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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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Manuscripts must be written in English, using British spelling. All manuscripts should be single-spaced, with wide margins and numbered pages. MS Word should be used for word processing, 12-point Times New Roman font.

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3. Final approval of the version to be published.

All people who meet the three criteria should be named as authors. Those who participate in the study but do not meet the requirements of authorship should be acknowledged as contributors.

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ACKNOWLEDGEMENTS

Individuals who supplied facilities, strains or reagents, or gave advice may be acknowledged. Also, supporting grants may be mentioned.

REFERENCES

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PHENOTYPIC DETECTION OF OXA-48-PRODUCING ENTEROBACTERIAL ISOLATES BY ROUTINE ANTIBIOGRAM

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ABSTRACT

Carbapenemase-mediated resistance in *Enterobacteriaceae* has increased in the last years, seriously compromising the management of life-threatening infections. Rapid detection of carbapenemase producers is essential for the control of their dissemination, for the successful treatment of infected patients and the preservation of carbapenem efficacy.

According to EUCAST methodology carbapenemase inhibition tests with boronic acid derivatives and dipicolinic acid/EDTA combined with a temocillin disc provide a reliable phenotypic confirmation method for class A, B and OXA-48 carbapenemases in *Enterobacteriaceae*. Using peculiar hydrolysis profile of OXA-48 enzymes and the high-level resistance to temocillin we developed a cost-effective disk-based method to screen for OXA-48- producers within routine antibiogram. The test is a potentially useful diagnostic tool especially for difficult-to-detect OXA-48-positive/ESBL-negative *Enterobacteriaceae*. It can provide important infection control information and help to ensure appropriate antibiotic therapy of the infected patients.

Key words: *Enterobacteriaceae*, carbapenemase, OXA-48

INTRODUCTION

Carbapenemase-mediated resistance in *Enterobacteriaceae* has increased in the last years, seriously compromising the management of life-threatening infections. Rapid detection of carbapenemase producers is essential for the control of their dissemination, for the successful treatment of infected patients and the preservation of carbapenem efficacy (1). Although molecular identification and differentiation of carbapenemase genes remain the reference standard, phenotypic detection may be indicated in diagnostic microbiology laboratories when molecular methods are not available. Several studies have shown that Ambler class A and B carbapenemases in *Enterobacteriaceae* may be detected using carbapenemase inhibition tests with boronic acid derivatives (BA) and dipicolinic acid (DPA)/EDTA, respectively (2). However, for OXA-48-like carbapenemases, no specific inhibitor is available. Such method is warranted because OXA-48-producing *Enterobacteriaceae* are associated with outbreaks, the prevalence is rapidly rising throughout Europe and they are frequently associated with serious therapeutic failures in hospital settings (3). Recently published data showed that OXA-48 confers high-level resistance to temocillin (4), and it has been suggested that temocillin may be a useful indicator of OXA-48 production (5). Moreover, these enzymes, unlike other carbapenemases, do not confer resistance to extended-spectrum cephalosporins, and are resistant to inhibition by clavulanic acid and tazobactam.

Taking into account the peculiar hydrolysis profile of OXA-48 enzymes and the high-level resistance to temocillin we developed a cost-effective disk-based method to screen for OXA-48- producers within routine antibiogram.

MATERIALS AND METHODS:

From January 2014 all *Enterobacteriaceae* isolates recovered from inpatients at a 252-bed oncology hospital in Sofia were routinely tested against temocillin, meropenem, imipenem, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, piperacillin/tazobactam and cefotaxime with disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (6). The results were interpreted following current CLSI-2014 recommendations (7). *Escherichia coli* ATCC 25922 was used as antibiotic-susceptible control. Species identification was carried out by using GNI cards on the VITEK 2 system (bioMerieux Vitek Inc., Hazelwood, MO).

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RESULTS AND DISCUSSION:

In March 2014, disk diffusion susceptibility tests, routinely performed in the hospital laboratory, revealed the presence of a phenotype highly suspicious for OXA-48-like enzyme in a peritoneal *Klebsiella pneumoniae* strain (**Fig.1**). This isolate had reduced susceptibility to imipenem (24 mm) and meropenem (23 mm), resistance to temocillin and penicillin/inhibitor combinations but was susceptible to extended-spectrum cephalosporins. According to the Bulgarian national guidelines on carbapenem non-susceptible isolates, the strain was submitted to the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance and confirmed by PCR as OXA-48-positive (8). Based on these results and susceptibility to fluoroquinolones, the treatment was carried out with intravenous combination of ceftazidime and ciprofloxacin. The infection prevention and control unit was timely notified and contact isolation precautions were maintained for the entire duration of the hospital stay. The patient was discharged home after three weeks antibiotic treatment in satisfactory general condition. As a result of the immediate implementation of contact precautions, no other patients were found to be colonised or infected with the *K. pneumoniae* strain PR2899.

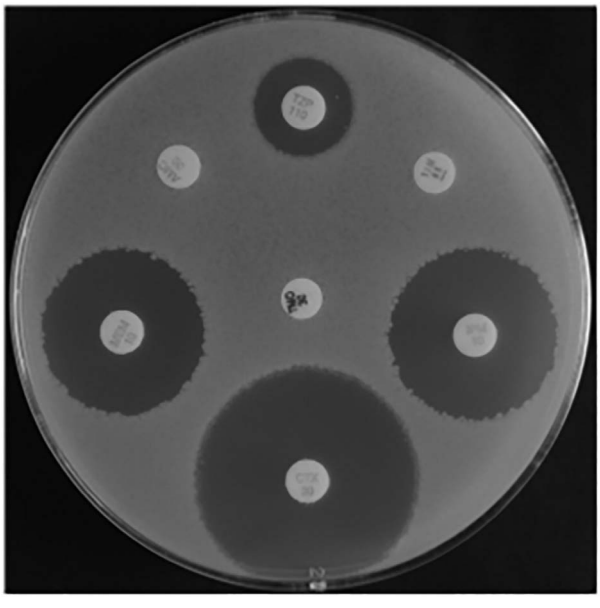

Figure 1: Antibiogram of OXA-48 producing *Klebsiella pneumoniae* PR2899. Clockwise: TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; IPM, imipenem; CTX, cefotaxime; MEM, meropenem; AMC, amoxicillin/clavulanic acid, TMO, temocillin - centre. Concomitant resistance to penicillin/inhibitor combinations and temocillin together with reduced susceptibility to carbapenems but susceptibility to extended-spectrum cephalosporins are indicative of OXA-48 production.

The prompt and accurate detection of OXA-48 is especially challenging because no specific inhibitor is available for class D carbapenamases. The investigated OXA-48 isolate in our study had meropenem zone diameter (23 mm) that would categorise it as susceptible according to CLSI interpretive criteria (7) and EUCAST clinical breakpoints (9) as well. The only commercially available inhibitor-based combination disk test did not detect OXA-48, as expected, and there are very limited published data on other phenotypical detection options (10). Therefore, the additional use of temocillin within routine antibiogram seemed to be a useful approach in further identification of these microorganisms (11). Our study suggests that concomitant resistance to penicillin/inhibitor combinations and temocillin together with reduced susceptibility to carbapenems but susceptibility to oxyimino-cephalosporins could be a sensitive screening tool for difficult-to-detect OXA-48-positive/ESBL-negative *Enterobacteriaceae*. The proposed disk configuration reflects the peculiar hydrolysis profile of OXA-48-like enzymes. The inclusion of temocillin disk in routine antibiogram prevents additional testing and additional overnight incubation.

CONCLUSION

Our study showed that meropenem CLSI susceptibility breakpoints by the disc diffusion method and EUCAST clinical breakpoints fail to detect OXA-48-positive/ESBL-negative *Enterobacteriaceae* exhibiting a cephalosporin-susceptible, carbapenem-non-susceptible phenotype. This observation is of concern since a large number of diagnostic microbiology laboratories in Bulgaria still rely on the disc diffusion method as the principal method for routine susceptibility testing. Concomitant resistance to penicillin/inhibitor combinations and temocillin together with reduced susceptibility to carbapenems but susceptibility to oxyimino-cephalosporins could be a sensitive screening tool for difficult-to-detect OXA-48-positive/ESBL-negative *Enterobacteriaceae*.

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UPDATES ON MOLECULAR EPIDEMIOLOGY OF S. TYPHI IN BULGARIA WITH REVIEW OF OUTBREAK AND SPORADIC CASES, 2008-2014

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ABSTRACT
This work provides an update in molecular epidemiology of typhoid fever in Bulgaria. A total of 15 patients were diagnosed with typhoid fever in Shumen region between 2008-2014. Another two cases occurred in Sofia in 2008 and 2012, but were imported from other countries. Fifteen *S*. Typhi isolates were confirmed with microbiological methods during the period 2008-2014, 10 of them were studied with pulsed-field gel electrophoresis (PFGE). Three main pulsotypes were identified allowing differentiation between local endemic (type I) and imported cases (types II and III). All isolates originating from Shumen, Bulgaria were further subdivided into four subtypes, which were closely related between themselves. Eighty percent of Bulgarian *S*. Typhi isolates were susceptible to a set of twelve antimicrobial agents including fluoroquinolones, which was favourable in terms of successful treatment of the patients. One of the imported *S*. Typhi isolates characterised by diminished zones to ciprofloxacin and resistance to nalidixic acid was negative for *qnr* and *qep*A genes.

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INTRODUCTION
Typhoid fever is one of the most serious forms of human infections caused by *S*. Typhi. The disease is encountered mainly in developing countries, but cases can emerge worldwide making diagnostic and treatment updates necessary at national and international levels (1, 2). In Bulgaria each case of typhoid fever is mandatory reported through the national surveillance system to the Ministry of Health. Isolates of *S*. Typhi are being sent to the National Reference Laboratory (NRL) of Enteric Pathogens, Sofia, Bulgaria for confirmation and further characterisation. Before 2013 the annual frequency of typhoid fever registered in the country did not exceed 1-2 cases per year (3). From May 2013 and until the end of June 2014 the total number of diagnosed cases increased to 11. This worrisome trend provoked extensive investigational work involving epidemiologists, microbiologists, clinicians and non-medical specialists. The objective of the present work was to study the molecular epidemiology of *S*. Typhi isolates collected at the National Reference Laboratory of Enteric Pathogens, Sofia, Bulgaria, between 2008-2014 and provide country surveillance updates.

MATERIALS AND METHODS

Patients
During the period 2008-2014 a total of 15 patients were primarily diagnosed with typhoid fever at the Multifunctional Hospital for Active Treatment (MHAT), Shumen. Two patients were diagnosed at the Specialised Hospital for Active Treatment of Infectious Diseases (SHATID), Sofia, one Bulgarian citizen with travel history to Eastern countries in 2012 and one Indian lama, who became ill during his mission in Bulgaria in 2008. Initially, all patients presented with high fever (> 39- 40^{o}C), shaking chills, pain in the back, bloody or cloudy urine. Three patients developed the following complications: acute interstitial nephritis (n=2) and bleeding duodenal ulcer (n=1) discovered at the first medical examination.

Table 1. Epidemiological data and origin of *Salmonella* isolates causing typhoid fever. Isolates are listed in retrospective order beginning from 2014. Laboratory ID numbers correspond with these at the NRL of Enteric Pathogens, Sofia, Bulgaria. Strains marked with an asterix (*) were studied with PFGE.

Isolate ref. number	Date of receipt to the NRL, Sofia, Bulgaria	Specimen	Age	Sex	National region
24*	14.2.2014	stool	40	male	Shumen
56	30.4.2014	blood culture	38	male	Shumen
64*	13.5.2014	blood culture	52	male	Shumen
65	13.5.2014	blood culture	50	male	Shumen
75*	28.5.2014	stool	58	female	Shumen
84	06.6.2014	stool	52	male	Shumen
85	08.6.2014	stool	52	female	Shumen
86	08.6.2014	blood culture	56	male	Shumen
59*	26.5.2013	blood culture	63	male	Shumen
133*	23.8.2013	blood culture	36	male	Shumen
176*	08.10.2013	stool	42	male	Shumen
177*	11.10.2013	blood culture	19	male	Shumen
32*	26.3.2012	stool	45	male	Sofia
2106*	15.8.2008	blood culture	11	male	Shumen
2418*	02.12.2008	stool	35	male	Sofia

S. Typhi bacterial isolates

Fifteen *S*. Typhi isolates collected between 2008-2014 at the NRL of Enteric Pathogens, Sofia, Bulgaria, were included in the current study. Their origin is represented in **Table 1**.

Microbiological characterisation of S. Typhi isolates

S. Typhi isolates were confirmed at the NRL of Enteric Pathogens, Sofia by culture on Deoxycholate citrate agar, biochemical tests (API 20 E, Biomerieux), agglutination with Vi, O9 and Hd-antisera (SSI, Denmark; Sifin, Germany; BulBio, Bulgaria) according to the White-Kauffmann-Le Minor scheme, 9th Edition, 2007 (4, 5).

Antimicrobial susceptibility was investigated with Kirby-Bauer Disc diffusion method for cefotaxime (30μg), cefoxitin (30μg), ceftazidime (30μg), ampicillin (10μg), amoxicillin/clavulanic acid (20/10μg), amikacin (30μg), gentamicin (10μg), tetracycline (30μg), chloramphenicol (30μg), ciprofloxacin (5μg), nalidixic acid (30μg), and trimethoprim/sulfamethoxazole (1.25/ 23.75 μg) (6).

The presence of *qnr* A, B, C, D, S and *qep*A genes was screened by *Multiplex EvaGreen PCR* in one of the three isolates with diminished zones to ciprofloxacin (*S.* Typhi # 32*) (7).

Diagnostic Widal reaction was performed to establish OD and Hd antibody titers in four patients, who were admitted to the MHAT Shumen with clinical symptoms of typhoid fever (8).

Pulsed-field Gel Electrophoresis (PFGE)

The PulseNet USA standardised laboratory protocol for molecular subtyping by Pulsed-field Gel Electrophoresis (PFGE) was applied to investigate ten *S*. Typhi isolates marked with an asterix in Table 1. The following apparatus and conditions were used: Chef DR-II (Bio-Rad), initial time 2.2 s, final time 63.8 s, voltage 200 V, run time 20 h (9).

RESULTS

All fifteen *S*. Typhi isolates demonstrated typical growth and biochemical features and were simultaneously Vi and O9 positive, which corresponded to the mixed v/w form of the causative agent (v/w stands for the German “viel”/”wenig”) (10).

ESBL-producing Enterobacteriaceae strains became an emerging problem in Bulgaria (11, 12), but none of the investigated *S*. Typhi strains proved to be ESBL producer. 12/ 15 (80%) of the bacterial isolates were susceptible to all antimicrobial agents tested. Three isolates (#2106*, Shumen, #32*, Sofia

and #2418*, Sofia) were characterised with resistance to nalidixic acid and diminished zones to ciprofloxacin (< 30 mm). Isolate #32* **(Table 1)** was screened for *qnr* and *qep* A genes, but revealed a negative result.

Acute and convalescent sera from two patients (D. M and J. K.) were tested with Widal reaction and their diagnosis was confirmed due to the dynamic increase in OD and Hd antibody titers. Single serum samples from two other patients (I. V and P. P) were also investigated. The first patient simultaneously had a positive culture for *S*. Typhi, while the second one had a borderline OD titer 1:100.

Molecular characterisation and comparison of genetic profiles of *S*. Typhi isolates was performed for the first time in our country **(Fig.1).**

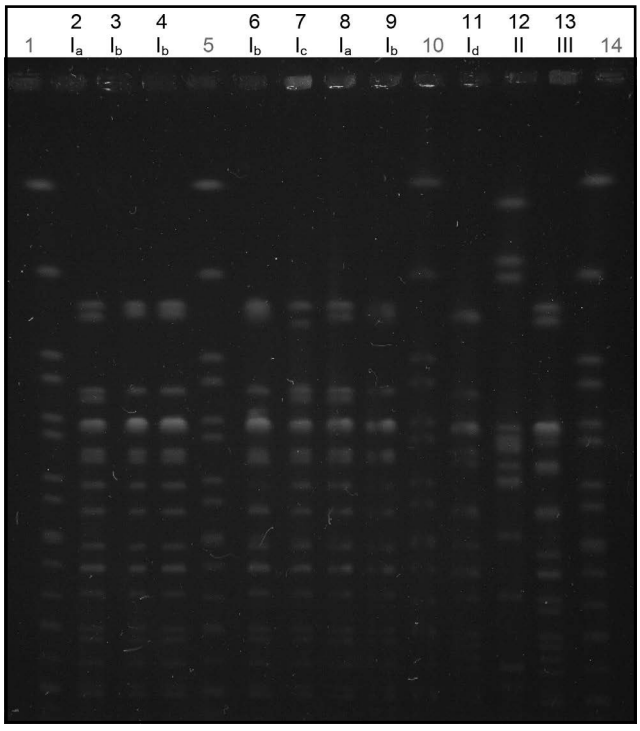

Fig. 1. PFGE typing of *S*. Typhi in Bulgaria. Strains marked with an asterix in Table 1: #24*- lane 2, Shumen; # 64*- lane 3, Shumen; # 75*- lane 4, Shumen; # 59*- lane 6, Shumen; # 133*- lane 7, Shumen; # 176*- lane 8, Shumen; # 177*- lane 9, Shumen; # 2106*- lane 11, Shumen; # 32*- lane 12, Sofia; # 2418*- lane 13, India. Lanes 1, 5, 10, 14 - S. Braenderup H2598 control strain.

Three main pulsotypes (I, II, III) were discovered based on the interpretative criteria recommended by the European Study Group on Epidemiological Markers (ESGEM) in 2007 (13). These pulsotypes differed by more than 5 bands and corresponded to three main locations: Shumen (I), Sofia (II), and India (III). All isolates originating from Shumen were further subdivided into four subtypes (Ia, Ib, Ic, and Id) (Fig. 1).

The comparison of the obtained PFGE profiles of *S*. Typhi during the outbreak in Shumen region, 2013-2014, revealed several important findings. The first of them was the discovery of 100% "indistinguishable" profiles in lanes 2 and 8 (subtype Ia), as well as the ones in lanes 3, 4, 6, and 9 (subtype Ib). These results proved the epidemiological link between the first case of typhoid fever for 2014 (lane 2) and a previous case from 2013 (lane 8). Another important finding was the 100 % similarity between *S*. Typhi in lanes 3 and 4. These two isolates originated from a construction worker and a healthy woman-carrier, respectively. The woman was a resident of a home for people with mental disorders and was identified during the epidemiological field investigation. We assumed that she had played a crucial role for the dissemination of bacteria in the community of her contacts.

The PFGE subtypes Ia, Ib and Ic were very closely related differing only by one band (Fig. 1). Although the high genetic similarity between human *S*. Typhi isolates from Shumen was suggestive of a common source outbreak, neither food nor water samples tested positive for the causative agent. Based on the findings of the investigation and following the specialists` recommendations, the sanitary conditions in the facility for mentally ill people were improved and the waste water systems reconstructed, which lead to the end of the outbreak. No new cases of typhoid fever have been reported through the national surveillance system since June 2014.

A previous *S*. Typhi isolate from Shumen (#2106*, lane 11, pulsotype Id) differed by 3 bands from the remaining outbreak strains, but still belonged to the same main pulsotype I. On the contrary, the isolates originating from the patients from Sofia and India (lanes 12 and 13, respectively) were assigned to two different pulsotypes II and III. According to our epidemiological, clinical, and genetic data, these two cases were imported in Bulgaria from other countries.

DISCUSSION

During the second half of the 20th century the reported incidence due to typhoid fever in Bulgaria was 7,1‰ with investigated clusters from Smoljan, Targoviste, Gabrovo, and Plovdiv regions (14). During that period, phage typing according to Craigie and Felix was the reference method for comparison of *S*. Typhi between countries. Bulgarian researchers determined two frequent phage types: E_1 which was predominant in Smoljan region and A_1- in Gabrovo region (14, 15). Similar phage type distributions have been discovered in Germany, Austria and the UK before 2005 (16). The history of documented cases of typhoid fever in Shumen region is relatively short and can be easily traced back in time. For the period 1997 – 1999 a total of 5 sporadic cases were reported among children (age range 7-13 years), another cluster occurred in the same region among three family members in 2002. This work provided the first molecular characterisation of Bulgarian *S.* Typhi isolates allowing differentiation between endemic local and imported cases.

Recent studies with advanced molecular methods have revealed that H58 is the globally dominating *S*. Typhi genotype (17). Nowadays, fluoroqiunolones are the preferred antimicrobial agents for treatment of adults with typhoid fever in Bulgaria as well as in many other countries (18). Clinicians have reported that symptoms successfully subside and relapses are rare with 7-14 days of ciprofloxacin regiments. Short-term 3 day courses are recommended for the residents of endemic regions. However, failures may occur, because of the increasing resistance of *S*. Typhi to fluoroquinolones due to a number of mutations. The most frequent are the double transitions in the *gyr*A gene (the obligatory Gly133-Glu and the second one at codons 83 or 87 Ser83-Phe or Asp87-Asn) (19). Previous investigations have shown that 79% of *S.* Typhi isolates from India belonged to H58 haplotype and were carrying *gyr*A mutations. In our work one *S.* Typhi was imported from India and its resistance profile indicated the presence of a chromosomal mutation of such type. Another kind of double mutations in *gyr*A/ *gyr*B genes are consistent with the so called non classical fluoroquinolone resistance. Isolates carrying such mutations are usually susceptible to nalidixic acid. They emerged in France, USA, England, but were primarily acquired from India (19). Unlike non-typhoid *Salmonella* serotypes *S*. Typhi do not frequently carry plasmid *qnr* genes, which was in line with our observations as well. Azithromycin or third generation cephalosporines are recommended for treatment of fluoroquinolone-resistant *S*. Typhi (20). All patients from the current study were successfully treated and dismissed from the hospital.

In conclusion: This is the first molecular characterisation of an endemic focus of typhoid fever, occurring in 2013-2014 in Shumen region, Bulgaria. PFGE profiles of local cases (type I) differed from the profiles of two imported cases (types II and III). Bulgarian *S*. Typhi isolates were susceptible to fluoroquinolones, which was the cornerstone for the successful treatment of the patients.

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PREVALENCE OF HELICOBACTER PYLORI SEROPOSITIVITY IN PATIENTS WITH PSORIASIS

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ABSTRACT

Aim: The aim of the present study was to determine the prevalence of *H. pylori*seropositivity and the presence of anti-CagA and anti-VacA antibodies in patients with psoriasis.

Materials and methods: Twenty five patients diagnosedwith psoriasis vulgaris and a control group of 24 healthy individuals were examined. *H. pylori,*CagA,and VacA serum antibodies were determined using ELISA and Western blot methods.

Results: Sixteen out of 25 (64%) patients were determined as seropositive with an average value of antibodies titer (IgG, anti-*H. pylori*) 149.2 ± 85.6 RU/ml. In the control group eight out of 24 (33.3%) healthy individualswere determined as seropositive with average values of antibodies titer 118.8±41.4 RU/ml. With regard to*H. pylori*seropositivity,significant difference between the two groups was found. Results obtained by Western blot method showed presence of antibodies against CagA antigen in six out of 16 (37.5%) and antibodies against VacA antigen in one out of 16 (6.3%) *H. pylori* seropositive patients.

Conclusion: In our study the degree of *H. pylori*seropositivityprevalencewashigher in patients with psoriasis than in healthy subjects. The presence of anti-CagA and anti-VacA antibodies was established in comparatively low levels. As the study was performed as a pilot with a relatively small number of patients,further investigations are necessary to explain the relationship between *H. pylori* and the pathogenesis of psoriasis.

Key words: *H. pylori*, psoriasis, antibodies

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INTRODUCTION

*Helicobacterpylori*is Gram-negative, spirally shaped flagellate bacterium. It ismicroaerophilic,fastidious and highly adapted to live in the gastric mucosa. Different parts of the stomach may be colonised, with the most common location –the gastric antrum. Protection against gastric acid is provided by the powerful urease activity of the bacterium. In this way it can successfully survive in the gastric acidic environment.

The global prevalence of *H. pylori* infection is more than 50% (1). It is implicated in some of the most common and socially significant gastroduodenal human diseases,-such as chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and MALT lymphoma.The infection caused is chronic and if untreated, persists throughout life (2). In the majority of infected cases (about 80%) there are no complaints reported, but a chronic gastritis can be detected histologically(3). Such persons are considered to be asymptomatically infected or colonised.

The immune response that develops as a result of*H. pylori*infection is generated-not only locally, but on a systemic level as well(4).In theserum, antibodies to different antigens of the bacteria are found and this determines the individuals as seropositive. Strains of *H. pylori* are associated with different virulence factors (5).Some ofthem possess genes of the so-called Islands of pathogenicity - cagA and vacA, encodingthe proteins CagA and VacA,which areconsidered to be the main factors of virulence (6). Their presence is associated with induction of strong gastric inflammation, synthesis of proinflammatory cytokines, carcinogenic potential,etc. There is astrong humoral immune response to these antigens which can characterise the degree of virulence of the strains(7).Although

H. pylori stimulates animmune response,-this is not efficient enoughand the infection persists (6).The microorganism produces a low-level inflammation at systemic level for decades. The increase in proinflammatory cytokines in the bloodcan act as a starting point in the stimulation of the inflammatory cascadewhich is possible predisposition for the development of chronic inflammatory diseases (4, 6).

Data from many studies find the relationship between *H. pylori* infection and diseases that are not related to the gastrointestinal tract (cardiovascular, metabolic, skin disorders) (8). The chronic inflammatory nature of *H. pylori* infection is considered to predispose patients to a number of skin diseases - rosacea, chronic urticaria, atopic dermatitis, psoriasis, etc. (9, 10).

Psoriasis is a chronic inflammatory dermatosis of unknown etiology. Data collected on the role of *H. pylori* infection in such patients is contradictory, but the well-knownability of the bacterium to induce systemic chronic inflammationat low grade,suggests its possible implication in the etiopathogenesis of the disease. In Bulgaria about 100,000 patients with psoriasis are registered (11).The rate of infectivity with*H. pylori*among this populationhas not been investigated. This study demonstrates the first results onseroprevalence of *H. pylori*and presence of the virulence factors CagA and VacA in Bulgarian psoriasis patients withoutgastrointestinal tractcomplaints.

MATERIALS AND METHODS

Patients

The study included 25 persons diagnosed with mild to moderate plaque form of psoriasis. The patients were diagnosed at theDepartment of Dermatology and Venereology, Medical University of Sofia,University HospitalAleksandrovska.The diagnosis was based on anamnesis and clinical criteria. The average age of the study group was 52.2 ± 19.4 years ranging from 20 to 88 years and withgender distribution of 17 menand eight women. A control group of 24 healthy individuals with an average ageof 47.1 ± 6.1 years, age range from 34 to 57 years and gender distribution of11 men and 13 women was studied as well.All members of both groups have no reported evidence of ulcer disease or gastrointestinal complaints. Blood samples were taken after a signed informed consent from all patients and controls and according to the regulations of the institution.

Determination of *H. pylori* specific antibodies in serum

For the purpose of this study during the period October 2011-December 2012sera were obtained, collected and stored at -20°C.Serological testing for two main factors was conducted as follows:

1. Establishment of IgG antibodies against *H. pylori* (frequency of infection/colonisation, respectively). The testing was performed with a commercial ELISA kit(*Helicobacter pylori*, bacterial lysate, strain ATCC 43504, Euroimmun, Germany). The antibodies being determinedare against the basic common antigens of bacterial lysate *H. pylori*, strain ATCC 43504.
2. Confirmation test for *H. pylori*seropositivity and determination of anti-CagA and anti-VacA antibodies (frequency of expression of CagA and VacA proteins, respectively) usingimmunoblotting method performed with a commercial western blot kit (Anti-Helicobacter pylori Euroline-WB,Euroimmune,Germany).

Statistical data processing

Processing of the data was performed by MS Office Excel software and the following statistical methodswere applied: variation analysis of quantitative variables, mean average, standard deviation, t-test. In order to determine the seropositive vs. seronegative cases distribution in different groups,a Fisher Exact Testwas used. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Study results on *H. pylori*seropositivity in patients with psoriasis and healthy control subjects, allasymptomatic for gastrointestinal tract complaints, are shown in **Table 1**.

Anti-*H.pylori*IgGseropositivity

Determination of anti-*H.pylori*IgGseropositivity was performed using ELISA method. The prevalence of *H. pylori*seropositivity in the group of psoriatic patients was 64% (16 out of 25). In the control group of healthy persons eightout of 24 (33.3%) testedindividuals were identified as seropositive, too. The difference between the two groups is statistically significant($P = 0.046$).

Table 1. IgGanti-*H. pylori* antibodies in patients with psoriasis.

	Patients with psoriasis n = 25	**Control group n = 24**	**P Patients/ controls**
Anti-*H. pylori*seropositivity	64% (16/25)	33.3%(8/24)	0.0465
Average titres anti-*H. pylori* (RU/ml)	149.2 ± 85.6	118.8 ± 41.4	NS
	Seropositive patientsn=16		
Anti-CagAseropositivity	37.5% (6/16)	-	-
Anti-VacAseropositivity	6.3% (1/16)	-	-

The serum antibodytiter(IgG, anti-*H. pylori*) had an average value of 149.2 ± 85.6 RU/ml in the patient group and 118.8 ± 41.4 RU/ml in the healthy individuals, i.e. there was no statistically significant difference between the two groups.

Anti-CagA and anti-VacAseropositivity

In the 16 psoriaticpatients, who were found seropositive for *H.pylori*,further examination was conducted in order to determine the presence of IgG antibodies to CagA and VacA antigen using Western blot method. Results showed the presence of anti-CagA antibodies in six (37.5%) and anti-VacA antibodies in one(6.3%) of the patients.

DISCUSSION

An assumption can be made that *H. pylori* infection may play a role as a trigger or exacerbation factor inthe etiopathogenesis of psoriasis. Results from different studies on this topic are summarised in many reviews(9, 10). Our investigation presents results on*H. pylori*seropositivity in patients with psoriasis. We used serological method as it is considered to be precise and specific for determining the infectivity (colonisation) with *H.pylori*in asymptomatic persons (12, 13).The results of our study showsignificantly higher value for theprevalence of *H. pylori*seropositivity (64%) in patients with psoriasis compared to healthy persons (33.3%), P <0.05. Serum titers of anti-*H.pylori* tend to show higher levels in the patients compared to the control individuals, but there is nostatistically significant difference between the two groups(149.2 ± 85.6 and 118.8 ± 41.4 RU/ml), P = NS. Other authors found similar results when using serological methods (14, 15). They demonstrate higher level of prevalence of *H. pylori* infection in patients with psoriasis, compared toa control group of healthy persons. G Fathy et al establishedthat higher values of seropositivity correlated with severe psoriasis and hypothesisedthat*H. pylori* could be at least the provoking factor behind psoriasis (15).

Some studies do not demonstrate significant difference between prevalence of *H. pylori* in psoriatic patients and healthy controls, but authorssuggestthat *H. pylori* seemsto be able to affect the clinical severity of psoriasis (16, 17).

In a study by Onsun Net alon a large group of psoriasis patients (300 patients and 150 healthy controls) using the stool antigen ELISA test,no statisticallysignificant difference of *H. pylori* prevalence between patients and controls was estimated. By studying the severity of psoriasis and the impact of therapy withacitretin, authorsconclude that *H.pylori* infection is related to the severity of clinical forms of psoriasis and its eradication has a positive effect on the on-going therapy with acitretin (16).

Using the ^{13}C urea breath test to evaluate the infection with*H. pylori*in a study on 210 patients with psoriasis and 150 healthy controls,Campanatiet al alsoshowed that prevalence of *H. pylori* was not higher in psoriasis,compared to the control group.

However patients infected with*H. pylori*demonstrated more severe psoriasis than the uninfected ones andsuccessful eradication of *H. pylori*infection showed improvement of the disease (17).

Results from some clinical cases also demonstrated benefit from antibiotic treatment of patients with psoriasis (18, 19, 20). Some authors reported resultsaccording to which there are no statistically significant differences between prevalence of *H. pylori* in patients with psoriasis and healthy subjects(21, 22, 23).Fabrizi G et al reported lack of evidence on the relationship between *H. pylori* infection and psoriasis in childhood (21).In the study performedbyAzizzadeh et al, 61 patients with psoriasis vulgaris and 61 healthy individuals were tested for *H. pylori* infection with serological methods. Results showed that there is no difference in the prevalence of infection and serum levelsof specific IgG antibodies between the two groups. Also, there is no significant relationship between psoriasis severity and serum level of anti-*H. pylori*IgG. (22). Reviewing the literature, Leontiadis GI et al made a conclusion that there is no evidence onthe association between*H.pylori* infection, psoriasis and other dermatological diseases (23).

The significance of the main virulence factors of *H. pylori* - expression of CagA and VacA proteins is well characterised in gastrointestinal diseases(2, 7). In the patients with psoriasis whotested seropositive for *H. pylori*,we identified the presence of IgG antibodies against virulence factor CagA in 37.5% and against VacA in 6.3% of infected persons. The results reflect a relatively low prevalence of these pathogenicity factors among patients with psoriasis.

In a similar type of study the authors found 54.5% CagA positivity in patients with psoriasis and 68.1% in a control group of subjects with peptic complaints,but no evidence of ulcer disease. The CagA status of these patients is not related with the severity of the disease, its progress or the presence of arthropathy, which is interpreted as a rather weak relationship between CagA and development of psoriasis (24).

Studies on the importance of *H. pylori*infectivity in patients with psoriasis reveal contradictory results. Significant differences in the design of the studies,such as methods used for determination of *H. pylori*infectivity, number of subjects tested and clinical forms of diseasemay explainthese findings. Also, globally different strains of *H. pylori* appear to be associated with differences in virulence(5).Most authors indicate thatthere is a need for broader and more unified studies in order to clarify the exact role of *H. pylori* infectionin patients with psoriasis.

Conclusion: Results obtained in our investigation show that*H. pylori*seropositivity in patients with psoriasis is higher compared to the healthy control group. The presence of anti-CagA and anti-VacA antibody is in low levels. The study was performed on a relatively small number of patients and is intended as a pilot. Further investigations are also necessary to explain the relationship between *H. pylori* and the pathogenesis of psoriasis.

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HIV-1 TRANSMITTED DRUG RESISTANCE IN BULGARIA

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ABSTRACT

Objectives: The aim of our study was to determine transmitted drug resistance (TDR) in Bulgaria.

Methods: The prevalence of TDR was determined in 305/1446 (21.1%) persons newly diagnosed with HIV/AIDS from 1988 - 2011. TDR mutations (TDRM) in protease (PR) and reverse transcriptase (RT) of the *pol* gene were defined using the WHO HIV drug mutation list.

Results: TDRM was found in 16/305 (5.2%) persons, eleven (3.6%) with resistance to nucleoside reverse transcriptase inhibitors (NRTIs), five (1.6%) with resistance to non-nucleoside reverse-transcriptase inhibitors (NNRTIs) and three (0.9%) with resistance to PR inhibitors. Dual class TDRM were found in three (1.0%) patients. TDRM were found in ten heterosexuals (HET), four men reporting sex with men (MSM) and two intravenous drug users (IDUs).

Conclusions: We found a low prevalence of TDR among antiretroviral naive patients in Bulgaria. Our results provide baseline data on TDR and support continued surveillance of high risk populations in Bulgaria to better target treatment and prevention efforts.

Keywords: antiretroviral mutations, Bulgaria, surveillance

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INTRODUCTION

The highly active antiretroviral therapy (HAART) has decreased the morbidity and mortality of HIV-1 infected patients and reduced HIV-1 transmission in some risk groups, including newborns and sexual partners (1). Nonetheless, antiretroviral therapy may select drug resistant strains which can be transmitted from person-to-person. Infection with drug-resistant HIV may negatively impact first-line antiretroviral regimens (2). Therefore, the International AIDS Society-USA and the European AIDS Clinical Society (EACS) guidelines recommend HIV drug resistance testing for drug naive patients, before the beginning of antiretroviral treatment (3, 4). The highest rates of TDRM have been reported in North America (14.6%), followed by Europe (10.9%), Latin America (6.3%), Africa (4.7%), and Asia (4.2%) likely correlating with the historic availability of treatment in those countries (5-7). TDR varies widely in some Balkan countries with 21.6% reported in Croatia, 14.75 % in Romania, 12.5% in northern Greece, 4.7% in Slovenia, 8.8% in Serbia and 7.6% in Turkey (8-13).

While antiretroviral therapy was initiated in Bulgaria in 1987 with azidothymidine monotherapy, followed by the addition of lamivudine in 1998 and inclusion of protease inhibitors in the regimen in 1999, very little is known about HIV-1 TDR in Bulgaria. In a preliminary study in 2008, we found genotypic evidence of TDRM in 9.1% (2/22) of drug naive patients (14, 15). Following these findings, we implemented

the European guidelines for resistance testing of antiretroviral naive patients to better monitor HIV-1 TDRM in Bulgaria. Our current study aims to further investigate TDRM prevalence in Bulgaria.

MATERIALS AND METHODS

Ethics statement

All patients provided written informed consent to participate in this study approved by the Ethical Committee at the National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria (NCIPD IRB 00006384). The CDC IRB determined participant consent was obtained for HIV analysis in this study.

Study design and specimen preparation

Blood samples were collected from 305 persons naive for antiretroviral therapy out of 1,446 patients diagnosed with HIV/AIDS in Bulgaria between 1998 and 2011 at the National HIV Reference Laboratory and/or in the clinics responsible for the management of patients with HIV in Sofia, Plovdiv and Varna. Patients were from 29 different cities and various risk groups, including heterosexuals (HET), men reporting sex with men (MSM), intravenous drug users (IDU), and patients with other sexually transmitted infections (STI).

Plasma samples were prepared at the National HIV Reference Laboratory as previously described and stored at -80°C (15-18). Specimens were linked to demographic and clinical data through an anonymous numerical code in accordance with the ethical standards of Bulgaria.

Sequence analysis

Plasma viral RNA was extracted using MagCor Nucleic Acid Extraction Kit (RBC Bioscience, Taiwan). PR and RT sequences of the HIV-1 *pol* gene were generated using the TruGene DNA Sequencing System (Siemens Healthcare, USA) following the manufacturer's protocol (15). HIV-1 drug resistance mutations were determined according to the WHO 2009 SDRM list (19) using the current Calibrated Population Resistance tool v5.0 of the Stanford University HIV Drug Resistance Database (http://cpr.stanford.edu/cpr.cgi).

RESULTS

Low prevalence of genotypic TDRM in Bulgaria

Three hundred and five of 1,446 (21.1%) registered HIV-1-infected persons naive to antiretroviral therapy participated in our study. The majority of tested (79.3%) were male and potential infection routes included HET (42.6%), IDU (27.5%), MSM (26.9%), mother-to-child (1.6%), MSM/IDU (1.0%), and blood transfusion recipients (0.3%) (Table 1). Infection was also distributed across other groups, including former prisoners (12.8%), sex workers (3.0%), persons with other STIs (2.0%), pregnant women (3.3%), and blood donors (6.2%). The majority of patients (89.8%) reported likely acquiring infection in Bulgaria.

The overall rate of TDRM in this population was 5.2% (16/305), including 13 (4.3%) men and three (0.9%) women (Table 2); 11/305 (3.6%) had resistance to nucleoside reverse transcriptase inhibitors (NRTIs), 5/305 (1.6%) to non-nucleoside reverse transcriptase inhibitors (NNRTIs) and 3/305 (0.9%) to protease inhibitors (PIs). The most prevalent mutations were T215C/D/S, M41L, K219Q and F77L for NRTIs; Y181C, K103N, V106M and G190E for NNRTIs, and D30N, N88D and M46L for PIs.

13/16 (81.3%) patients had mono TDRM while dual class TDRM was identified in 3/16 (18.8%) men, two of whom were MSM and one was HET. Two patients had both NRTI and PI mutations and one had NRTI and NNRTI resistance mutations (Table 2). TDRM prevalence across risk groups was highest among HETs (10/130, 7.7%), followed by MSM (4/82, 4.9%) and IDUs (2/84, 2.4%). TDRM was found in 5/31 (16.1%) patients who reported likely HIV-1 infection while travelling or living abroad (Table 2). TDRM was not detected in samples from sex workers, blood transfusion recipients, STI patients, pregnant women or HIV-1-positive newborns.

Although there was no data available for determining the date of infection, most of the patients` blood specimens were collected shortly after HIV/AIDS diagnosis. 49.8% of the patients` specimens were analysed during the year of diagnosis, 36.1% between one and three years after diagnosis, and 14.1% four or more years after diagnosis.

Table 1. Study population characteristics[a]

Characteristic	Prevalence in 16 naive patients with TDRM (%)	Prevalence in 305 HAART naive patients (%)	Total HIV-1 positive patients (%)	P[b]
Gender				
Men	13 (81.3)	13/242 (5.4)	1085 (75.0)	
Women	3 (18.8)	3/63 (4.8)	361 (25.0)	>0.05
Likely route of HIV infection[4]				
HET	10 (62.5)	10/130 (7.7)	851 (58.9)	>0.05
IDU	2 (12.5)	2/84 (2.4)	342 (2.7)	>0.05
MSM	4 (25.0)	4/82 (4.9)	203 (14.0)	<0.001
Mother-to-child	0	5	14 (1.0)	>0.05
MSM+IDU	0	3	13 (0.9)	>0.05
HET+IDU	0	1	4 (0.3)	>0.05
Blood transfusion	0	0	19 (1.3)	
Likely country of infection				
Bulgaria	12 (75.0)	12/274 (4.4)	1219 (84.3)	
Other	4 (25.0)	4/31 (12.9)	227 (15.7)	<0.05
Other epidemiological data				
Individuals reporting incarceration	2 (12.5)	2/39 (5.1)	149 (10.3)	>0.05
Sex worker	0	9	41 (2.8)	>0.05
STI Patient	0	6	40 (2.8)	>0.05
Pregnant women	0	10	48 (3.3)	>0.05
Blood donors	1 (6.3)	1/19 (5.3)	94 (6.5)	>0.05

[a] Prevalence of each population characteristic provided for 16 patients with TDRM and HAART naive patients separately.
[b] *p*, is the probability for differences between the total HIV-1 population (n=1446) and the studied patient group (n=305), concerning the frequency of basic demographic and epidemiological characteristics (Chi-square test, GraphPad Prism 4.0).

Table 2. Characteristics of HIV-1 patients with genotypic transmitted drug resistance mutations

Patient code	Gender	Reported country of infection	Likely route of infection	Year of diagnosis	Year of specimen collection	Antiretroviral mutations		
						NRTI	NNRTI	PI
08BG460	M	Bulgaria	HET	2004	2008	**M41L, T215D**	-	**D30N, N88D**
09BG534	M	Spain	HET	2005	2009	-	**Y181C**	-
09BG651	M	Bulgaria	HET	2006	2008	**M41L**	-	-
09BG862	F	Bulgaria	HET	2008	2009	**K219Q**	-	-
11BG892	M	Bulgaria	IDU	2008	2011	-	**G190E**	-
08BG893	M	Germany	MSM	2008	2008	**M41L**	-	-
08BG914	M	Dominican Republic	HET	2008	2008	**F77L**	-	-
09BG944	M	Bulgaria	HET	2009	2009	**T215S**	-	-
10BG1100	F	Bulgaria	HET	2009	2010	-	-	**M46L**
10BG1101	M	Country not reported	HET	2010	2010	-	**V106M**	-
10BG1109	M	Turkey	MSM	2010	2010	**F77L, T215C**	**Y181C**	-
11BG1119	M	Bulgaria	MSM	2010	2011	**M41L, T215D**	-	**D30N, N88D**
11BG1318	F	Bulgaria	HET	2011	2011	**K219Q**	-	-
11BG1362	M	Bulgaria	IDU	2011	2011	-	**K103N**	-
11BG1373	M	Bulgaria	MSM	2011	2011	**T215D**	-	-
11BG1429	M	Bulgaria	HET	2011	2011	**V75M**	-	-

Discussion

Although HIV-1 was introduced more than 28 years ago in Bulgaria, and the country has one of the highest HIV infection rates among the Balkan countries, very little is known about the characteristics of the epidemic (20). Assessment of TDRM and viral diversity are key parameters for monitoring the HIV-1 epidemic and optimising the first-line therapy for long-term management of HIV-1 infection in Bulgaria (21). Herein, we found that TDRM in Bulgaria differ from those in other European and Balkan countries. The 5.2% TDRM prevalence in Bulgaria is about half of that we have previously reported (15), and the one reported across Europe (10.9%) (5), and is generally lower than that of the majority of neighbouring Balkan countries (4.7 - 21.8%) (8-12). In Western Europe, a 10% TDRM rate was reported, and it was higher in MSM (5, 7). This may be due to the fact that in Western Europe HIV-1 was initially introduced and transmitted among MSM who have the longest history of treatment with antiretroviral drugs and thus typically have higher TDRM. In contrast, the lower TDRM prevalence in Bulgaria may be due to the fact that HIV-1 was first introduced among HETs in Bulgaria from persons travelling mostly in non-Western European countries who have lower TDRM rates (15). The decrease in TDRM seen in drug-naive patients in our current study compared to that reported in 2008 is a likely result of having identified only 2/22 HAART naive persons in the initial study. TDRM was present in about 1/3 of persons that had reported acquiring the infection abroad, demonstrating that TDRM is also being imported into Bulgaria. In the last years, the HIV-1 epidemic in Bulgaria has seen dramatic prevalence increases among MSM and IDUs which may cause a future increase of TDRM among IDUs and MSM with the concomitant introduction of TDRM into other risk groups.

As in our previous report (14), most TDRM were to RT inhibitors, including those in patients with dual resistance. The majority of patients had non-polymorphic mutations selected by the thymidine analogues

azidothymidine or stavudine, including M41L and K219Q. The revertant TDRMs T215C/D/S were also found in five patients. These mutations usually occur in individuals primarily infected with strains containing the primary resistance mutation T215Y/F and which can also be transmitted. The common NRTI resistance mutation V75M, which occurs predominantly in CRF01_AE infection, was found in one patient with this subtype. Three patients who had reported acquiring the infection abroad, had NNRTI TDRM. In this study, we found E138G/A/D/K variants, which are related to rilpivirine resistance. However, these mutations are also encountered as polymorphisms and therefore are not considered as a certain indicator of transmitted resistance according to the WHO definition (19). Three patients had PI TDRM although PI TDRM was not observed in our previous study (14). One individual was infected with the M46L mutation selected by various PIs. Sequences from two other patients infected in Bulgaria had the D30N and N88D mutations, both of which are selected by nelfinavir and commonly occur together but have little clinical impact on first line therapy. Although nelfinavir has not been used in clinical practice for years, these two mutations have already been detected in Bulgaria in our previous study (14) and one of these two patients was diagnosed in 2004 when nelfinavir was still in use.

Analysis of the geographical distribution of TDRM showed that half of the cases were found in two major cities, Sofia and Plovdiv, where the largest number of HIV-1 patients in our study were registered. However, TDRM were also identified in persons from five cities in remote locations across Bulgaria demonstrating that TDRM is widespread in the country, though the overall prevalence is low. In addition, about one-third of TDRM cases were found in individuals reporting they acquired infection abroad suggesting that some TDRM is likely being imported into Bulgaria.

We did not find any association between the time from diagnosis and TDR in our study; however, the numbers of resistance mutations found are too low for statistically supported assumptions.

Our findings are limited by the lack of longitudinal follow-up to determine whether TDRM persists or reverts to wild-type virus which might influence the level of TDRM observed in persons from whom samples were collected after one year of diagnosis. This is especially relevant for comparisons with results from Western countries where genotyping and drug resistance testing occurs at the time of diagnosis. Also, the results from a cross-sectional study design may not be truly representative of the other 80% of reported cases in Bulgaria. For example, a comparison of the demographic and epidemiological characteristics of the subset of patients (n=305) studied in the current report and all HIV-1 diagnosed persons (n=1446) in Bulgaria, showed the TDRM in MSM and persons acquiring HIV-1 abroad might have been overestimated (Table 1). The findings may also be limited by the use of only standard population-based sequencing which may not detect minority TDRM present in less than 20% of the viral population in plasma (19). Finally, self-reporting of epidemiological data used in the study could introduce recall biases concerning the country origin of infection or the route of HIV-1 transmission especially for those reporting infection abroad and for non-MSMs, which could affect.

CONCLUSIONS

We found a low TDRM prevalence in treatment naive HIV-1-infected persons in Bulgaria. The contribution of TDRM acquired outside Bulgaria by migrants, and the increasing number and size of local transmission clusters among the high risk groups, raises public health concerns. Combined, our findings provide baseline TDRM data and support the need for further surveillance of TDRM in Bulgaria, especially in high risk populations like the emerging MSM and IDU sub-epidemics.

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CD4/CD8 RATIO FOR MONITORING THE IMMUNOLOGICAL RESPONSE TO COMBINED ANTIRETROVIRAL THERAPY IN BULGARIAN HIV+ PATIENTS

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ABSTRACT

Background: HIV viral load (VL) and CD4 absolute count (CD4AC) are traditionally used to monitor combined antiretroviral therapy (cART) of HIV-1+ patients. Accumulating data indicate that additional parameters might contribute to the prognosis and evaluation of cART effects.

We analysed the effects of cART in Bulgarian HIV-1+ patients treated during 2003-2013, comparing CD4AC and CD4/CD8 as parameters for immune monitoring.

Material and methods: The study included 643 treatment-naive HIV-1+patients (male/female: 475/168), started on cART between January 2003 – December 2013. Immunologic (CD4AC, CD4/CD8) and viral response were evaluated in the course of 5 years by multicolour flow cytometry (BD Biosciences), and RT-PCR (Roche-Diagnostics). At least 40 cases were analysed for each time point.

Results: According to baseline CD4AC (cells/μl), subgroups with low-L (<200, n=347), medium-M (200–350, n=143), and high-H (>350, n=119) count were defined. CD4AC change (∆CD4) and viral success (VS, VL<1.6) rates in the subgroups at 6, 24 and 60mo were comparable (Fischer's p>0.05). Average CD4AC entered reference range in 36mo at latest (L) while CD4/CD8 remained low even at 60mo. Baseline ratio independently of CD4AC was associated with better early immune response (p<0.05). Baseline CD4/CD8 predicted cART effect independently of CD4AC, and improved predictive value of CD4AC only in subgroup L. Regardless of VS, low CD4/CD8 at 6, 24, and 60mo was significantly associated with poor immune recovery (ΔCD4, p<0,001).

Conclusions: In addition to CD4AC, CD4/CD8 is an important surrogate marker of immune recovery most probably reflecting accelerated immune senescence in conditions of ongoing low-level activation.

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INTRODUCTION:

Although combined anti-retroviral therapy (cART) has considerably improved prognosis and life quality of HIV+ patients, its effects are limited by persistent undetectable viral load, and a growing risk of therapy resistance. Strict monitoring of indicative laboratory parameters is indispensable for taking the right therapeutic decisions and for evaluating therapy success, and disease progression. HIV plasma viral load (VL) and peripheral blood CD4 T cell absolute counts (CD4AC) have been the guideline monitoring parameters ever since the introduction of cART (1). Viral load is the most direct indicator for cART effect in terms of viral suppression, while CD4AC is related to immune reconstitution and the risk of opportunistic infections (2, 3). However, accumulating data indicates that patients with comparable baseline viral loads and CD4AC may have completely different response to cART, and long-term restoration. In general, failure to suppress VL is associated with poorer CD4AC recovery. However, incomplete immunological recovery may also occur when plasma VL is sustained below detection limits. Low level immune activation may have significant effects in the settings of long-term cART and increased life-expectancy of treated HIV+ patients.

Therefore additional parameters are needed for precise evaluation of immune recovery, and long-term prognosis.

A number of phenotypic or functional parameters may contribute to prognosis and evaluation of cART effects: the quantitative expression of CD38 by CD8 T cells was shown to correlate perfectly with viral replication (4), a high baseline expression of CD160 by CD8 T Ly was related to a better HIV-specific activity of CD8 T cells (5) and better immune restoration in the course of ART (6), increased CD39+Treg subset predicted independently quick evolution to AIDS (7), soluble plasma IL-6 levels – to correlate with residual HIV viremia in ART-controlled patients (8), etc. None of them has been adopted for routine monitoring since most require more expensive and/or complicated technical approaches. In this line, CD4/CD8 ratio is one of the most straightforward parameters. "Inverted" CD4/CD8 ratio (<1.0) is as a hallmark of any active viral infection inducing a dominating CD8 effector response, and a manifestation of the principle of 'blind' T-cell homeostasis (a constant number of T lymphocytes is normally maintained without regard to CD4+ or CD8+ phenotype) (9). While it may be obvious that a decreasing CD4/CD8 ratio would be associated with CD4 depletion in the settings of uncontrolled HIV-infection, studies on its predictive power are not unanimous. Absolute CD4AC, CD4 lymphocyte percent, and CD4:CD8 ratio were shown to be similar (10, 11); superior (12, 13) or inferior than the CD4+ T-cell count or other markers in their ability to predict disease progression and the development of AIDS (14). CD4/CD8 is even more intriguing in the settings of HIV control, since it has been identified in the general HIV-negative population as a hallmark of immunosenescence and a surrogate of all-cause mortality (15, 16). Indeed, small cohort analysis in HIV+ patients with stable cART mediated viral control has shown that CD4/CD8 ratio is independently associated with T-cell activation, a skewed T cell phenotype toward terminally differentiated CD8+ T cells, and replicative senescence (17, 18).

The aim of our study was to compare CD4AC and CD4/CD8 ratio as parameters for monitoring and predicting the immunologic response to cART in a large cohort of treatment-naive Bulgarian HIV+ patients.

MATERIALS AND METHODS:

Study design and patients groups. The study is a retrospective analysis of immunophenotyping data obtained during routine immune monitoring of HIV+ patients registered at the Specialised Hospital for Active Treatment of Infectious and Parasitic Diseases "Professor Ivan Kirov", Sofia. 643 (M 475, F 168) treatment-naive HIV-1+ patients that have started cART between Jan 2003 and Dec 2013 were included in the study. cART regimens comprised 2 nucleotide or nucleoside reverse transcriptase inhibitors (NRTI) plus either another NRTI or a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a protease inhibitor (PI), or a ritonavir-boosted protease inhibitor (PI/r). The immunologic and viral response to cART has been evaluated following the corresponding guideline requirements according to CD4AC and VL at least 4 times per year.

Definitions: As ***baseline CD4AC*** was defined the last one determined before the start of cART. The ***change of CD4AC (∆CD4AC)*** was the difference between CD4AC at the time point of monitoring and the baseline CD4AC. ***Virologic response*** was defined as suppression of VL to below 50 copies/ml during the first year after the start of cART. ***Virologic success*** was defined as the absence of 2 consecutive plasma viral load measurements > 50 copies/ml within the monitored period.

Immunophenotyping and virology tests: Blood samples (5 ml peripheral blood in BD Na Heparin Vacutainer tubes and 5 ml peripheral blood in BD K-Na EDTA Vacutainer tubes) were obtained after informed consent in the course of routine monitoring of HIV+ patients at the National Reference Laboratory of Immunology, National Centre of Infectious and Parasitic Diseases (NCIPD). Absolute counts and percentage of lymphocyte subsets were determined with a BDMultitest 6-Color TBNK reagent (CD45/CD56+16/CD4/CD8/CD19) using standard lysis–no–wash procedure

and TRUCount tubes. (BD Biosciences). At least 5000 events in the lymphocyte gate were collected and analysed using BD FACSCanto II flow cytometer and DIVA 1.2.1 software. HIV infection was confirmed by the National Reference Laboratory of HIV/AIDS at NCIPD. HIV-1 RNA levels (VL) were determined on EDTA-anticoagulated plasma by RT-PCR (using Amplicor (Real-Time PCR - COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0; Roche-Diagnostics).

Statistics: Between-group and inter-group statistically significant differences were evaluated by non-paired and paired T-tests, after confirming normal distribution of analysed data. P-values equal or less than 0.05, were considered significant (GraphPad Prism software, v.5.5).

RESULTS:

1. Immunologic and virologic response to cART according to baseline CD4AC.

According to the European and national guidelines at the time of the study, cART was recommended in general at CD4AC 350 cells/μl as the probability for immune restoration below that limit was considered to decrease very quickly; and the limit of 200 cells/μl was associated with severe immune deficiency, and AIDS-related events. Therefore, to assess the importance of baseline CD4AC for the effect of cART, we defined subgroups with low (< 200 cells/μl, L); medium (200 – 350 cells/μl, M), and high (> 350 cells/μl, H) baseline CD4AC. No significant differences were established between the subgroups according to age and gender distribution, presumable disease duration or baseline viral load (**Table 1**, p>0.05 for all).

The average ΔCD4AC and the rate of virologic success (% of patients with suppressed VL) in each group were analysed and compared at 6, 12, 24, 36, 48 and 60 months after the start of cART **(Fig.1A, B).**

According to our results, CD4AC dynamics (ΔCD4AC) was quite similar in the subgroups, without significant differences at the examined time points, (p>0.05). The rate of VS increased with cART duration, but no statistically significant differences

Table 1. Demographic and laboratory characteristics at baseline of HIV+ patients from groups L, M and H.

Characteristics \ Groups		L <200 CD4 cells/μl	M 200-350 CD4 cells/μl	H > 350 CD4 cells/μl
Number of patients	All	340	204	99
Gender, number (%)	Male	251 (73%)	157 (76%)	67(67%)
	Female	89 (27%)	47 (24%)	32 (33%)
Age at baseline, (years)	Mean Min-max	36 (17 - 71)	38 (19 - 65)	36 (18 - 65)
Infection duration[1] (years)	Mean Min-max	2 (0 – 19)	2 (0 – 18)	2 (0 – 9)
Baseline CD4 AC (cells/μl)	Mean Min-max	72 (0.66 – 199)	273 (200 – 347)	515 (362 – 936)
Baseline HIV VL (log RNA copies/ml)	Mean Min-max	5.1 (1.6 – 7.0)	4.5 (1.6 – 6.3)	4.3 (1.6 – 6.2)

[1] **Presumable duration of HIV infection was estimated according to the date of initial diagnosis**

were detected between the subgroups, either (p>0.05). In the context of cART, CD4AC increased with stable rates in the subgroups, the only difference being the values attained at each time-point; in group M the average CD4AC reached the lower reference limit already at 6mo, while in L this took 36 months (**Fig.1C**). Finally we looked at CD4/CD8 ratio at each time point. Importantly, in spite of the stable rate of CD4AC increase, it was hardly restored. After 36 months of cART the average CD4/CD8 ratio reached the lower reference limit only in subgroup H, and after 60mo of cART it was below the lower reference limit for all subgroups **(Fig.1D).** We concluded that baseline CD4/CD8 ratio is a more sensitive indicator of immunologic restoration/deterioration than CD4AC, and might be informative as a predictor of cART success.

2. Early immunologic and virologic response to cART according to baseline CD4/CD8 ratio

Next, we divided the studied cohort according to the median baseline CD4/CD8 value into patients with low (<0.5), and those with relatively high baseline CD4/CD8 ratio (>0.5) before the start of cART. The analysis of the early immunologic and virologic response (after 6mo of cART) showed that the average ΔCD4AC was significantly higher in the high CD4/CD8 subgroup, in comparison to the low ratio one: mean (SEM) =165 (8) vs. 118 (6), p<0.0001, **(Fig.2A).** In addition, patients with high baseline CD4/CD8 suppressed HIV replication significantly better as compared to patients with low baseline ratio: mean (SEM) of VL = 2.6 (0.1) vs.3.96 (0.1), p<0.0001, **(Fig.2B)**. Undoubtedly, a higher CD4/CD8 ratio at baseline was associated with a better early immunologic and virologic response to cART.

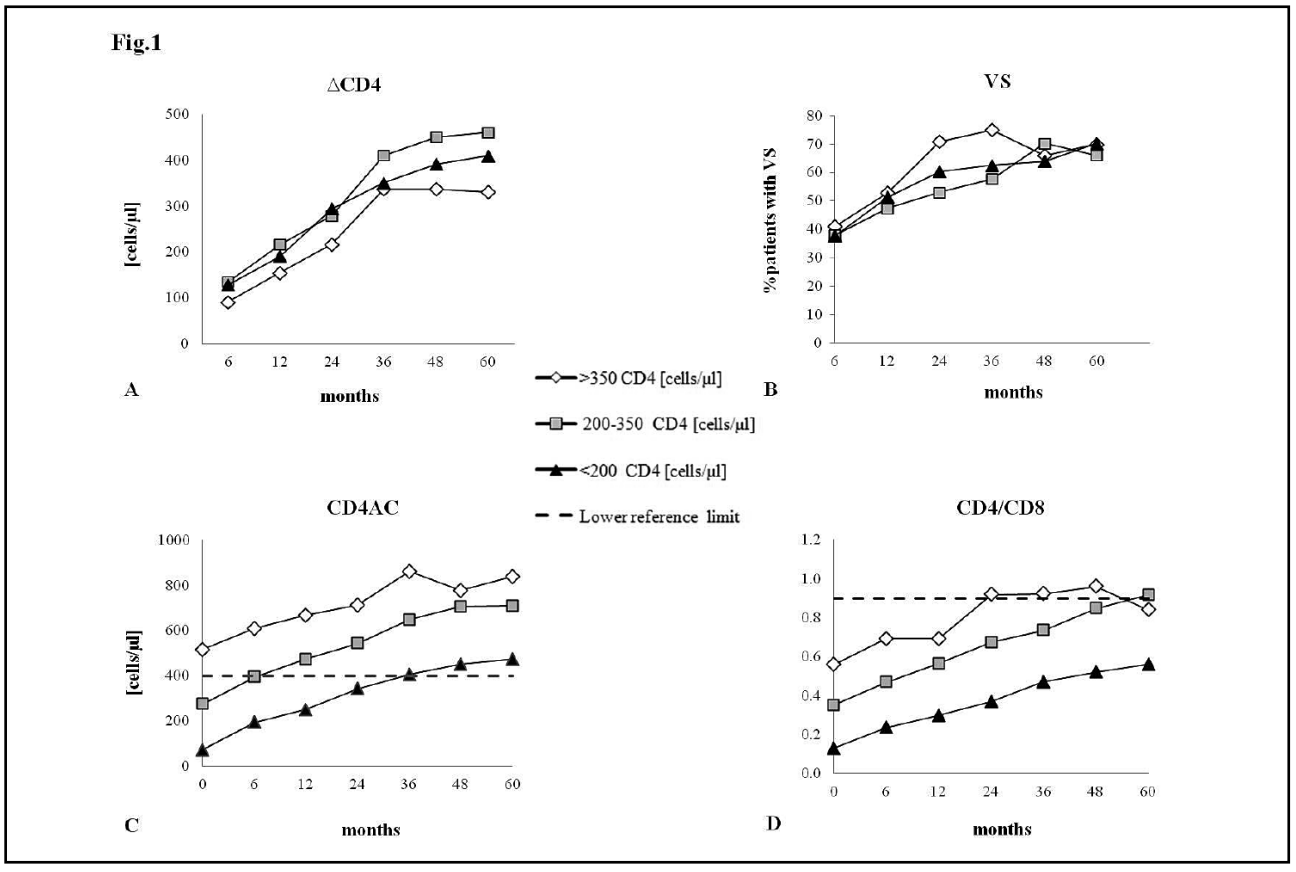

Fig.1 Dynamics of the immunologic and virologic effect of cART in the subgroups of HIV+ patients defined according to baseline CD4AC. The mean ΔCD4AC (**A**), viral success (**B**), CD4AC (**C**) and CD4/CD8 ratio (**D**) were determined at 6, 12, 24, 36, 48 and 60 months after the start of cART in the patients' subgroups defined according to baseline CD4AC. At least 40 patients were analysed for each time point. The lower reference limits for CD4AC **(C)** and CD4/CD8 **(D)** are presented with dashed line.

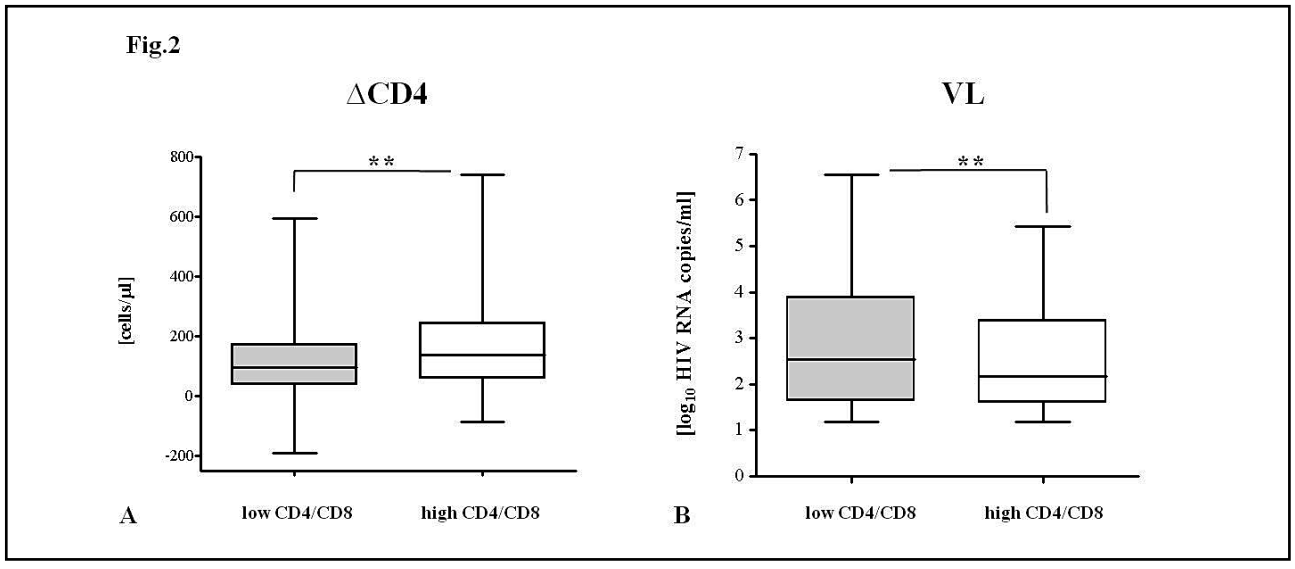

Fig.2 Early immunologic and virologic effect of cART in patients groups defined according to median baseline CD4/CD8 ratio. ΔCD4AC (**A)** and VL (**B**) were determined at 6mo after the start of cART in the subgroups with low (grey) and high (white) baseline ratio.

3. Immunologic response to cART in patients with VS according to CD4/CD8 ratio after 6, 24 and 60 months of cART

Since CD4/CD8 seemed a more sensitive indicator of cART effect than CD4AC, we asked whether it could better characterise the groups with controlled viral load at different time-points after the start of therapy. To this end, we stratified patients with viral success after 6, 24 and 60mo of cART according to their median CD4/CD8 ratio at those time points (0.5; 0.4; 0.5 respectively). The changes in CD4AC (ΔCD4 AC) were compared between the low and high ratio subgroups at each time point (**Fig. 3A, B and C)**.

Our results showed that the average ΔCD4 AC values were significantly higher in the high ratio subgroups, in comparison with low ratio ones for the three examined time points: mean (SEM): 173 (17) vs 118 (10); 370 (25)

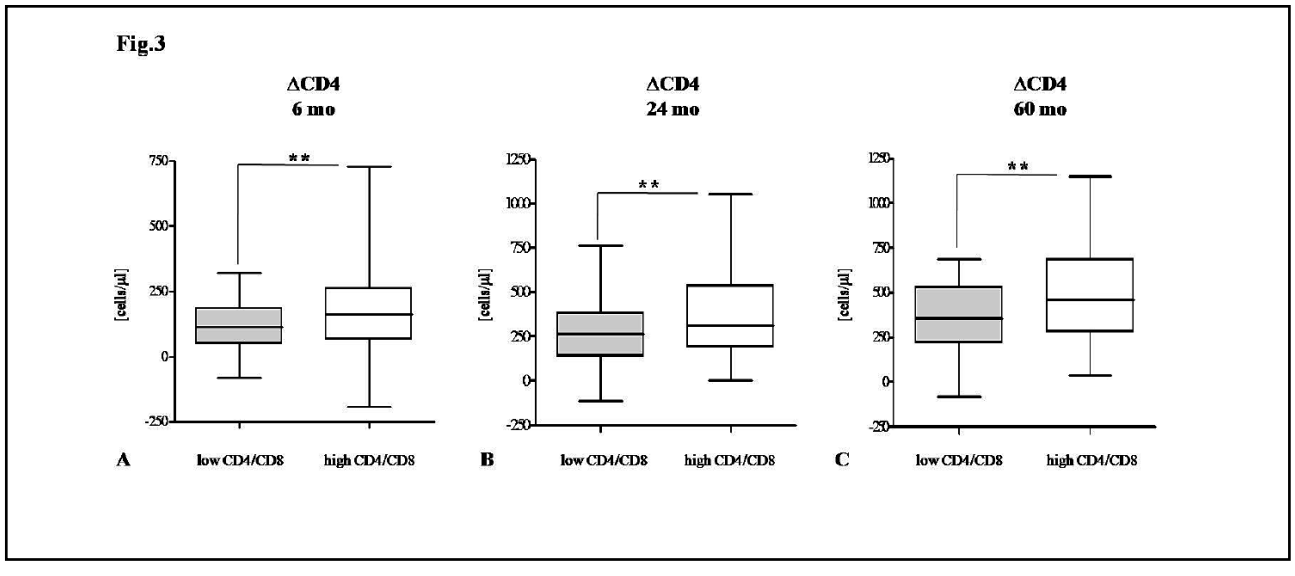

Fig.3 Immunologic response to cART in patients with viral success after 6mo, 24mo and 60mo of cART according to the CD4/CD8 ratio. ΔCD4AC at 6mo **(A),** 24mo **(B)** and 60mo **(C)** after the start of cART was determined in patients' subgroups with low (grey) and high (white) CD4/CD8 ratio at those time points.

vs. 280 (21), and 509 (39) vs. 380 (37), respectively; p<0.05 for all comparisons. These results indicated that independently of HIV suppression, a consistently low CD4/CD8 ratio was significantly associated with poor early and long-term immunologic response (∆CD4).

4. Baseline CD4AC in combination with baseline CD4/CD8 ratio may better predict the response to cART.

We checked whether the simultaneous determination of CD4/CD8 and CD4AC at baseline would better predict the response to cART. To this end, the subgroups defined according to baseline CD4AC (H, M, L) were further stratified according to the median baseline CD4/CD8 ratios. Immunologic recovery (CD4AC, and ∆CD4AC) was compared between patients with high and low baseline ratio in the corresponding subgroups, for each time point. In the subgroups with comparatively preserved CD4 pool (M and H, CD4AC > 200), no significant differences in the rate and magnitude of immunologic response were established in association with CD4/CD8 (data not shown). However, in patients with advanced immune deficiency (L, CD4 < 200 cells/µl), a higher baseline CD4/CD8 ratio (> 0.1) was significantly associated with a better early recovery rate: mean (SEM) ∆CD4AC 145 (9) vs. 114 (6), p<0.05 **(Fig.4A).** Consequently, the same patients experienced a significantly better long-term immune restoration: mean (SEM) CD4AC at 24mo - 396 (18) vs. 289 (15), p<0.05 **(Fig.4B).**

DISCUSSION

In this study, we compared CD4AC and CD4/CD8 ratio as surrogate markers for immune monitoring of treatment-naive HIV+ patients. Based on monitoring data from a large cohort of Bulgarian HIV+ patients we demonstrate that CD4/CD8 ratio significantly contributes to both predicting the short- and long-term effects of cART, and to characterising the quality of immune restoration.

The immediate goal of cART is to suppress efficiently viral replication, resulting in the 'rescue' of the CD4 pool by both preventing a further depletion, and by promoting its regeneration. Therefore the HIV VL and CD4AC have served traditionally as the primary measures for cART effects. However, the long term goal of treatment is to increase life expectancy while reducing AIDS and non-AIDS related morbidity. It is now clear that although treatment-mediated increase in peripheral CD4 count is associated with reduced morbidity and mortality, compared to HIV-negative age-matched individuals, those on ART have a higher risk of morbidity and mortality. This risk is predicted in part by the on therapy CD4 count, although achiev-

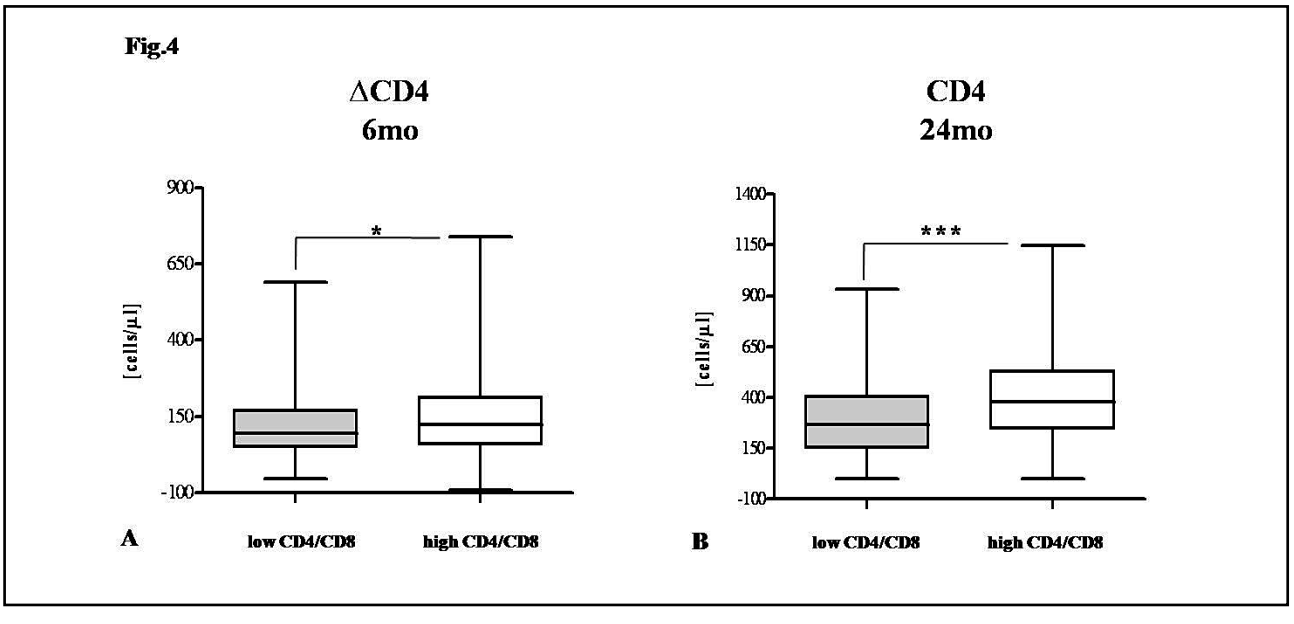

Fig.4 Immunologic response to cART in the patients with low baseline CD4AC (L, CD4 <200 cells/ µl) at 6mo and 24mo after the start of cART, additionally divided according to CD4/CD8 ratio. ∆CD4AC at 6mo **(A)** and CD4AC at 24mo **(B)** were determined in subgroups with low (grey) and high (white) CD4/CD8 ratio.

ing an apparent normal CD4 count may not fully restore health (19-21).

First, we observed similar CD4AC restoration rates in patients with significantly different baseline CD4AC levels. This confirmed ours and other authors' data indicating that nadir CD4 counts alone may not predict correctly response to cART. In a previous study on a cohort of 55 HIV+ patients subjected to cART for 2 years, the retrospectively defined groups with sustained and transitional response to cART did not differ significantly according to baseline CD4AC, either. Although circulating CD4 T cells may account to a certain extent for the quantity and quality of the total CD4 pool, they are only vaguely proportional especially in the settings of important immune activation when considerable share of immune cells are retained at the level of intestinal mucosa and peripheral lymph nodes, which is the situation in uncontrolled HIV infection. Markers reflecting immune activation are expected to be especially instrumental in such case.

Our second important observation was that CD4/CD8 ratio was unexpectedly "resistant" to cART effects. It was striking to observe that over 50% of those starting cART "on time" (i.e. CD4AC >350) could not attain the lower reference limit after 5 years of cART, and in spite of sustained HIV suppression. Clearly, the definition of immunological response to cART is infinitely more complex than the absolute CD4AC increase, and certainly depends on pathological factors additional to actively replicating HIV.

We have previously demonstrated that a sustained response to cART was associated not only with a balanced restoration of the CD4 pool in terms of Th1/Th2; naive/memory and effector subsets (22) but, importantly – of the CD8 pool, including: decreasing absolute counts, decreasing share of terminally differentiated CD57+CD28- CD8 T cells, reduced quantitative expression of CD38 activation-related molecules, improved phenotypic structure and functionality of non-HIV antigen-specific CD8 (23, 24). CD4/CD8 ratio has the advantage to summarise events in both CD4 and CD8 pools. A low ratio is reflecting either CD4-depletion, or activation-related CD8 increase, or both.

In the current study, we demonstrated two independent connotations of CD4/CD8 ratio: at baseline, just before the start and – in successfully treated patients. In the untreated, a low CD4/CD8 would reflect both HIV-mediated CD4 depletion and CD8 immune activation, with the second dominating in advanced disease. Therefore, CD4/CD8 increased the predictive value of baseline CD4 count only in the subgroup with advanced CD4 depletion. In the ART-suppressed, a low CD4/CD8 ratio would be rather associated with low-level immune stimulation related to the phenomena of compromised intestinal mucosal barrier and microbial translocation, or with latent HIV reservoirs.

In the last years a lot of evidence has accumulated in favour of the theory of "immunosenescence" in the settings of persistent infections, characterised by events similar to those observed in the very old: expansion of CMV-specific CD8+ T cells, enrichment for CD28− and PD-1+ T cells, reduced T cell telomere lengths and a low CD4/CD8 ratio (25). Each of these immunologic characteristics is present in untreated HIV infection, proposing that HIV might as well accelerate the aging of human immune system (25, 26). In a very recent study (11) using bioinformatical methods, markers of CD4(+) and CD8(+) T cell activation, exhaustion, senescence and differentiation were analysed in HIV-infected individuals, and phenotypes corresponding to "combined T cell pathogenesis," were defined. Importantly, CD4/CD8 ratio was correlated with more pathological T cell populations than any other "routine" laboratory parameters Sergio Serrano-Villar et al demonstrated, that low CD4/CD8 ratio correlated with markers of immune activation and with the presence of T cells with terminally differentiated phenotype. In line with this, a low CD4/CD8 ratio in HIV+ untreated individuals with variable CD4 counts has been associated with increased risk of AIDS-related events and death (27). In the settings of contemporary cART, researches have focused on disease progression in successfully suppressed patients, and proposed that an expansion of CD8 T cells reflected in a low CD4/CD8 ratio may identify individuals with persistent innate and adaptive immune activation at greater risk of serious non-AIDS events (18). Based on these results CD4/CD8 would be useful, in addition to CD4AC, to determine

the risk of therapeutic failure in patients with advanced deficiency at the start of cART, and to monitor the residual immune activation in treated patients with viral success. Tracking CD4/CD8 ratio may be instrumental in evaluating novel therapeutic approaches for ongoing immune dysfunction in cART-treated patients. In addition, CD4/CD8 is compatible with the appeals for simplified immune-monitoring approaches in the limited resource settings. Basic flow cytometry implies usually a CD45/CD3/CD4 or at least a CD3/CD4 combination that automatically permits CD4/CD8 calculation. In addition, a volumetric single platform image cytometer (SP ICM) dedicated to count both CD4(+) and CD8(+) T lymphocytes for HIV monitoring in resource-constrained settings has been recently elaborated (19).

In conclusion, CD4/CD8 is an important surrogate marker of immune recovery most probably reflecting the accelerated immune senescence in conditions of ongoing high- or low-level activation. A really successful response to ART should include both normalisation of peripheral CD4 count and restoration of CD4/CD8 ratio.

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HEPATITIS B AND HEPATITIS C AS CO-INFECTIONS IN PATIENTS WITH HIV/AIDS

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ABSTRACT

One third of HIV/AIDS patients are typically co-infected with viral hepatitis B and C (HBV and HCV). This rate is even higher, reaching up to 60-90%, in intravenous drug users. There is a significant double risk for HIV-positive patients co-infected with hepatitis to develop liver failure with lethal outcome. **Objective:** The aim of the present study was to perform clinical and epidemiological analysis of HIV/AIDS patients with hepatotropic virus co-infection and also to investigate the correlation between the degree of immune suppression and the level of liver damage. **Materials and Methods:** 86 HIV/AIDS patients co-infected with hepatotropic viruses were included in the study. During the period January 2010 - May 2014 they followed-up at the Clinic of Infectious Diseases and Parasitology, St. George University Hospital, Plovdiv subsequently after treatment. Epidemiological analysis and clinical monitoring was conducted. Laboratory examinations included 12 different parameters, determination of CD4 and viral load (VL). **Results:** Out of 164 HIV/AIDS patients in total, 86 (54.2%, 80 men and six women) had hepatitis co-infection. Six patients had HIV/HBV, 14 had HIV/HBV/HCV, and 66 were co-infected with HIV/HCV. Hospital admission rates for these patients were higher and most of them were drug abusers. A definite correlation was established between CD4, VL counts and the degree of liver damage and aminotransferase level. Between January 2010 and May 2011 lethal outcome was registered in 31 patients, 29 of whom had co-existing infection with hepatotropic viruses. **Conclusion:** Co-infections with HBV and HCV among HIV-positive individuals were present in more than 50% of the studied patients. They pose an important risk factor for morbidity, frequent hospital admissions and lethality.

Keywords: co-infection, viral hepatitis, HIV/AIDS.

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INTRODUCTION

HIV replicates in $CD4^+$ T cells at mucosal sites and draining lymph nodes. Following systemic dissemination, a major reservoir of infection is established in the gut-associated lymphoid tissues (GALT), eventually leading to depletion of GALT $CD4^+$ T cells, altered mucosal defences, gut mucosal injury, and microbial translocation (1). Despite host immune responses and antiviral therapies that suppress viral replication, viral reservoirs persist in long-lived memory $CD4^+$ T cells, including a stem-cell like population (Tscm). Additional reservoirs of viral persistence are established in macrophages in the brain and other macrophage-rich tissues. Chronic immune activation, a hallmark of HIV infection, is a strong predictor of disease progression, morbidity, and mortality that has also been linked to accelerated aging and age-related end-organ comorbidities

involving the cardiovascular system, kidneys, liver, brain, endocrine system, and bone. Underlying causes of chronic immune activation include ongoing viral replication, persistently elevated interferon responses, and microbial translocation. Cofactors associated with the HIV epidemic, such as HCV and other co-infections, and substance abuse, can also increase innate immune activation and end-organ comorbidity (1).

Hepatitis C virus (HCV)-related liver disease has emerged as one of the most important causes of morbidity and mortality in HIV-infected persons. Compared to persons with just one infection, HIV/HCV co-infected persons have a higher incidence of all liver complications and even some extrahepatic conditions raising the possibility of multiple pathogenic interactions (2).

Studies of some authors show that one third of patients with HIV/AIDS are infected with viral hepatitis C (HCV) and viral hepatitis B (HBV). This proportion reaches 60-90% in the population of intravenous drug users (IVDUs) (3, 4). HIV co-infected patients are at much higher risk for developing liver failure and death (5, 6).

The aim of the study was to perform clinical and epidemiological analysis of patients with HIV/AIDS and existing co-infection with hepatotropic viruses and also to investigate the correlation between the degree of immune suppression and the level of liver damage.

MATERIALS AND METHODS

The study included 86 HIV/AIDS patients co-infected with HBV and/or HCV monitored at the Clinic of Infectious Diseases, Medical University of Plovdiv, during the period January 2010 – May 2014. The survey employed epidemiological research methods, clinical surveillance and laboratory analyses. The number of CD4 cells was determined with fluorescence spectroscopy, using BD Multitest TM 6-color TBNK reagent and fluorescence apparatus BD FACSCanto™ II at the Department of Microbiology, Virology and Immunology, Medical University of Plovdiv. HIV diagnosis confirmation and viral load (VL) determination with PCR was performed at the National Centre of Infectious and Parasitic Diseases, National Reference Laboratory of HIV.

The etiological diagnosis of HBV and HCV infection was established by detection of HbsAg and anti-HCV in patients` sera, respectively, using commercial diagnostic ELISA kits (BioELISA HBsAg 3.0 and BioELISA HCV 4.0, Biokit, Barcelona, Spain). HCV results were confirmed with ELISA HCV Ab kit (Dia. Pro Diagnostic Bioprobes srl, Italy). All analyses were performed strictly according to the manufacturer`s instructions and the results were calculated with Multiskan reader.

Statistical analysis was performed using SPSS software version 18.

RESULTS

During the study period 164 HIV-positive patients were monitored monthly at the Clinic of Infectious Diseases, Medical University of Plovdiv. 86 patients out of 164 (54.2%) were co-infected with hepatitis viruses (HBV and/or HCV). They were aged 22 - 43 years (mean age 27.8+/-4.3), among whom 80 were male and 6 female with male-female ratio of 13.3:1.

Risk factor survey determined that 82 (95.35%) were intravenous drug users, 38 (44.19%) reported a history of numerous unprotected sexual contacts, and three (3.49%) were sex workers. Lethal outcome was registered in 27 patients with co-infections, where lethality reached 16.46%.

Eight of the patients (4.88%, all male) were HIV/HBV co-infected, 14 patients (8.54%) were with triple infection (HIV/HBV/HCV), and 64 (39.02%) were with HIV/HCV co-infection (**Diagram 1**). All HIV/HCV patients were intravenous drug users.

Results from laboratory examinations (CD4 cells, VL, ALT, AST, TPROT, ALB, TBIL, CHOL, WBC, RBC, HGB, and PLT) of patients with HIV/HBV are shown in **Table 1**.

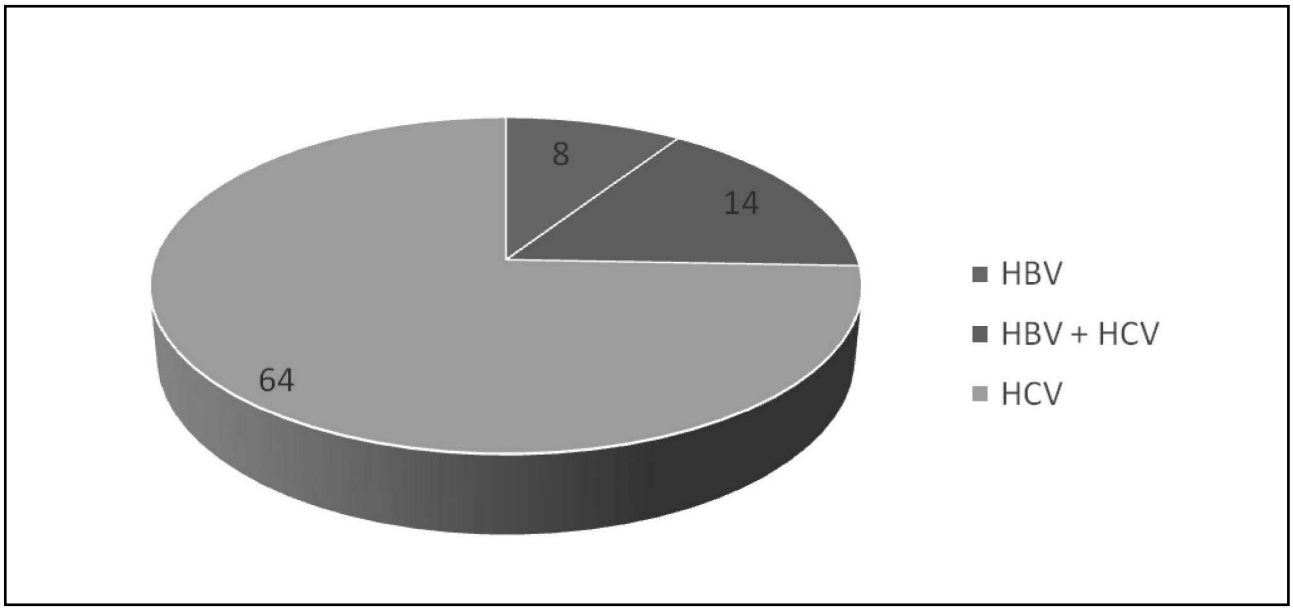

Diagram 1. Distribution of hepatitis co-infection among HIV-positive patients.

Table 1. Patients with HIV/HBV

No	CD4	VL	ALT	AST	TPROT	ALB	TBIL	CHOL	WBC	RBC	HGB	PLT
1	864	20	54	39	83	48	9.4	4.2	6.9	5.6	161	267
2	600	20	24	18	77	49	10.1	5.2	5.6	4.8	151	219
3	481	22800	9	19	86	47	18.0	4.2	6.0	4.6	145	202
4	261	20	23	20	83	46	6.3	5.6	7.1	5.2	164	168
5	452	1780	89	121	73	36	12.1	4.8	5.7	4.3	143	255
6	352	223000	9	9	75	37	10.4	2.8	7.6	3.6	112	194
7	568	49000	30	28	78	42	12.6	2.6	4.4	4.1	103	231
8	680	24900	45	42	80	40	10.5	3.4	5.4	4.2	124	201

The number of CD4 cells varied from 261 to 864, and the VL from 20 to 223 000. Aminotransferase level, especially ALT, was slightly elevated in two patients – 54 E/l and 89 E/l, whereas AST was elevated in one patient – 121 E/l.

HBV was not active and we did not find correlation with the low level of CD4 cells.

Patients with triple infection (HIV/HBV/HCV) were 12 men and two women, aged 22 to 43 years and with mean values of CD4, VL, ALT, AST, and PLT presented in **Table 2**.

Table 2. Mean values of CD4, VL, ALT, AST, and PLT among patients with HIV/HBV/HCV.

Parameter	Mean value
CD4	**228.3 +/-92.1**
ALT	**42.1 +/- 23.4**
AST	**40.0 +/- 20.3**
PLT	**290 +/-65.2**
VL	**126284.6 +/- 123.5**

The mean value of CD4 cells among HIV/HBV/HCV patients was 228.3 with a range from 80 to 740. The mean value of VL was 126284.6 with a range from 70 to 780 000. Aminotransferase level was slightly elevated, as one patient had ALT level of 160 E/l. The mean value of ALT was 42.1 E/l (reference value: 35 E/l).

Mean values of VL, CD4, ALT, AST, and PLT of HIV/HCV co-infected patients are shown in **Table 3**.

Table 3. Mean values of VL, CD4, ALT, AST, and PLT among patients with HIV/HCV.

Parameter	Mean value
VL	**73808.6 +/- 234.7**
CD4	**360.2 +/- 98**
ALT	**51.4 +/- 31.0**
AST	**40.0 +/- 24.5**
PLT	**210.7 +/- 59.4**

The mean values of aminotransferase activity among patients with HIV/HCV were: ALT – 51.4 +/- 31.0, AST – 40 +/- 24.5. PLT ranged from 169.5 to 325.6 with a mean value of 210.7 +/- 59.4. The mean value of CD4 cells was 360.2 +/- 98.3 and the mean VL was 73808.6+/- 234.7.

During the clinical course of all 27 co-infected patients with fatal outcome the levels of CD4 cells progressively decreased, while the VL increased.

Pearson correlation analysis was performed on laboratory results data from 40 patients with HIV/HCV co-infection to examine the relationship between the stage of liver damage and the severity of HIV infection measured as the number of CD4 cells (more or less than 500). Results are shown in **Table 4**.

Table 4. Correlation analysis data

Parameter	CD4	N	Mean+/-SE	SD	u	Pu
ALT	< 500	29	90.2+/-13.7	73.9	3.8	<0.001
ALT	> 500	11	36.3+/-3.6	12.5	3.8	<0.001
AST	< 500	29	86.9+/-11.9	64.3	2.9	<0.01
AST	> 500	11	45.6+/-7.0	23.9	2.9	<0.01
PLT	< 500	29	173.5+/-11.9	64.4	2.8	<0.01
PLT	> 500	11	243.9+/-21.8	72.4	2.8	<0.01

Pearson correlation analysis showed that the stage of liver damage, measured by the value of aminotransferase level (ALT, AST) and the decrease of PLT, significantly influenced on the severity of HIV infection, as demonstrated with the decrease of CD4 cells bellow 500 (Pu<0.001).

The opposite relationship was also examined, or how the severity of HIV infection influenced on the stage of liver damage. Correlation analysis data are shown in **Table 5**.

Table 5. Correlation analysis of the relationship between the severity of HIV infection and the stage of liver damage.

Dependence	Parameters	ALT	AST	PLT
CD4	Pearson	-0,45	-040	0.62
CD4	P/sig/	<0.001	<0.01	<0.001
CD4	N	40	40	40

A significant relationship was found between the level of CD4 cells and ALT, AST, and PLT values. A moderate reverse correlation was also found on the relationship between the level of CD4 cells and aminotransferase activity, and high right correlation dependence between CD4 cells and PLT ($P<0.001$).

DISCUSSION

Our study established co-infections with hepatitis viruses (HBV and HCV) in more than 50% of HIV-positive patients contributing to morbidity, frequent hospitalisations and mortality. Similar results were obtained by other authors as well (7).

There was a certain correlation between the level of immunosuppression and the severity of liver damage. The same correlation was observed by Soriano et al and Tien (8, 9). Compared to HCV mono-infected, HIV/HCV co-infected patients show rapid progress to cirrhosis and hepatocellular carcinoma. The same risk has been discussed in the work of Sulkowski and Dieterich (10, 11). HCV-associated immune activation accelerates HIV infection and HIV-mediated immune suppression stimulates replication of hepatitis C virus and deteriorates the immune-mediated HCV clearance, as described also by Thomas (12).

Our data differ significantly from the data of Diwe et al (13) who established that among 404 patients with HIV, HBsAg-positive were nine (2.2%) while three (0.7%) were positive for HCV and there were not any subjects with HIV/HBV/HCV triple infection.

Human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) exact a high toll worldwide. Both can lead to chronic disease, cancer, death, and neither can be eradicated with the use of current therapies. Antiviral drug resistance often develops after patients have received treatment for some time and is usually followed by the loss of clinical benefit. Co-infection with the two viruses exacerbates the negative effects (14). According to the Joint United Nations Program on HIV/AIDS (UNAIDS), about 33 million people are infected with HIV worldwide, and the majority of them live in Asia and Africa. Approximately 10% of the HIV-infected population has concurrent chronic hepatitis B with co-infection more common in areas of high prevalence for both viruses. In countries where the viruses are highly endemic, the rate can be as high as 25%. In areas where HBV is less endemic (North America, Europe, and Australia), HBV and HIV are most often acquired during adolescence or adulthood through sexual transmission or injection-drug use. The prevalence of HIV/HBV co-infection in these regions is generally less than 10% of the HIV-infected population (14). Our study confirmed these data, HIV/HBV co-infections among our patients were 8/164 (4.88%) and HIV/HBV/HCV triple infection was established in 14/164 (8.54%). The highest co-infection rate was with HIV/HCV. This may be due to the HBV immunoprophylaxis and the significant decrease in the incidence rate of HBV during the last 20 years in Europe and also in Bulgaria (15).

CONCLUSION:

HCV and HBV co-infection in HIV-positive patients is one of the most important risk factors influencing HIV infection control. The incidence of co-infection cases with fatal outcome is not rare. We consider that HIV, HCV, and HBV screening tests should be performed together. If co-infection is confirmed antiretroviral therapy should be initiated immediately as every delay can be fatal.

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CONFLICT OF INTEREST STATEMENT (AUTHORS)

I certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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CONFLICT OF INTEREST STATEMENT (REVIEWERS)

I certify that have no personal or financial conflict of interest with authors of the manuscript provided me for review.

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STATEMENT ABOUT PROTECTION OF HUMAN SUBJECTS
AND ANIMALS IN RESEARCH

I certify that this study involving human subjects is in accordance with the Helsinky declaration of 1975 as revised in 2000 and that it has been approved by the relevant institutional Ethical Committee.

Author name Date Signature

I certify that this study involving animals followed the institutional and national guide for the care and use of laboratory animals.

Author name Date Signature

