

In Vitro Activity of Colistin and Trimethoprim/Sulfamethoxazole Against Consortia of Multidrug Resistant Non-Fermenting Gram-Negative Bacilli Isolated from Lower Respiratory Tract

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Abstract

Background: Multidrug resistant (MDR) *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* have a leading role in nosocomial infections, including lower respiratory tract (LRT) infections. When polymicrobial infection by these three bacteria occurs, colistin against MDR *P. aeruginosa* and *A. baumannii* and trimethoprim/sulfamethoxazole (SXT) against *S. maltophilia* can be an optional antimicrobial strategy.

Objectives: The aim of this study was to investigate the potential synergic effect of colistin-plus-SXT against those MDR *P. aeruginosa*, *A. baumannii* and *S. maltophilia* isolates that were isolated at the same time, from the same LRT sample of patients.

Methods: Sixty connected isolates from 20 different patients were collected in a two-year study period. The checkerboard method and time-kill assays were used for synergy testing.

Results: All *P. aeruginosa* and *A. baumannii* strains were susceptible to colistin, whereas all *S. maltophilia* isolates were resistant to it. Fifteen percent of MDR *A. baumannii* strains and all *S. maltophilia* isolates were susceptible to SXT. By the checkerboard method, colistin-plus-SXT showed synergy in 50%, 35% and 45% of *S. maltophilia*, MDR *P. aeruginosa* and MDR *A. baumannii* strains, respectively. Antagonistic effect was not found. A time-kill assay was performed on strains which showed synergy by the checkerboard method: 70%, 57% and 56% of *S. maltophilia*, *P. aeruginosa* and *A. baumannii* strains showed the same results. Synergic activity of the combination was already detected after 6 h incubation in 86% of *S. maltophilia* isolates and 50% of *P. aeruginosa* strains. Regrowth of *A. baumannii* after 24 hour in the presence of colistin was prevented by the combination. The results gained by CB and TKA methods correlated in 61% of cases, but the Σ FIC values did not correlate with the rate of log₁₀ decrease in TKA. Colistin-plus-SXT combination had synergic effect on 35% of *S. maltophilia*, 20% of *P. aeruginosa* and 25% *A. baumannii* strains by both methods.

Conclusions: According to our *in vitro* results, colistin-plus-SXT combined therapy can be used efficiently in clinical practice as no antagonistic effect was detected. In certain cases colistin-plus-SXT has a synergic effect against MDR *P. aeruginosa*, *A. baumannii* and *S. maltophilia*.

Keywords: Colistin, Trimethoprim Sulfamethoxazole Drug Combination, Drug Synergism, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*

1. Background

Among the non-glucose-fermenting bacteria, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* have a leading role in nosocomial infections, especially in lower respiratory tract (LRT) infections in mechanically ventilated patients and in bacteraemia. While *S. maltophilia* has intrinsic resistance to many antibiotics, limiting treatment options to trimethoprim/sulfamethoxazole (SXT), fluoroquinolones and few other antibiotic agents, *P. aeruginosa* and *A. baumannii* often show a high level of acquired resistance in a hospital environment, often making colistin therapy necessary. The biofilm-forming ability of these bacteria makes antibiotic treatment even more challenging.

Polymicrobial colonization of the LRT is frequently observed in patients treated in intensive care units (ICU) or in patients suffering from chronic respiratory tract diseases with frequent hospital care. Polymicrobial infection can develop from previous polymicrobial colonization; however, it is difficult to decide whether the infection is really polymicrobial or caused by just one member of the bacterial consortia. Furthermore, differentiation between polymicrobial colonization and infection of the LRT in ventilated patients with serious underlying diseases is also difficult.

Stenotrophomonas maltophilia is often part of polymicrobial infections. In our centre 58% of *S. maltophilia* isolated from LRT specimens was cultured as co-pathogen or co-colonizer in 2013 - 2014. *Pseudomonas aeruginosa* was

found to be the most frequent co-pathogen, but *A. baumannii* was also a significant co-habitant. Although co-infection/co-colonization by multidrug resistant (MDR) *P. aeruginosa*, *A. baumannii* and *S. maltophilia* in LRT is rare, we cannot treat it as a unique and therefore marginal problem. A rapid and efficacious antimicrobial therapy against this MDR bacterial consortium is essential.

In a meta-analysis it was demonstrated that colistin was efficacious and safe for treatment of patients with pulmonary infection caused by MDR *P. aeruginosa* or *A. baumannii* (1). However, considering the low penetration of colistin in the lung parenchyma after intravenous administration, there is a certain level of clinical reluctance to its use for treatment of respiratory tract infections. Inhalational use of colistin provides a high concentration in airways, and therefore represents a promising therapy approach (2). Trimethoprim/sulfamethoxazole is the first-line antimicrobial agent for *S. maltophilia* infections.

In cases of patients with MDR *P. aeruginosa*, MDR *A. baumannii* and *S. maltophilia* co-infection in LRT, colistin against MDR *P. aeruginosa* and *A. baumannii* plus SXT against *S. maltophilia* looks to be an optional or obligatory antimicrobial strategy. Colistin-plus-SXT is a combined monotherapy and not an unconventional combination therapy in such cases. The question is whether this 'combination' has synergy or antagonism on *S. maltophilia*, *P. aeruginosa* or *A. baumannii*.

In recent years combination antibiotic therapy has become an important option against MDR bacteria. Physicians should be supplied with *in vitro* synergy testing data, but most of the testing methods (checkerboard method, time-kill assay) are labour-intensive, therefore they are rarely performed in routine diagnostic laboratories. Furthermore, results gained by different techniques can be controversial and difficult to interpret. Especially non-fermenting bacteria demonstrate the methodical difficulties (3).

2. Objectives

Objective of this study was to determine the *in vitro* activity of the colistin-plus-SXT combination, using different synergy testing methods, against MDR *P. aeruginosa*, MDR *A. baumannii* and *S. maltophilia* strains isolated at the same time from the same LRT samples.

3. Methods

In a two-year study period (2013 - 2014) 392 consecutive non-duplicate *S. maltophilia* strains were isolated from LRT samples. In 58% of cases, other pathogens were isolated

next to *S. maltophilia*. In 7% of cases, both *P. aeruginosa* and *A. baumannii* were co-isolated and in 5% of cases (n = 20) *P. aeruginosa* and *A. baumannii* fitted the criteria of multidrug resistance. This study included these 20 MDR *P. aeruginosa*, 20 MDR *A. baumannii* and 20 *S. maltophilia* isolates collected in the Diagnostic Laboratory of Clinical Microbiology, Institute of Laboratory Medicine, Semmelweis University (Budapest, Hungary). The bacterial 'triplets' were isolated at the same time from the same sample (tracheal aspirate or bronchoalveolar lavage sample) of different patients. Isolates were identified by the MALDI-TOF mass spectrometry technique (Bruker Daltonics, Germany). All strains were isolated from patients treated at ICUs.

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) was used for molecular typing of isolates, as described by Silbert et al. (4). Bacteria were suspended in 100 μ L of PCR-grade water and heated at 100°C for 15 minutes. After centrifugation at 12,000 rpm for 2 minutes, supernatant was removed. One μ L of the supernatant was used as DNA for PCR. Primers of ERIC1 5'-ATGTAAGCTCTGGGGATTAC-3' and ERIC2 5'-AAGTAAGTACTGGGGTGAGCG-3' (Biocenter, Hungary) and REDTaq Ready Mix PCR reaction mix (Sigma-Aldrich, USA) were used for DNA amplification, in 50 μ L final PCR reaction volume. PCR conditions were the following: initial denaturation at 95°C for 2 minutes, 30 cycles at 90°C for 30 seconds, 52°C for 1 minute, 65°C for 8 minutes. Electrophoresis in 1.5% agarose gel stained with 0.01% GelRed (Biotium, USA) was performed. Isolates that differed by two or more bands were interpreted as unrelated.

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method in cation-adjusted Mueller-Hinton II broth (Becton Dickinson, USA) (5). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains. Colistin (Sigma-Aldrich, USA) was tested in the range 1 - 512 mg/L in case of *S. maltophilia* strains and at 0.06 - 32 mg/L in case of *P. aeruginosa*, *A. baumannii* strains. The MIC values of SXT (Ratiopharm, Hungary) were tested at 0.5 - 256 mg/L and 2 - 1024 mg/L in case of *A. baumannii* and *P. aeruginosa* strains, respectively, and at 0.06 - 32 mg/L in case of *S. maltophilia* isolates. To interpret MIC results, EUCAST species-specific breakpoints were applied, except for the colistin MIC of *S. maltophilia*, when the *Pseudomonas* sp.-specific breakpoint was used (6).

Antibiotic combination of colistin-plus-SXT was analysed initially by a checkerboard technique (CB). Mueller-Hinton II broth was used. *Stenotrophomonas maltophilia* isolates were tested in 7 doubling dilutions of colistin and 11 doubling dilutions of SXT, whereas *P. aeruginosa* and *A. baumannii* strains were tested in 7 doubling dilutions of SXT and 11 doubling dilutions of colistin. Microbroth plates

were inoculated with bacteria to yield 5×10^5 CFU/mL in the 100 μ L final volume. Plates were incubated at 35°C for 18 - 22 hours. Fractional inhibitory concentration indices (Σ FIC) were calculated following the formula: $FIC(A) + FIC(B) = \Sigma FIC$, where $FIC(A) = MIC$ of antibiotic agent A in combination/ MIC of antibiotic agent A alone and $FIC(B) = MIC$ of antibiotic agent B in combination/ MIC of antibiotic agent B alone (7). The Σ FIC of two antibiotics tested defines the effects of antimicrobial agent combinations as antagonistic ($\Sigma FIC > 4$), indifferent ($0.5 < \Sigma FIC \leq 4$) or synergistic ($FICI \leq 0.5$).

When synergy was detected by CB, a time-kill assay (TKA) was performed at 1xMIC following a previously published method (8). When MICs were above the therapeutic level, SXT was used at 8 mg/L and colistin at 4 mg/L, which fits the peak serum levels of these agents (9). Twenty ml of SXT, colistin and SXT-plus-colistin containing Mueller-Hinton II broth were inoculated with bacteria to yield a density of 106 CFU/mL in the final volume. Tubes were incubated at 37°C with constant agitation. After 1, 2, 4, 6 and 24 hours incubation aliquots were removed, serially diluted in 0.9% sodium chloride solution and plated on sheep blood agar plates (BioMerieux, France). Colony-forming units (CFUs) were counted on agar plates after 24 hours incubation at 37°C. The lower limit of detection by this method was 20 CFU/mL. Synergy was defined as a ≥ 2 log₁₀ decrease in CFU/ml at 24 h for the antibiotic combination compared with its more active constituent (8).

4. Results

According to ERIC-PCR, isolates in the same species were from different genotypes. All *P. aeruginosa* and *A. baumannii* strains were susceptible to colistin, with MIC₅₀ 1 mg/L and MIC₉₀ 2 mg/L. Fifteen percent of *A. baumannii* strains were susceptible to SXT, MIC₅₀ 32 mg/L and MIC₉₀ 128 mg/L was found. *Pseudomonas aeruginosa* strains showed a high level of intrinsic resistance to SXT, with MIC₅₀ 256 mg/L and MIC₉₀ 512 mg/L. All *S. maltophilia* strains were sensitive to SXT, with MIC₅₀ 0.25 mg/L and MIC₉₀ 1 mg/L, and resistant to colistin, with MIC₅₀ 256 mg/L and MIC₉₀ > 512 mg/L. Results of colistin-plus-SXT combination tests performed by CB method are summarized in Table 1. As summarized in Table 2 synergic and indifferent effects were found, but an antagonist effect was not.

Strains showing synergy by CB method were further examined by TKA. The tested *S. maltophilia*, *P. aeruginosa* and *A. baumannii* strains showed synergy in 70%, 57% and 56% of cases, respectively. The rates of log₁₀ decrease after 6 and 24 h are summarized in Table 3 considering that for most combinations with colistin against Gram-negative species

initial killing is usually dramatic, but is followed by significant regrowth. Synergic activity of the combination was already detected after 6 hours incubation in 86% of *S. maltophilia* isolates and 50% of *P. aeruginosa* strains. In the case of *A. baumannii*, synergy was detected just after 24 hours incubation. The results gained by CB and TKA methods correlated in 61% of cases, but the Σ FIC values did not correlate with the rate of log₁₀ decrease in TKA. The results of different *in vitro* synergy testing must be synthesized and carefully interpreted. Colistin-plus-SXT combination had synergistic effect on seven *S. maltophilia* (35%), four *P. aeruginosa* (20%) and five *A. baumannii* (25%) strains by both methods.

5. Discussion

The potential synergic effect of colistin-plus-SXT against MDR *P. aeruginosa*, MDR *A. baumannii* and *S. maltophilia* isolates was investigated in this study. The isolates were connected as each one of the three species was isolated at the same time from the same LRT sample of patients. Colistin-plus-SXT therapy is an obligatory antimicrobial strategy in LRT co-infections caused by the discussed three bacteria.

Co-colonization of patients with carbapenem-resistant Enterobacteriaceae and *A. baumannii* or *P. aeruginosa* has been shown to be associated with increased antibiotic resistance and mortality (10). As potential interspecies interactions may enhance bacterial virulence and antibiotic resistance, co-colonization or co-infection of patients with the intrinsically carbapenem-resistant *S. maltophilia* and *A. baumannii* or *P. aeruginosa* might be associated with increased antibiotic resistance and mortality. This hypothesis was not considered in previous studies. In our study the patients' overall mortality in hospital was 50%. This did not differ significantly from a previous study where all-cause mortality of 45% was found in 100 *S. maltophilia* infections, of which 62 cases were pneumonia (11). The high mortality underlines the need for a rapid and effective antimicrobial therapy.

The folate synthesis inhibitor SXT is the first-line antimicrobial drug for *S. maltophilia* infections. All *S. maltophilia* strains were sensitive to SXT in our study, which supports the current antimicrobial guidelines. Colistin was found to have weak *in vitro* activity against the studied *S. maltophilia* isolates: high level of colistin resistance (MIC₅₀ 256 mg/L) was detected. This shows that colistin should not be used alone either in *S. maltophilia* infection or in *S. maltophilia* co-infection, but it can have synergic activity in combination, as reported in previous studies (12). The effect of colistin in antibiotic combination is based on its detergent-like property: it interacts with surface LPS

Table 1. Summary of Results Gained by CB Method; Effect of Colistin-Plus-SXT Combination was Tested on 20 *S. maltophilia*, 20 MDR *P. aeruginosa* and 20 MDR *A. baumannii* Strains; Strains were Connected as Each One of the Three Species was Isolated at the Same Time from the Same LRT sample^a

	<i>Stenotrophomonas maltophilia</i>					<i>Pseudomonas aeruginosa</i>					<i>Acinetobacter baumannii</i>				
	AB1	AB2	AB1 + AB2	AB2 + AB1	ΣFIC	AB1	AB2	AB1 + AB2	AB2 + AB1	ΣFIC	AB1	AB2	AB1 + AB2	AB2 + AB1	ΣFIC
1	0.25	256	0.062	32	0.375	256	1	32	0.25	0.375	32	1	2	0.25	0.312
2	0.062	256	0.062	4	1.015	128	1	8	1	1.06	8	2	1	0.25	0.25
3	1	512	0.25	8	0.265	512	2	8	1	0.52	128	1	8	0.5	0.56
4	1	8	0.5	2	0.75	128	2	8	1	0.56	64	2	4	0.25	0.187
5	0.25	32	0.125	4	0.625	8	2	1	0.062	0.156	64	0.5	2	0.25	0.53
6	0.25	256	0.062	8	0.281	512	2	8	1	0.52	32	2	4	0.5	0.375
7	0.06	64	0.015	8	0.375	128	1	32	0.125	0.375	64	2	8	0.25	0.25
8	0.5	512	0.062	16	0.151	16	2	0.5	1	0.53	32	2	16	0.25	0.625
9	0.25	256	0.062	16	0.312	512	2	8	0.5	0.265	128	0.5	8	0.25	0.56
10	0.5	32	0.062	2	0.187	512	2	8	1	0.52	32	1	2	0.5	0.56
11	0.25	128	0.125	2	0.52	512	2	16	1	0.53	1	1	0.25	0.125	0.375
12	0.125	256	0.125	16	1.06	512	1	32	0.5	0.56	1	1	0.25	0.125	0.375
13	0.25	64	0.125	16	0.75	512	2	8	0.5	0.265	64	1	8	0.25	0.375
14	0.5	512	0.062	16	0.25	8	1	1	0.25	0.375	128	0.5	4	0.25	0.53
15	0.25	512	0.25	8	1.015	256	0.5	8	0.5	1.03	4	0.5	1	0.25	0.75
16	0.25	512	0.25	8	1.015	512	1	16	0.5	0.53	2	0.5	1	0.25	1
17	0.062	128	0.062	8	1.06	128	0.5	64	0.125	0.75	4	0.125	0.5	0.125	1.125
18	0.25	256	0.25	8	1.03	1024	1	16	0.25	0.265	64	1	32	0.062	0.56
19	0.5	128	0.125	8	0.375	16	0.5	4	0.25	0.75	64	0.5	8	0.125	0.375
20	1	64	0.062	4	0.125	512	1	64	0.5	0.625	16	0.5	1	0.5	1.06

^a AB1, MIC value of SXT; AB2, MIC value of colistin; AB1 + AB2, MIC value of SXT in combination with colistin; AB2 + AB1, MIC value of colistin in combination with SXT; ΣFIC values in bold means synergism.

Table 2. Summary of Results Gained by CB Method; Colistin-plus-SXT Combination was Tested on 20 MDR *P. aeruginosa*, 20 MDR *A. baumannii* and 20 *S. maltophilia* Strains

Colistin + SXT Combination Tested by CB Method	No. (%) of Strains Showed	
	Synergy	Indifferent Effect
<i>S. maltophilia</i>	10 (50)	10 (50)
<i>P. aeruginosa</i>	7 (35)	13 (65)
<i>A. baumannii</i>	9 (45)	11 (55)

and phospholipids, disturbing membrane permeability. Colistin exposure leads to increased permeability to large or hydrophobic compounds such as SXT (8). Synergic effect of colistin and SXT against *S. maltophilia* was found in 47% of isolates by Giamarellos-Bourboulis et al. (13). This is in concordance with our CB results (synergy in 50% of isolates). When CB and TKA results are evaluated together, the rate of synergic effect is only in 35%.

In current medical practice SXT is not recommended for treatment of MDR *Acinetobacter* infections. In the majority of studies regarding MDR *Acinetobacter* spp., the non-susceptibility rate was > 70%. In our study 85% of MDR *A. baumannii* strains were resistant to SXT. Only single case reports evaluated SXT for *A. baumannii* infections, mainly in combination therapy. Though they considered

therapeutic success, clinical evidence has failed so far (14). Recent publication report that SXT combined with colistin might represent an effective therapy for severe carbapenem-resistant *A. baumannii* infections (15). In concordance with previously published data, colistin-plus-SXT was found to display a synergic effect against *A. baumannii* isolates: synergy was found in 45% by CB method, but in 25% when results gained by the two methods were synthesized. Similarly to the findings of Nepka et al. the regrowth of *A. baumannii* after 24 hours was prevented by colistin-plus-SXT (15). In case of colistin-resistant *A. baumannii* strains colistin-plus-SXT combination demonstrated limited synergism (16).

Pseudomonas aeruginosa is a poor target for therapy with SXT (6). Strains showed high level of intrinsic resistance to SXT. The combination of colistin-plus-SXT was synergistic against 20% of *P. aeruginosa*. In contrast with our results, Vidailiac et al. found no activity of colistin-plus-SXT against their tested colistin-susceptible *P. aeruginosa* strains (8).

Discrepancies between our results gained by CB and TKA indicate that different methods to assess synergic effects do not provide necessarily comparable results (17). Nevertheless, the probability of synergy is high in those cases when a synergic effect is proved by two different techniques. An important finding of our study is that colistin-

Table 3. Summary of the Synergic Results Gained by TKA; Colistin-Plus-SXT Combination was Tested on Strains Which were Previously Tested by CB and Σ FIC was < 0.5

Colistin + SXT Combination Tested by TKA	No. (%) of strains showed synergy by TKA			Previously Determined Σ FIC Values
	Synergy by TKA	Difference in log ₁₀ after 6 h	Difference in log ₁₀ after 24 h	
<i>S. maltophilia</i>	7 (70)	5.9	2.4	Sm#1 Σ FIC = 0.375
		7.7	3.8	Sm#3 Σ FIC = 0.265
		7.5	3.9	Sm#6 Σ FIC = 0.281
		2.4	4.7	Sm#7 Σ FIC = 0.375
		2.7	2.9	Sm#9 Σ FIC = 0.312
		6.3	3.1	Sm#10 Σ FIC = 0.187
		6	6	Sm#20 Σ FIC = 0.125
<i>P. aeruginosa</i>	4 (57)	7	4.2	Pa#1 Σ FIC = 0.375
		3.4	2.3	Pa#5 Σ FIC = 0.156
		6.7	4.1	Pa#14 Σ FIC = 0.375
		7.2	3.7	Pa#18 Σ FIC = 0.266
<i>A. baumannii</i>	5 (56)	3.1	7.3	Ab#2 Σ FIC = 0.25
		3.3	5.3	Ab#4 Σ FIC = 0.094
		3.1	7.3	Ab#6 Σ FIC = 0.375
		2.7	4.8	Ab#11 Σ FIC = 0.375
		3.6	5.5	Ab#12 Σ FIC = 0.375

plus-SXT combination can be used efficiently as no antagonistic effect was detected. Furthermore, synergism can be observed in 20% - 35% of isolates. Regrowth of *A. baumannii* after 24 hour in the presence of colistin can be prevented by colistin-plus-SXT combination. Of note, previous studies tested each species separately, whereas in our study MDR bacteria were investigated in their complex 'triplet' as they were isolated from a LRT sample. Two 'triplets' out of 20 showed synergy verified by both methods. In these cases patients had obvious benefit from combined colistin-plus-SXT therapy.

The potential interspecies interaction between these bacteria has to be highlighted. Dominantly in cystic fibrosis several studies focused on interaction of *P. aeruginosa* with other bacterial species, but only a few have been published on the interaction between *P. aeruginosa* and *S. maltophilia*. It was found that *S. maltophilia* increases the risk of resistance of *P. aeruginosa* to polymyxin; beta-lactamase leaking from *S. maltophilia* enhances the growth of *P. aeruginosa* in the presence of beta-lactam antibiotic agents; *S. maltophilia* might confer a selective fitness advantage to *P. aeruginosa* and increase the virulence of *P. aeruginosa* (18). The interaction of *A. baumannii* and *S. maltophilia* is not discussed in the literature, except for their ability to increase each other's biofilm production (19). It was reported that a Burkholderia cenocepacia subpopulation highly resistant

to polymyxin B can protect a sensitive *P. aeruginosa* from polymyxin B in broth co-culture (20). Similarly, it can be hypothesized that *S. maltophilia* highly resistant to colistin can protect a sensitive *P. aeruginosa* or *A. baumannii* from colistin in broth co-culture. Co-culturing of these bacteria in sessile form - like they growth together in LRT biofilms - can be suitable to detect this presumed interaction. Further investigations are needed to elucidate this hypothesis.

Further *in vitro* pharmacokinetic/pharmacodynamic experiments and animal studies are required to evaluate the combination of colistin with SXT against MDR Gram-negative pathogens. Evaluation of the clinical significance of our observation has to be performed also. The dose-response relationship of the colistin-plus-SXT combination must be clarified.

In conclusion, according to our *in vitro* results we can state that colistin-plus-SXT combined therapy can be used efficiently in clinical practice as no antagonistic effect was detected. In certain cases colistin-plus-SXT has a synergic effect against MDR *P. aeruginosa*, *A. baumannii* and *S. maltophilia*.

Footnotes

Authors' Contribution: Study concept and design: Emese Juhasz, Katalin Kristof; aquisition of data: Emese

Juhasz, Andrea Kovacs; analysis and interpretation of data: Emese Juhasz, Andrea Kovacs; drafting of the manuscript: Emese Juhasz; critical revision of the manuscript for important intellectual content: Emese Juhasz, Katalin Kristof; statistical analysis: Emese Juhasz, Andrea Kovacs, Miklos Ivan; administrative, technical and material support: Emese Juhasz, Andrea Kovacs, Miklos Ivan, Julia Pongracz; study supervision: Emese Juhasz, Katalin Kristof.

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