Delta	Max weekly COVID-19 incidence - Delta	Max weekly COVID-19 incidence - Omicron
Blagoevgrad	384.2164	502.4877	1369.039
Kardzhali	225.658	135.2684	330.5858
Montana	364.5641	749.6004	761.4113
Razgrad	253.6353	388.1252	398.054
Sliven	402.4571	392.1377	949.9291
Targovishte	277.6926	306.5438	544.5661
Haskovo	288.0386	354.1677	705.2286
Bulgaria	369.4895563	481.6239185	900.3547733

Table 4. Share of population within the age groups 0-14 and 30-64 years old in selected regions in 2020 presented as percentage of the respective region's population.

Region	Share of population 0-14 y.o. (%)	Share of population 30-64 y.o.(%)
Blagoevgrad	14.74	51.47
Montana	13.59	46.60
Kardzhali	14.06	52.31
Razgrad	13.57	50.08
Sliven	18.53	45.53
Targovishte	14.56	48.97
Haskovo	14.51	49.08
Bulgaria	14.44	49.56

Table 5. Percentage of population covered by primary complete vaccination against COVID-19 in the selected regions.

Region	Population covered by COVID-19 vaccine (%)
Blagoevgrad	25.55
Montana	21.44
Kardzhali	33.85
Razgrad	29.08
Sliven	26.44
Targovishte	23.70
Haskovo	24.54
Bulgaria	32.23

Table 6. Population covered by influenza vaccination in the respective years (%).

Region	2017	2018	2019	2020	2021	2022
Blagoevgrad	0.124723	0.101926	0.930312	1,89082746	2.045928	2.854654
Kardzhali	0.155513	0.114474	1.997177	2,07860382	2.664655	3.19982
Montana	0.075635	0.134992	0.173227	0,22249691	3.09464	3.219364
Razgrad	0.417715	0.271766	2.35583	1,81222111	1.791879	3.319256
Sliven	0.107731	0.099735	0.273736	0,77457806	1.602261	1.793262
Targovishte	0.054235	0.013441	0.016229	1,68504094	2.118076	2.54883
Haskovo	0.242135	0.138073	2.708628	2,64415875	3.784918	3.517661
Bulgaria	0.178808	0.145442	1.231493	1,5715354	2.107886	2.63889

regions, has the highest maximal weekly incidence (749.60) compared to the others at this specific period of the pandemic. According to the demographics of Montana, the region has lower share of both the age groups 0-14 and 30-64 y.o.

When Omicron took over, the epidemic wave was the most intensive in Blagoevgrad and Sliven regions – the same regions that were the most affected during the pre-Delta period.

The region of Kardzhali appeared to be the least affected by any of the COVID-19 waves. The Kardzhali region also differed by the considerably higher proportion of the population covered with immunizations against COVID-19.

In the region of Razgrad we observed a lower intensity of the COVID-19 waves similar to the one in the region of Kardzhali. Demographically, there are some similarities between the two regions. The shares of the children group are similar but the group of 30-64 y.o. is better represented in Kardzhali as compared to the region of Razgrad. The vaccination coverage in the Razgrad region was lower than the country average but much higher in comparison with the other regions analysed in this study.

The regions Targovishte and Haskovo were characterized by COVID-19 waves with intermediate intensity as compared to the country one, and the distribution of the population within the age groups 0-14 and 30-64 y.o. there is comparable to the one for Bulgaria as a whole.

CONCLUSION

COVID-19 prevalence in the regions with the highest Influenza/ARI incidence varied significantly. Within the 7 regions fulfilling the criteria for extremely high circulation of Influenza/ARI in the 2017-2022 period we had both Blagoevgrad and Kardzhali included. These are the regions where the COVID-19 maximal incidence at regional level was recorded (Blagoevgrad) and where the maximal COVID-19 incidence remained 3 times lower than the value for the country total (Kardzhali). Therefore, we conclude that although COVID-19 and Influenza/ARI are both respiratory infections, they differ in their epidemiology and no common pattern can be found. Our hypothesis that differences in the demographic structure of the regions might be associated with a higher prevalence of COVID-19 or Influenza/ARI, was not confirmed by the data from the 7 analyzed regions.

Differences in vaccination coverage, however, might be determining for the intensity of COVID-19 waves as the two regions that presented with the lowest maximal COVID-19 incidence during the Omicron period had also the highest vaccination rates among the 7 filtered regions.

Although we concluded that the epidemiology of the two viruses is very different, it should be recognized that their transmission paths are essentially the same. Therefore, anti-epidemic measures are strongly recommended to limit the circulation of both respiratory pathogens.

LIMITATIONS

Other factors that were not a subject of this study might also influence the incidence values. First, the analysis worked with the official numbers of registered residents. Thus, we did not account for the people who had moved and had not registered the changes in their home address.

Another limitation of this study concerns the innate differences of surveillance models used for the two infections. While data for Influenza/ARI is taken from sentinel sites and represents only regional cities, data for COVID-19 incidence represents the region as a whole.

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RARE CASE OF ASCARIASIS DETECTED BY COLONOSCOPY ON THE BACKGROUND OF ELEVATED LEVELS OF FECAL CALPROTECTIN

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ABSTRACT

Introduction: The causative agents of ascariasis in humans are two species: *Ascaris lumbricoides* and *Ascaris suum*. For diagnosis, a fecal sample is most often examined. In some cases, the parasite can be identified when coming out with the intestinal passage, and very rarely up on colonoscopy.

Aim to present a rare case of ascariasis where the diagnosis was made by colonoscopy on the background of elevated levels of fecal calprotectin (f-CP).

Case presentation: A colonoscopy was performed on a 52-year-old female patient due to elevated f-CP. The patient had no complaints. The colonoscopy did not detect pathological changes of the intestinal mucosa, but documented larval stages of *Ascaris spp*. freely moving in the lumen of the large intestine. The patient was treated with albendazole. Subsequent parasitological examinations of fecal samples were negative.

Discussion: In developed countries, the transmission of *Ascaris lumbricoides* is greatly reduced. On the background of a very limited transmission of *Ascaris lumbricoides*, many authors consider that most of the sporadic cases of ascariasis are due to *Ascaris suum*. In the case described by us, the f-CP levels normalized after the treatment, and for this reason, we cannot categorically reject the relationship between *Ascaris* infection and elevated f-CP levels.

Conclusion: The presented clinical case is of interest due to the unusual way of diagnosi ascariasis. In the absence of clinical symptoms, and pathological changes of blood and biochemical parameters, except for elevated fecal calprotectin, inflammatory colon disease was suspected and was colonoscopy performed on this occasion.

Key words: Ascariasis, *Ascaris lumbricoides*, *Ascaris suum*, faecal calprotectin, colonoscopy

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INTRODUCTION

Human ascariasis is one of the most widespread parasitic infections on a global scale, and belongs to a group of diseases defined by the WHO as Neglected Tropical Diseases. The causative agents are nematodes from the genus *Ascaris*. Due to the specifics of their life cycle, they are also classified as soil-transmitted helminths, and the disease - as soil-transmitted helminth (STH) infections (1). The causative agents of human ascariasis are two species of *Ascaris*: *Ascaris lumbricoides* and *Ascaris suum*, which are genetically very close species, and according to some studies can interbreed and even produce fertile (albeit with reduced fertility) offspring (2).

According to a recent meta-analysis study excluding the non-endemic areas as North America, Europe, Australia and New Zealand, in 2021 732 million people in endemic tropical areas had ascariasis and the total population parasitemia in these areas was 11.01%. The highest levels of prevalence were recorded in Central and South-Eastern Asia (10.01–16.13%), South America and the Caribbean (10.75–14.88%), and Sub-Saharan Africa (10.56–12.81%). The lowest prevalence was found in North Africa and Western Asia (1.47–2.70%) (3).

Of the soil-transmitted helminths (*Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, and *Trichuris trichiura*), only *Ascaris lumbricoides* and *Trichuris trichiura* are locally distributed in Bulgaria (4). On the average, about 700 cases of ascariasis and 100 cases of trichuriasis are registered in the country each year (5). Most of the ascariasis cases are asymptomatic and are mainly diagnosed during prophylactic examinations. The country

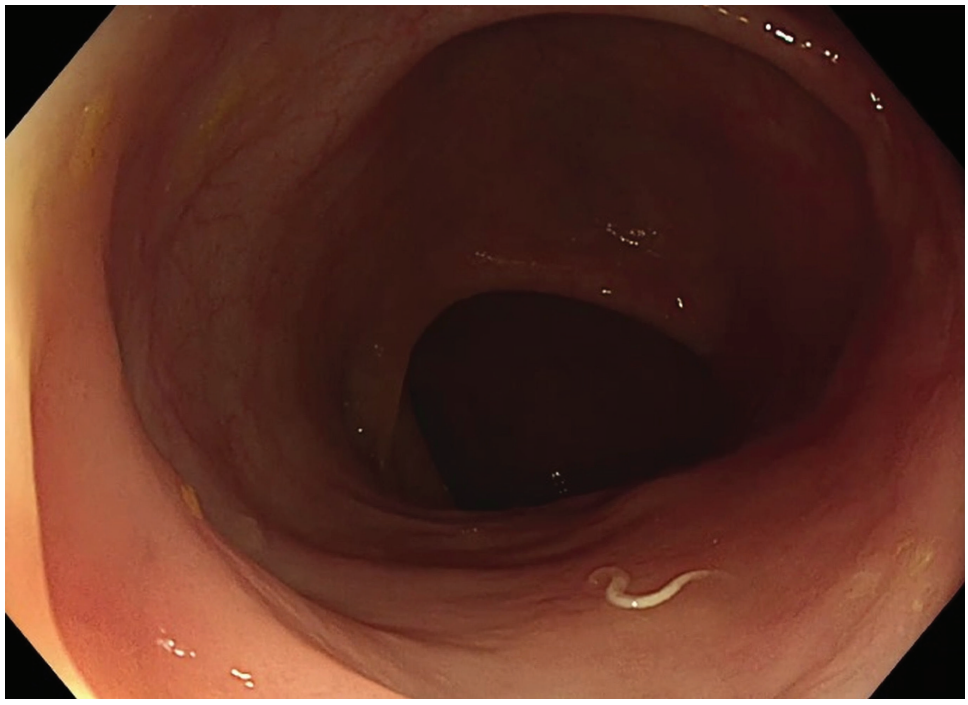

Figure 1. A larval stage of *Ascaris* spp. in large intestine

burden of Bulgaria for ascariasis and trichuriasis is relatively low and preventive chemoprophylaxis with albendazole/mebendazole is not conducted. Calprotectin is a protein that is primarily released by neutrophils cells. Faecal calprotectin (f-CP) is a marker of intestinal inflammation and is used as a biomarker in gastrointestinal disorders (6). In recent years, its use in various enteric infections has been increasing, especially as correlate of clinical severity in the evaluation of bacterial and viral pathogens (7). Our aim was to present a clinical case of Ascariasis, diagnosed by colonoscopy that was performed on the basis of high calprotectin values.

CASE PRESENTATION

The report concerns52-year-old woman regularly attending preventive examination. During the last such examination, an elevated level of f-CP was found (361.5 μg/g feces; reference value <50 μg/g). The patient had no complaints. All other tests, including complete and differential blood count, biochemical indicators and urine, were without deviations from reference values. On the recommendation of a gastroenterologist, the patient underwent a colonoscopy. The examination did not reveal any pathological changes in the intestinal mucosa. However, but on one episode of the colonoscopy, lasting about 30 seconds (out of a total of about 20 minutes of recording), an actively moving nematode was visualized in the lumen of the large intestine (Fig. 1).

On this occasion, the patient was referred for a consultation with a medical parasitologist at the National Center of Infectious and Parasitic Diseases, Sofia. The examination of a fecal sample did not prove the presence of *Ascaris* eggs or other parasitic pathogens. Based on the external morphological features and the size of the nematode, it can be reasonably assumed that it is a larval stage of *Ascaris* spp. The patient was prescribed a single dose of 400 mg of albendazole. Two more tests performed 2 weeks apart were also negative. Three months after the first dose of albendazole, the patient was given a second dose. As already mentioned, during all this time she had no subjective complaints. One month after the treatment the faecal calprotectin level was within the reference range.

DISCUSSION

There is nothing unusual about the etiology and clinical presentation of the described case. Although the prevalence of ascariasis in Bulgaria has been reduced to insignificant levels, single cases of infection are registered each year. However, several

questions of interest arise.

The first issue regards the causal parasite species. The high living and sanitary standards, as well as the established personal hygiene in developed industrial countries have significantly limited people's contact with human faecal waste. As a result the transmission of *Ascaris lumbricoides* has been significantly reduced. In most reported cases of ascariasis in these countries, a low infectious burden with development of single specimens is observed, as was in our case: only one exemplar of *Ascaris.* Since most of those countries have also well developed pig farming, many authors consider that the sporadic cases of ascariasis are primarily due to *Ascaris suum* (8, 9).

The classical diagnostic methods for ascariasis are based on the microscopic detection of parasite eggs in a faecal sample or the morphological characteristics of a spontaneously excreted preimaginal or imaginal form (10). The eggs of both species are morphologically indistinguishable, while the adults differ only in the shape of the lips and teeth which can be detected by electron microscopy (11). Because of their morphological similarities, it is currently debated whether *A. lumbricoides* and *A. suum* were the same species (2) or rather - different species, based on genetic evidence (12). Eggs of the parasite can be found in a faecal sample effectively only after the expiration of the pre-patent period, which for *A. suum* is 24 days and for *A. lumbricoides* - 67 days. In early ascariasis, the symptoms related to the migration of the parasite are indicative of the diagnosis. Eosinophillia is observed in the peripheral blood. Sputum examination also reveales eosinophillia and the presence of Charcot-Leyden crystals. Eosinophilic Loeffler infiltrates develop in the lung, and increased levels of total IgG and IgE are found in the serum. In rare cases, such as the one described by us, the diagnosis is macroscopic based on the detection of *Ascaris* is in the intestinal passage during colonoscopy.

Another important question is whether f-CP could serve as a non-specific biomarker of the parasitic disease. There are a few published studies of this kind. To establish the role of faecal occult blood (FOB) and f-CP as potential markers of intestinal morbidity in soil-transmitted helminth infections (trichuriasis, with or without association with ascariasis and/or hookworm infections), Patel et al. (2021) studied a total of 1034 *T. trichiura* infected cases (mostly mild infections) and 157 STH negative controls for f-CP and FOB. No statistically significant relationship was found between *T. trichiura* infection or *Ascaris lumbricoides* co-infection and f-CP concentration (7). The results of another relatively large cross-sectional study in children also did not establish a correlation between STH and f-CP values (13). In contrast, a study by Salman et al. found a significant correlation between some protozoal infections of the gastrointestinal tract and f-CP levels (14). In the present case, f-CP levels normalized after the treatment, and therefore we cannot categorically reject a relationship between *Ascaris* infection and elevated f-CP levels. Moreover, no evidence of bowel inflammation or other co-infection was found, and any subjective complaints were absent. Therefore, additional targeted studies are needed in this area.

Conclusion

The present clinical case is of interest due to the unusual way of diagnosing ascariasis: in the course of colonoscopy performed on the occasion of elevated f-CP values, and in the absence of clinical symptoms, or major blood count and biochemical deviations.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical considerations: The study was conducted in accordance with the Declaration of Helsinki. Ethical review and approval for this study by the Ethics Committee of National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria, is not necessary due to it being a retrospective single case report and the absence of any personal data.

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NONTUBERCULOUS MYCOBACTERIA DIVERSITY IN KARST WATERS AND BIOFILMS IN BULGARIAN CAVES

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ABSTRACT

Background: Nontuberculous Mycobacteria (NTM) are emerging pathogens causing opportunistic infections in humans and animals. Their distribution in the waters and caves of Bulgaria is poorly studied. Climatic changes are associated with changes in the amplitudes of ambient and water temperature, as well as changes in the amount of precipitation which play an essential role in the creation of reservoirs of some types of NTM in the environment.

Material and Methods: We optimized the methods for successful isolation of environmental NTM and then used molecular genetic methods for identification.

Results: A total of 235 samples (karst water, sediments, soil, bat guano) were collected in some caves of the following karst regions: 203 in Vratsa Karst area, 204 in Ponor Karst area, 205 in Bezdenski area and 303 in Karst and caves of Bosnek region. Primary isolation of mycobacteria by Löwenstein–Jensen at room temperature was more successful than on liquid media at 37°C. We identified NTM in 10% (n=24) from these materials. Diverse NTM included: *M. chelonae* (n=3), *M. gordonae* (n=2), *M. intermedium* (n=3), *M. scrofulaceum* (n=1), *M. szulgai* (n=4), *M. fortuitum* group (n=4), NTM mix culture (n=5), *M. terrae* complex (n=1), *Mycobacterium sp*. (n=1). Rapidly growing NTM (*M. chelonae, M. fortuitum* group) were the most common. The isolates belonged to group of environmental saprophytes (Risk group 1) and potential pathogens (Risk group 2).

Conclusions: We successfully implemented a procedure for decontamination and isolation of NTM from the environment. For the first time in the country, NTM species were identified in biofilms, karst waters, soil and bat guano within caves. The presence of NTM in cave ecosystems represents a potential source for human infection.

Keywords: Nontuberculous Mycobacteria, geographical diversity, Bulgarian caves.

INTRODUCTION

Genus *Mycobacterium* includes *M. tuberculosis* complex, *M. leprae* and nontuberculous mycobacteria (NTM). NTM are represented by over 190 species and are the fastest growing group of the genus. In the recent years, an increased incidence of NTM infections has been reported worldwide. NTM cause most often chronic lung infections, but they are also involved in central nervous system diseases, and skin/soft tissue infections in children, adults, and especially in patients with immunocompromised conditions (1, 2). NTM are not transmitted from person to person, but rather from environmental sources. They have been found in various ecological niches worldwide. NTM can be isolated from soil, dust and water sources, including surface, recreational, ground, waste, tap water. Biofilms can also serve as a reservoir for these bacteria. *M. gordonae* is more commonly isolated from water sources using treated surface water, while *M. nonchromogenicum* predominates in water sources fed by chlorinated groundwater (3).

The presence of NTM in the environment of Bulgaria has been poorly investigated. A study conducted in 2015 did not detect NTM in the waters of the Iskar Dam and the Black Sea (4). Analysis of bat guano in eight European countries - the Czech Republic, France, Hungary, Italy, Romania, Slovakia, Slovenia, and Bulgaria, performed by a Czech team from Mendel

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Figure 1. A map of karst regions in Bulgaria. The borders of the karst areas from which materials were collected are colored in blue.

University in Brno (MENDELU) without Bulgarian participation, detected NTM representatives in eight Bulgarian samples (5).

These studies and the published data did not clarify the situation, and the question about the primary sources of NTM in the country's environment remained unsolved. The exploration of cave biofilms and karst waters for NTM is important because these habitats can determine the above-ground and subsurface environments, and water is the main source of NTMs distribution (6).

In Bulgaria, karst zones occupy 22.7% of the territory, and so far about 5100 caves have been discovered (6). They offer a unique living environment - a specific microclimate (almost constant temperature and humidity), limited or completely absent daylight (6). Caves are an independent and complete eco-geographic area in which a dynamic equilibrium is established under unique conditions. Caves and above-ground structures are interconnected and strongly influence each other. The stable conditions of deep dark cave areas reveal a wide variety of bacteria, algae and fungi living on the rock walls and speleothems, in the sediments and temporary pools. The adaptation mechanisms used by cave microorganisms are complex, with increasing potential to affect humans (7).

METHODS

A total of 235 samples from environmental materials (water, soil, sediment, biofilms, bat guano) were collected from caves. We used the cave zoning of the country's territory accepted by geologists (8). According to it, the collected materials were as follows: 203 Vratsa karst region (from 12 caves), 204 Ponor karst region (from 3 caves and 3 karst springs and rivers), and 303 Bosnek karst region (from 4 caves) **Fig. 1**. Collection was carried out in sterile containers, biofilms were collected using sterile swabs. Sampling requirements included: 5 g of soil from at least 3 cm depth from the surface; 50 ml of water. They were placed in clean transparent self-sealing envelopes, and labelled with the type of specimen, location and date. Samples were stored in refrigerator at +6° C for up to 48 hours. All of the samples were decontaminated in order to destroy

the accompanying bacterial and fungal microflora, homogenized and inoculated on a specific liquid and solid medium (MGIT - Mycobacteria Growth Indicator Tube (BACTEC MGIT 960, Becton Dickinson, USA) and egg-based medium – Löwenstein–Jensen (Becton Dickinson, USA)). The reagents used for decontamination, are described in detail in the Results and Discussion section as we feel that the successful isolation of NTM strains is one of the merits of our work. Samples were incubated for 76 days at room temperature and at 37°C, with continuous monitoring to detect fast-growing NTM. When growth was visible, a smear was made by the Ziehl-Nielsen technique to detect acid-fast bacteria (AFB). If the presence of AFB was confirmed, phenotypic, immunochromatographic (Capilia™ TB Neo) and molecular genetic determination of the isolates were performed. Geno Type® Mycobacterium CM and AS (Hain Lifescience GmbH, Nehren, Germany) tests (PCR tests known as LPA based on DNA strip technology) were used for identification of the most common and relevant to human pathology NTM. Genotypimg was performed after DNA isolation from a pure culture (GenoLyse® Hain Lifescience GmbH, Nehren, Germany) with subsequent amplification of the 23S rRNA gene and reverse hybridization to specific oligonucleotides immobilized on a membrane strip.

RESULTS AND DISCUSSION

Isolation of NTM from environmental materials is a serious challenge. Samples from natural habitats are rich in bacterial flora. The presence of contaminating spore-forming bacteria and moulds destroy the medium in a short time, and supress or mask the growth of NTM. It is necessary to destroy the other bacteria and fungi, in order to obtain visible colonies of NTM. Specific procedures to ensure homogenization and decontamination of the collected materials are used. The purpose of homogenization is to free the bacteria from the mucus, cells or tissues in which they are infiltrated. Decontamination is based on the high resistance of mycobacteria to acids and hydroxides. Relevant protocols have been successfully established for clinical material, however working with environmental samples requires the consideration of different variables depending on the conditions and type of specimen being processed. The procedure can cause a loss of up to 70% of the target NTMs from each sample, which would result to an underestimation of the amount and species diversity of NTM and possibly the inability to isolate strains because they could be sensitive to the decontaminating agents. (9) According to Kazda et al. 2009 (9) the conditions for successful cultivation of ecological NTM are not fully known. For this reason, for *in vitro* isolation of NTM it is imperative to select and use appropriate decontamination procedure, isolation medium and incubation temperature. The decontamination method we used was an adaptation of Parashar D. (10). Water samples were concentrated before decontamination, and soil samples were dissolved in 20 ml of sterile distilled water. The procedure of decontamination was two-step. First we are using 4% NaOH for 20 min followed by 5% oxalic acid for 30 min. The materials thus treated were inoculated into two tubes of liquid and solid medium (MGIT and Löwenstein–Jensen), which were cultured in parallel in the dark at room temperature and at 37°C. Once growth was observed, a Ziehl Neelsen microscope slide was made, and AFB-positive cultures were subcultivated on Löwenstein–Jensen and used for phenotypic, immunochromatographic and molecular genetic identification. Typically, rapidly-growing NTM took from one week to 10 days to grow, while slow-growing ones often formed visible colonies in a month (11). For this reason, it is also important to clearly and accurately distinguish specific growth from contaminants. There are very limited studies aimed at the detection of NTM in caves. (12, 13). According to literature data, the most frequently isolated NTM from cave waters and sediments are the species - *M. avium, M. mucogenicum, M. chelonae and M. fortuitum* (14). These data concurred with our established species diversity, according to which the fast-growing NTMs predominate among the isolates. From 235 samples that we collected in 19 caves and 3 karst springs from the above-mentioned Bulgarian karst regions, 10% (n=24) gave growth to NTM strains, that were isolated according to the described procedure. They are presented in Table 1. Most of NTMs were identified from bat guano (67%, n=16), the other materials positive for NTMs were: water and biofilm (13%, n=3 each), sediment and clay (3%, n=1 each). The mycobacterial species diversity

Table 1 Diversity of NTM isolates by site.

Name of Cave and Locality	Number of isolates	Type of specimen	Isolated NTM species
Vrazhite dupki 2431 ВД№9; Vratsa karst region	**2**	guano (n=1) sediment (n=1)	*M. chelonae* mix cilture
Dupkata pod asfalta Bosnek karst region	**1**	guano (n=1)	*M. fortuitum* group
Dushnika Iskrets, Ponor karst region	**2**	guano (n=2)	*Mycobacterium sp.* *M. terrae* complex
Zidanka Lakatnik, Vratsa karst region	**1**	biofilm (n=1)	mixed NTM culture
Opushenata nisha Lakatnik, Vratsa karst region	**1**	clay (n=1)	*M. szulgai*
Pepelyanka Bosnek karst region	**2**	biofilm (n=2)	*M. gordonae* *M. szulgai*
Svinska dupka Vratsa karst region	**13**	guano (n=12) water (n=1)	2 x *M. chelonae* 6 x *M. fortuitum* group 4 x *Mycobacterium sp.* from water -*M. scrofulaceum*
Proboinitza hut; Ponor karst region	**1**	water (n=1)	*M. gordonae*
Fountain in front town hall Buchin prohod; Ponor karst region	**1**	water (n=1)	mixed NTM culture

identified in the samples included: *M. chelonae* (n=3), *M. gordonae* (n=2), *M. intermedium* (n=3), *M. scrofulaceum* (n=1), *M. szulgai* (n=4), *M. fortuitum* group (n=4), mixed culture (n=4), *M. terrae* complex (n=1), one species was determined as belonging to the genus *Mycobacterium* without the possibility of a more precise identification because of the limitation of the test.

The distribution of NTM isolates by cave region was as follows: 73% (n=11) from 203 (Vratsa), 20% (n=3) from 204 (Ponor) and 7% (n=1) from area 303 (Bosnek). Interestingly, an isolate of NTM was detected far from the entrance of the cave and from the often visited area. The respective were samples collected approximately 104 meters from the cave entrance. This was a mixed culture of NTM species that remains to be precisely characterized.

CONCLUSION

Our study proved that NTM were spread in natural habitats in the country and that their species diversity was comparable to the most frequently isolated NTM species in Europe. The presence of NTM in cave ecosystems represents a potential source for human infection. We had successfully implemented a working protocol for decontamination and isolation of NTM species from the environment. For the first time in the country, NTM species were isolated from caves (biofilms and karst waters, sediments and bat guanos) and their geographical distribution were analyzed by habitat mapping of the most frequently isolated NTM. The species of the isolated NTM will be clarified by whole genome sequencing. The results obtained will form a database for tracking the trend of NTM distribution in the country and determining their clinical significance.

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DISCLOSURE OF CONFLICT OF INTEREST

There is no conflict of interest to declare.

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DECODING MICROBIOME DYSBIOSIS THROUGH METAGENOMIC ALPHA DIVERSITY. IMPLICATIONS FOR SARCOIDOSIS AETIOLOGY

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ABSTRACT

Background: Sarcoidosis is a chronic inflammatory disease that can affect multiple organs. The aetiology of sarcoidosis is not fully understood, but there is increasing evidence that the microbiome may play a role. The blood microbiome is a collection of microorganisms that live in the bloodstream. It is a complex and dynamic community that is influenced by a variety of factors, including the host's lifestyle and pathology. Recent studies have shown that people with sarcoidosis have alterations in their blood microbiome. These alterations include changes in the diversity, richness, and evenness of the microbial community. The abundance measures by which the blood microbiome diversity may detect instances of dysbiosis related to sarcoidosis aetiology. It should be clearly distinguished from microbiome changes related to unspecific inflammation or sepsis. However, the available evidence suggests that the microbiome may be a promising target for therapeutic interventions.

Aim: The primary goal of this review was to assess and compare the existing metrics of microbiome composition and diversity as established by metagenomic analyses. Additionally, we aim to elucidate the potential causal relationship between these measures, the phenomenon of blood microbiome dysbiosis and the pathogenesis of sarcoidosis.

Conclusion: In the present review, we investigated alpha diversity measures as characteristics of microbiome communities, examining their potential as indicators of dysbiosis, and the probable mechanisms of microbiome participation. A descriptive qualitative comparison was conducted between lung microbiome data of sarcoidosis patients and blood microbiome data of healthy adults. This comparison elucidates common taxa between the two microbiomes and identifies taxa potentially involved in sarcoidosis.

Key words: sarcoidosis, dysbiosis, blood microbiome, alpha diversity

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INTRODUCTION

Sarcoidosis is characterized by the formation of small inflammation areas (granulomas) in various organs, most commonly the lungs and lymph nodes [1]. Current theories on sarcoidosis aetiology suggest a complex interaction between genetic susceptibility, immune response, and exposure to specific environmental, occupational, or infectious agents, but the precise pathogenesis remains unclear [2,3]. Corticosteroids are commonly used as first-line therapy, but a significant proportion of patients may require additional treatment due to refractory disease or adverse effects, pointing out the necessity for novel therapeutic strategies [4].

The blood microbiome is now being recognized as potentially affected by various systemic and inflammatory diseases, as microbial components and metabolites were identified in the blood and can directly interact with the immune system [5]. There is a growing body of evidence suggesting that alterations in microbiome, or dysbiosis, could play a role in sarcoidosis [6,7]. Dysbiosis may influence sarcoidosis development through several mechanisms, including immune dysregulation, metabolic shifts, or increased permeability of mucosal barriers that allow the translocation of bacteria or bacterial products into

the bloodstream [8–10]. These alterations in the microbiome could contribute to the granulomatous inflammation observed in sarcoidosis, suggesting a possible link between microbial dysbiosis and the pathogenesis of the disease. Future studies investigating the blood microbiome in patients with sarcoidosis could provide valuable insights into the disease's aetiology and offer novel therapeutic targets [11].

The composition of blood microbiome is mainly assessed through metagenomic sequencing, a method that allows a comprehensive survey of the microbial community within a given sample [12]. This high-throughput technique provides a detailed picture of the diversity of microbial community [13]. Metagenomics can identify both known and novel microorganisms, including bacteria, viruses, fungi, archaea, and eukaryotic unicellular and multicellular parasites, that would otherwise be missed with traditional culture techniques [14]. This technique has significantly advanced our understanding of blood microbiome, and its role in health and disease [15].

The primary goal of the present review was to assess and compare the existing metrics of microbiome composition and diversity as established by metagenomic analyses. Additionally, we aim to elucidate the potential causal relationship these measures hold with the phenomenon of blood microbiome dysbiosis and the pathogenesis of sarcoidosis.

MEASURES OF MICROBIOME ABUNDANCE

Metrics for analysing microbiome's composition and diversity using metagenomic data primarily come from ecological studies. These metrics can be generally classified into alpha diversity and beta diversity measures [16]. The primary focus of this review is alpha diversity, as various measures of alpha diversity provide increasingly detailed insights into the structure of microbial communities. This information is vital for evaluating the health of a community, and is crucial for identifying signs of microbiome dysbiosis. In contrast, beta diversity, in all its forms, serves as a straightforward measure that essentially quantifies the numerical distance between two communities — the higher the beta diversity, the greater the distance — requiring no further interpretation [16].

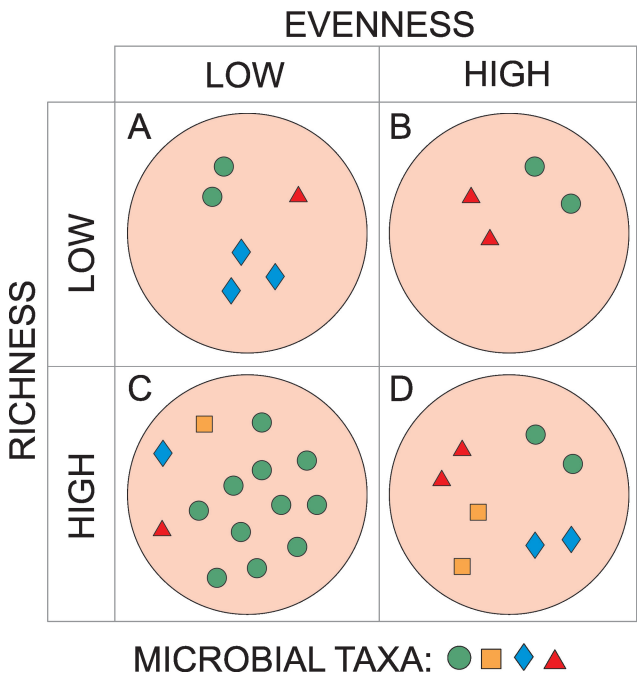

Figure 1. Schematic representation of four distinctive states of microbiome alpha diversity assessed by the measures of evenness and richness; A) low evenness vs. low richness; B) low richness vs. high evenness; C) low evenness vs. high richness; D) high evenness vs. high richness.

Alpha diversity is a key measure used to describe the complexity of microbial communities within a particular sample. It represents both the richness (number of different species) and evenness (distribution of individual species) of the community (**Figure. 1**) [16]. Different alpha diversity indices can provide various insights into the composition of the microbiome and potential dysbiosis. (1) Richness: The total number of unique species in a sample provides a basic measure of diversity. **Richness** is the simplest measure and only takes into account the number of different species, without considering their relative abundance. As such, it might overlook important shifts in the distribution of individual species. If there is a substantial reduction in the richness of the blood microbiome it may suggest a loss of beneficial microorganisms or overgrowth of a few species, characteristic of dysbiosis. (2) **Evenness** typically ranges within an interval of 0 to 1; 0 indicates complete unevenness (with one taxon dominating the entire community), while 1 signifies

perfect evenness (all taxa are equally abundant within the community). Such a community maintains a balanced distribution of species abundances, with no single taxon overshadowing others. This scenario represents a healthy and stable microbiome, fostering a more resilient community. A low evenness value suggests that the microbiome is dominated by one or a few species, with others present in much lower abundances. Consequently, the community might be more susceptible to disturbances; in other words, low evenness could be indicative of microbiome dysbiosis. (3) Shannon Index and Simpson Index: These are more complex indices that consider both **richness** and **evenness**. **The Shannon Index and Simpson Index** combine both richness and evenness into a single measure. The Shannon Index places more weight on richness, while the Simpson Index places more weight on evenness (dominance). Therefore, they complement each other and offer a more comprehensive overview of diversity. For instance, a microbiome sample might have high richness but low evenness due to the overrepresentation of a particular species. This could lead to a low Shannon Index value but a high Simpson Index value. Understanding these nuances can help detect subtle changes in microbiome composition, such as those that may occur during dysbiosis [17,18]. A decrease in the Shannon index or an increase in the Simpson index can both indicate a decline in diversity and potential dysbiosis. For example, decreased alpha diversity reflecting reduced richness and/or evenness (measured by the Shannon index) has been associated with inflammatory bowel disease, indicating a less diverse and potentially dysbiotic microbiome [19]. An increase in the Simpson index indicating the dominance of certain species might be associated with conditions like periodontitis [20]. By assessing alpha diversity, researchers can get a sense of the overall health of the microbiome. Changes in alpha diversity can indicate shifts towards dysbiosis, and studying these changes over time could help to elucidate the onset and progression of diseases linked to microbiome alterations.

While some overlap exists between the various alpha diversity metrics, they primarily serve as complementary tools that capture different aspects of microbial diversity. Indeed, the use of these various metrics in tandem allows researchers to capture different aspects of microbiome complexity. For instance, observing high richness but low evenness might suggest that while a large number of species are present, the community is being dominated by a few species, potentially indicating dysbiosis. Similarly, a low richness but high evenness could suggest a more balanced community, but with fewer species present, indicating a possible loss of beneficial microorganisms [21]. Metrics like the Shannon and Simpson indices which integrate both richness and evenness, could help identify more nuanced shifts in the microbiome. Therefore, the combined application of these metrics offers a multi-faceted view of the microbial community, capturing its richness, balance, and overall diversity. This comprehensive approach is crucial for a thorough understanding role of microbiome in health and disease, and the potential implications of microbiome dysbiosis.

MICROBIOME DYNAMICS

Regardless of the mechanisms by which the microbiome affects health, it is crucial to develop reliable methods to describe and differentiate alterations in the blood microbiome. Microbiome alterations could be graded as random fluctuations, microbiome dysbiosis and infection or sepsis.

(1) **Random fluctuations** occur even in healthy individuals. The composition of microbiome may naturally fluctuate due to factors such as diet, sleep, stress, and other environmental factors [22]. Therefore, discerning between these random fluctuations and disease-related dysbiosis is a critical challenge. Longitudinal studies that track individual microbiomes over time can help to set a baseline for these natural fluctuations [23]. Random fluctuations in microbiome composition are natural variations that can occur due to factors such as daily diet, short-term illnesses, minor changes in the environment, or even the circadian rhythm. These fluctuations typically do not lead to significant shifts in the overall structure of the microbial community, and the latter tends to return to its original state (baseline) once the influence of the transient factors ends. Alpha diversity metrics can reflect these fluctuations as random and transient changes over time. Similarly, the relative abundances of different species may

fluctuate slightly due to random variations, while the overall evenness of the community should remain stable unless a certain species starts to consistently dominate or become marginalized. The combined measures of richness and evenness can be sensitive to random fluctuations. However, if the community is resilient, these indices should return to baseline levels once the temporary influencing factor is removed. Thus microbiome random fluctuations are highly unlikely to contribute to sarcoidosis aetiology.

(2) **Microbiome dysbiosis,** or chronic conditions often involves sustained and significant shifts in the microbiome composition. Differentiating these shifts from random fluctuations requires a detailed understanding of the diversity and abundance of microbial species, often achieved through metagenomic sequencing [13]. Microbiome dysbiosis refers to a state where the natural balance of the microbial community is disrupted, often in association with a disease or a pathological condition. Alpha diversity metrics can help detect and quantify such disruptions. In a state of dysbiosis, the richness of the microbiome, or the total number of different species might decrease significantly. This is because certain species may outcompete others or some may not survive the altered conditions. A decrease in richness may indicate that beneficial species have been lost or that pathogenic species have overgrown. During dysbiosis, the evenness of the microbiome can also be affected as some species become overrepresented while others become underrepresented. This means that even though many species may still be present, their distribution is uneven, often favouring pathogenic or opportunistic species. As previously mentioned, Shannon and Simpson's indices combine richness and evenness into a single measure, and changes in these indices can indicate dysbiosis. A decrease in the Shannon index or an increase in the Simpson index suggests a decrease in diversity and an indication for dysbiosis. Therefore, alpha diversity metrics can help detect shifts in the microbiome associated with dysbiosis, providing valuable insights into the microbiome's role in health and disease.

(3) **Infection or sepsis**. Sepsis represents a clear disturbance of blood microbiome usually linked to the proliferation of a particular pathogen. Rapid diagnostic tools like PCR or next-generation sequencing can help identify pathogens directly from blood samples [24]. Sepsis is a severe, systemic response to infection that can lead to organ failure and death. The dysbiosis that accompanies sepsis represents a significant disruption of the normal microbial community structure with potentially life-threatening consequences. Alpha diversity metrics can provide insights into these microbial changes. In sepsis, a decrease in species richness can occur due to the overwhelming presence of a particular pathogen, leading to the reduction or elimination of other microbial species. This can also be the result of broad-spectrum antibiotic treatment commonly used in sepsis management, which can indiscriminately kill both harmful and beneficial microorganisms [25,26]. Similarly, the evenness of the microbiome is likely to decrease in sepsis as the pathogen causing the infection dominates the microbial community, or as antibiotics alter the relative abundance of various species. A significant decrease in the Shannon index or an increase in the Simpson index could indicate a state of dysbiosis associated with sepsis. There is increasing evidence that microbiome analysis, including the use of alpha diversity metrics, may provide valuable insights for sepsis diagnosis and prognosis. For instance, a study by Yin and colleagues [27] found that lower diversity (assessed using the Shannon index) of gut microbiome was associated with a higher six-month mortality rate in patients with sepsis. However, while these metrics can provide a snapshot of the microbial community at a given point in time, they do not capture the dynamic changes of microbiome over time.

ASSOCIATION OF MICROBIOME DYSBIOSIS AND HOST PATHOGENESIS

(1) **Developing pathology.** Changes in blood microbiome may be a result, rather than a cause of disease development. From this perspective, the disease process causes systemic changes, including immunological or metabolic shifts that subsequently lead to dysbiosis. Thus dysbiosis is an effect of the disease rather than its initiator [28]. For example, changes in gut microbiome composition have been observed in numerous diseases such as obesity and diabetes, and it was suggested that those changes may be a reflection rather than the cause of the

Table 1. Members of the extended microbiome included used in the metagenomic analysis of BAL of sarcoidosis patients

Taxon/Species	Phylum	Alpha Diversity Measure	Richness Measure
Streptococcus	Firmicutes	Shannon	Sequence count [6]
Corynebacterium	Actinobacteria	Shannon	Sequence count [6]
Neisseria	Proteobacteria	Shannon	Sequence count [6]
Atopobium	Actinobacteria	Shannon	Bacterial burden [7]
Fusobacterium	Fusobacteria	Shannon	Bacterial burden [7]
Mycobacterium	Actinobacteria	Shannon, Simpson, Inverse Simpson	Sequence count [33]
Cutibacterium	Actinobacteria	–	Sequence count vs. total number of sequences [34]

altered metabolic state [29].

(2) **Unlocking pathology**. Conversely, there is substantial evidence suggesting that dysbiosis can contribute to the development and progression of a disease. From this perspective microbial imbalance is a trigger event that unlocks pathological processes. For example, alterations in the gut microbiome can disrupt the gut barrier, leading to translocation of bacteria and bacterial products into the bloodstream. This in turn can trigger systemic inflammation, a common feature of various diseases [30]. Similarly, changes in blood microbiome could contribute to disease by triggering an inappropriate immune response or causing direct tissue damage [5]. Dysbiosis in the subgingival microbial plaque is the reason for the development of periodontitis. The specific mechanisms for development of periodontitis are not sufficiently well understood, but microbiome dysbiosis, as a cause of immune dysregulation, has its place in the general picture. In this case, the relationships between the oral, intestinal, and blood microbiome are not sufficiently well studied. *Porphyromonas gingivalis* is the causative agent of chronic periodontitis and has been identified in the brain of patients with Alzheimer's disease [31]. Toxic proteases from the bacterium called gingipains have also been identified in the brains of Alzheimer patients and their levels correlate with tau and ubiquitin proteins in pathology [31]. Microbial translocation to the blood is evident, but whether the oral or the intestinal microflora is the primary source of pathogen, is not clear [32].

ASSOCIATION OF PULMONARY MICROBIOME DYSBIOSIS AND THE ETIOLOGY OF SARCOIDOSIS

Currentlyin sarcoidosis research, bronchoalveolar lavage (BAL) is the primary sample type used for comparing microbiome composition in patients with sarcoidosis and control subjects [6,7,33]. **Table 1**. shows microbial genera with specifically increased their alpha abundance in BAL samples of sarcoidosis patients. Overall, such samples were characterized by high richness and low evenness values (**Figure. 1 C**).

BLOOD MICROBIOME AND THE ETIOLOGY OF SARCOIDOSIS

Blood microbiome is a complex community of bacteria, fungi, viruses, and other microorganisms. The composition of this microbiome is influenced by various factors, including diet, age, gender, and overall health status. While the exact composition can vary from person to person, certain common microbial species have been identified. To our knowledge, two studies have specifically addressed the characterization of blood microbiota in healthy adults:a study conducted by Paise et al., in 2016 [35], and another by Panaiotov et al., in 2021 [9]. Both investigations reported similar findings regarding

taxon compositions and proportions at both the phylum and genus levels. The dominant bacterial classes identified were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria, while the prevalent genera included *Staphylococcus, Micrococcus, Corynebacterium, Acinetobacter, Streptococcus, Fusobacterium, Pelomonas, and Rothia*. A scrutiny of the prevalent genera in sarcoidosis BAL reveals a comparable taxon composition [6, 7, 33, 34]. Notably, the presence of potentially pathogenic microbial species such as *Mycobacterium* and *Neisseria spp.*, as well as commensals associated with sarcoidosis like *Atopobium* [7] and *Cutibacterium*, have been documented.

In light of the high similarity in taxon composition and the presence of commensals, several implications can be drawn. Firstly, the data suggest a potential interaction between lung and blood microbiomes, indicating a complex interplay between the microbial communities inhabiting these sites. Secondly, the findings raise the possibility that microbiome dysbiosis could significantly contribute to the aetiology of sarcoidosis, highlighting the need for further investigations into the role of microbiome dysbiosis in the aetiology of this condition. Lastly, the presence of Mycobacterium spp. hints at the potential contribution of latent infections to the disease process, suggesting a nuanced role of these microbial species in sarcoidosis, which deserves deeper exploration.

Based on the above data, one can propose a hypothetical scenario associating sarcoidosis aetiology with the blood microbiome, based on our current understanding of microbiome dysbiosis. In sarcoidosis, the immune response is thought to be triggered by an unknown antigenic stimulus, which could potentially be linked to the bloodstream or dysbiosis in the blood microbiome. Less compelling alternatives, such as the absence of certain taxa or those with reduced abundance, do not align so well in this context. This is because the contribution of microbiome to health conditions is more likely linked to the presence and proliferation of certain microbes, rather than their absence or reduced presence. In the context of sarcoidosis, it could be hypothesized that genera typically associated with pathogenic traits, such as *Staphylococcus*, *Streptococcus*, *Corynebacterium*, and *Pseudomonas*, might be overrepresented. Conversely, genera that are typically associated with a healthy microbiome, such as *Cutibacterium*, *Prevotella*, *Veillonella*, and *Fusobacterium*, might be transformed by microbiome interactions into opportunistic pathogens and also increase in their abundance. However, it is important to emphasize that this is a hypothetical scenario and actual research may show different results. The relationship between blood microbiome and sarcoidosis, and the potential role of specific microbial genera, need to be confirmed through empirical studies.

CONCLUSIONS

Microbiome dysbiosis in the blood is the main scope of this review because of its hypothesized influence on chronic inflammatory conditions and sarcoidosis in particular. Microbiome dysbiosis represents a significant shift in the relative abundance and diversity of different microbial species that populate the body's ecosystems, and these changes can be particularly evident in the blood microbiome. For instance, in cardiovascular diseases, there has been growing evidence of alterations in the blood microbiome composition, with a relative abundance of specific bacteria such as Proteobacteria and decreased diversity observed in patients with atherosclerosis [36]. Similarly, in autoimmune conditions such as rheumatoid arthritis, dysbiosis of the blood microbiome has been identified, with an increase of rare or pathogenic species and an overall decrease in diversity as compared to healthy controls [37]. Chronic kidney disease has also been linked to blood microbiome dysbiosis, with increased levels of circulating bacterial DNA and a predominance of certain bacterial genera, such as *Staphylococcus* and *Pseudomonas,* in the bloodstream [38]. Given the significant associations between blood microbiome dysbiosis and various chronic conditions, the deciphering of these microbial changes could potentially lead to the identification of novel diagnostic markers and therapeutic targets. Still, comprehensive metagenomic sequencing studies are required to better understand these complex relationships and their implications for human health [13]. In particular, measures of microbiome

abundance could play a crucial role in evaluating the aetiology of sarcoidosis. Not only might they help identify specific microbial taxa associated with the disease, but they could also shed light on the dynamic interplay between the host and its microbiome. These insights could, in turn, enhance our understanding of how sarcoidosis develops and progresses, resulting inmore effective strategies for prevention and treatment.

CONFLICT OF INTERESTS:

The authors declare no conflict of interest.

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