

## Evaluation of *Neisseria meningitidis* Carriage with the Analysis of Serogroups, Genogroups and Clonal Complexes among Polish Soldiers

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### Abstract

*Neisseria meningitidis* is an etiological factor of invasive meningococcal disease (IMD). This Gram-negative diplococcus is transmitted from person to person via droplets or through a direct physical contact with secretions of infected patients or asymptomatic carriers. The latter account for 5–10% of the general population. The aim of the study was to estimate the actual *N. meningitidis* carriage rate in the military environment with identification of serogroups, genogroups, sequence types and clonal complexes of the isolates detected among Polish soldiers. The study was conducted during winter seasons of 2015 and 2016 and involved 883 professional soldiers from the Armoured Brigade in Świętoszów, Poland. The material for testing were nasopharyngeal swabs obtained from study participants. The samples were tested using standard microbiological methods (culture, incubation, microscopy, biochemical and automated identification). *N. meningitidis* isolates were subjected to slide agglutination test (identification of serogroups), the bacterial DNA was extracted and allowed to determine genogroup, clonal complexes and sequence types. 76 soldiers were found to be carriers of *N. meningitidis*, they accounted for 8.6% of the study group. The meningococcal isolates mostly belonged to serogroup B. Sequence types ST-11439, ST-136, ST-1136 and the clonal complex 41/44CC were found to be predominant. Clonal complexes responsible for IMD were detected in 15.8% of carriers and 1.4% of the whole study participants. Carriage rates of *N. meningitidis* among Polish soldiers were found to be similar to those reported in the general population.

**Key words:** *Neisseria meningitidis*, serogroups, genogroups, clonal complexes, soldiers

### Introduction

*Neisseria meningitidis* is an etiological factor of invasive meningococcal disease (IMD), which is an acute, infectious illness characterized by a severe clinical course, even if antibiotics are administered at an early stage and intensive care is provided (Rosenstein et al. 2001). This Gram-negative diplococcus colonizes the nasopharyngeal mucosa and is transmitted from person to person via droplets or through a direct physical contact with secretions of infected patients or asymptomatic carriers, who account for 5–10% of the general population (Soriano-Gabarro et al. 2011). It is estimated, that carriage rates of *N. meningitidis* in closed environments (dormitories, military units) may be significantly higher and reach >40% (Tyski et al. 2000). Until now, twelve meningococcal serogroups have been described (they are distinguished according to biochemical constitution of the polysaccharide capsules of bacteria). The groups of *N. meningitidis* mostly associated with IMD are A, B,

C, W and Y (Skoczyńska and Hryniewicz 2012). Serogroup incidence varies depending on the geographical area (Jafri et al. 2013). Groups B and C are a main cause of IMD in Americas and Europe. It is estimated that there are 1.2 million new IMD cases worldwide every year, with mortality reaching 10% or even 70% in cases of a septic shock (Rosenstein et al. 2001). In Poland, IMD incidence was found to be 0.43/100 000 in 2016 and 0.58/100 000 in 2017 (NIZP-PZH 2018). IMD cases have also been reported among the Polish military personnel: four cases in the military base in Skwierzyna, including two deaths in 2006 (Grecki and Bienias 2006), 15 cases in a military base in Warsaw, including two deaths in 2007 (Kadhubowski et al. 2007), and one death from IMD in the Polish Military Contingent in Afghanistan (Konior and Korzeniewski 2016). The incidence of IMD in US military personnel, historically far above that in the general population, has decreased >90% since in early 1970s, when the first vaccine against meningococcal infections was introduced (Broderick et al. 2015).

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Over the last decade, incidences of IMD in the military and US general population have become equivalent (Broderick et al. 2012).

Meningococcal carriage studies conducted among European conscripts over the last several decades demonstrated high carriage incidence, often exceeding 30%. The studies by Tyski et al. (2001) carried out in the last decade of the 20<sup>th</sup> century revealed that *N. meningitidis* carriage incidence among Polish conscripts was 60%. However, in 2009 Poland suspended conscription and transformed its national army into a fully professional organization. Recruits have been replaced by professional soldiers, whose socio-demographic profile as well as the character of work/service they perform is different from that of young conscripts who used to serve on a 24/7 basis and were permanently assigned to a given military facility. The first study presenting the general prevalence of *N. meningitidis* carriage in professional soldiers in Poland was conducted in winter season of 2016. The overall carrier rate among 1246 soldiers from the Armoured Brigade amounted to 5.2% (single examination), with the serogroup B being predominant (Korzeniewski et al. 2017).

The aim of the present study was to estimate the actual *N. meningitidis* carriage rate in the military environment with identification of serogroups, genogroups, sequence types and clonal complexes of the isolates (including clonal complexes responsible for IMD) detected among Polish soldiers.

## Experimental

### Materials and Methods

**Study population.** A total of 1766 biological samples from nasopharyngeal mucosa were taken from 883 professional soldiers serving in the 10<sup>th</sup> Armoured Brigade in Świętoszów, Poland (the first sample was collected in 2015, the second in 2016 from the same individual). The soldiers were joining the study group during the winter season (January–March); all of the participants had to provide informed consent and complete a socio-demographic and behavioral questionnaire including information on their military rank, age, sex, place of residence, cigarette smoking, medications taken on a regular basis, and vaccinations received against meningococcal serogroups A, C, W-135, Y (18% of examined soldiers were vaccinated between 2008 and 2014). Only professional soldiers, men or women aged 21–59 years (median 31 years), with 1–26 years of military service, in a good health condition were involved in the study. Soldiers with any anatomical or pathological lesions in the nasopharynx, which could prevent the collection of samples were excluded from the study.

**Ethical Procedure.** A research task was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland (Decision No. 24/WIM/2014 of 18 Aug 2014).

**Identification of isolates.** The specimens obtained from the nasopharynx were transported to the microbiological laboratory in the Military Institute of Medicine, Warsaw, where they were plated onto the appropriate medium, i.e. Columbia Agar with 5% sheep blood and PoliVitex VCA3 and incubated under elevated CO<sub>2</sub> concentration at 37°C for 48 hours. After incubation, the colonies grown were macroscopically evaluated. Colonies morphologically similar to *N. meningitidis* phenotypes were isolated onto Columbia Agar with 5% sheep blood and were incubated under elevated CO<sub>2</sub> concentration at 37°C for 24 hours. The incubated pure colonies of bacteria were used to prepare Gram-stained preparations, which were then observed under a light microscope. Catalase and cytochrome oxidase tests were performed. All Gram-negative strains were identified based on their biochemical features using Vitek 2 NH card. The strains identified as *N. meningitidis* were stored frozen in a temperature of –20°C and then transported to the National Reference Center for the Diagnostics of Bacterial Infections of the Central Nervous System (KOROUN) in Warsaw, Poland for further diagnostics.

**Serogrouping.** The strains delivered to KOROUN were revived by placing them onto Columbia Agar medium; they were incubated in elevated CO<sub>2</sub> atmosphere at 37°C for 24 hours. Serogroups of all isolates were identified with a slide agglutination test using a set of primers in compliance with the manufacturer's instructions (detection of capsular antigens of *N. meningitidis*). The specific reagents covered the following serogroups: A, B, C, Y and W (Remel), E29 (Bio-Rad), X and Z (Becton Dickinson). Typing was performed on colonies isolated from the Mueller-Hinton agar medium following the identification of the strains.

**Genogrouping.** Chromosomal DNA was isolated from meningococcal isolates with Genomic DNA Prep Plus (A&A Biotechnology) following the manufacturer's recommendations. Genogroups were identified with the PCR assay using genogroup-specific oligonucleotide primers *orf-2*(A), *siaD*(C), *siaD*(W135) and *siaD*(Y) described by Taha (2000) and *siaD*(B) described by Guiver et al. (2000). The products were analyzed on the agarose gel. Genogroups A, B, C, E29, W, Y were identified.

**Sequencing.** Sequencing was performed on seven DNA fragments of the isolated strains. The chromatograms were analyzed using the Seqed 10.3 programme (Applied Biosystems) and the sequences obtained from the specimens were compared with the sequences available in the international database (<http://pubmlst.org/>

neisseria/). Individual loci were assigned to the right alleles. We identified the sequence type (ST) and clonal complex (CC) of the isolates which had a complete allelic profile (seven loci). In the case of isolates whose allelic profile was incomplete, only the clonal complex was identified.

**Statistics.** Statistical analysis was performed using STATISTICA PL version 12.0 and Microsoft Excel 2013. The quantitative variables were counted by arithmetic mean  $\pm$  SD or median and 95% confidence interval. The qualitative variables were introduced in the absolute or percentage terms. Significance of differences between two groups was processed with the t-Student or U Mann-Whitney test. In all the calculations *p*-value under 0.05 was considered statistically significant.

## Results

A nasopharyngeal culture sample was collected twice during winter seasons (in 2015 and then in 2016) from 883 soldiers. 76 of the subjects tested were found to be carriers (at least one of the single samples collected in 2015 and 2016 was positive for *N. meningitidis*); the carriers accounted for 8.6% of the study group. In 2015, genogroups were determined for 46 isolates: B (*n* = 33, 61.1%), E29 (*n* = 6, 11.1%), C (*n* = 4, 7.4%), Y (*n* = 2, 3.7%), W (*n* = 1, 1.9%). Eight isolates were non-groupable (NG, 14.8%) with the primers used. In 2016, genogroups were determined for 46 isolates: B (*n* = 24, 45.3%), E29 (*n* = 8, 15.1%), C (*n* = 6, 11.3%), Y (*n* = 6, 11.3%), W (*n* = 2, 3.8%); genogroups of 7 isolates (NG, 13.2%) could not be determined with the primers used (Fig. 1).

Based on the MLST analysis, 21 different sequence types were identified among the isolates analyzed. Although the analysis was repeated several times, the sequence type and clonal complex of one of the isolates

of genogroup B could not be determined in 2016. In both studies, 12 clonal complexes (CC) were identified among 62 *N. meningitidis* strains (25 isolates were identical to each other in both years, 12 were isolated once only). The most common clonal complexes included: 41/44CC (*n* = 6), 1136CC (*n* = 2), 53CC (*n* = 2). Only five strains which were found in both studies (in 2015 and again in 2016) and two strains isolated in 2016 were not assigned to any clonal complex. In six carriers the authors observed a change in the sequence type at the second collection and in four carriers also a change in the clonal complex. In one carrier genogroup B was found at the first collection and was replaced by genogroup Y at the second collection (Table I).

The mean age of *N. meningitidis* carriers in the study group was  $30.4 \pm 4.7$  years and of non-carriers it was  $32.1 \pm 5.3$  years. Carriers were significantly younger (*p* = 0.0083). No statistically significant differences were found between carriage prevalence and sex or residence. There were significantly more tobacco smokers among *N. meningitidis* carriers than in the non-carrier group (51.3% vs. 32.8%; *p* = 0.0012). The distribution of military ranks in both groups (carriers vs. non-carriers) was found to be statistically significant (*p* = 0.0088), the corps of privates being the largest. There were no statistically significant differences between carrier state and prior vaccination against *N. meningitidis* (Table II).

## Discussion

*N. meningitidis*, the etiological factor of invasive meningococcal disease (IMD), can be the cause of meningitis and/or sepsis. In most cases, meningococcal carriage does not lead to invasive disease, but is limited to asymptomatic carrier state which is normally found in 5–10% of the general population (Tzeng and

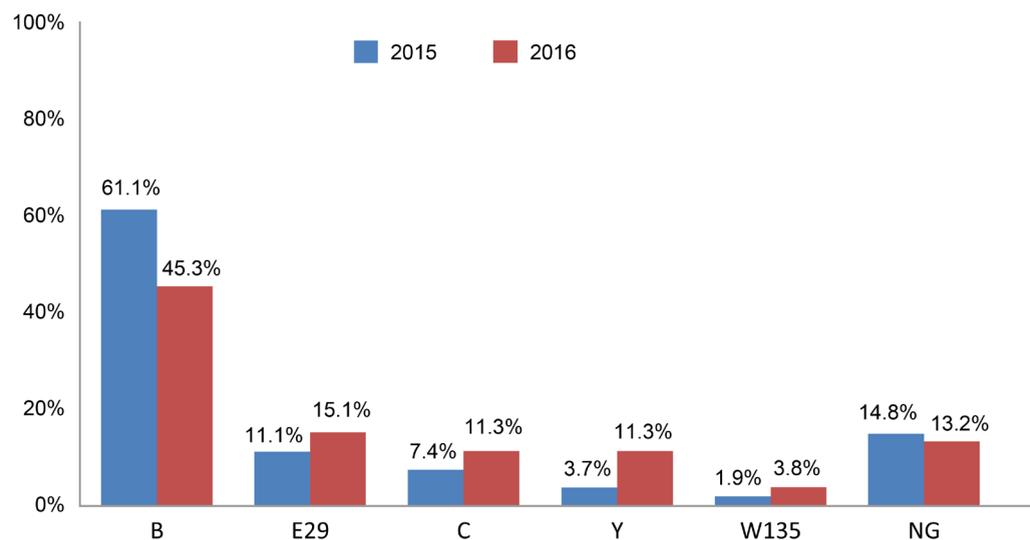


Fig. 1. Percentage distribution of *N. meningitidis* genogroups among Polish soldiers tested in years 2015–2016.

Table I  
Distribution of sequence types (ST), clonal complexes and genogroups  
of the *N. meningitidis* isolates tested in years 2015–2016.

ST 2015	Clonal complex 2015	ST 2016	Clonal complex 2016	
Genogrup B				
136	ST-41/44 complex/Lineage 3	136	ST-41/44 complex/Lineage 3	
136		136		
136		136		
1097		1097		
2840		2840		
11442		8107		ST-213 complex
1732		negative		negative
112		negative		negative
973		negative		negative
35		ST-35 complex		35
35	negative		negative	
35	negative		negative	
35	negative		negative	
162	ST-162 complex	162	ST-162 complex	
162		4509	–	
162		negative	negative	
162		negative	negative	
11433	ST-213 complex	11433	ST-213 complex	
213		negative	negative	
33	ST-32 complex	negative	negative	
33		negative	negative	
32		negative	negative	
11440	ST-1136 complex	11440	ST-1136 complex	
2126	ST-53 complex	2126	ST-53 complex	
198	ST-198 complex	198	ST-198 complex	
1001	ST-18 complex	11446	–	
11436	ST-364 complex	negative	negative	
5133	ST-103 complex	negative	negative	
9316		9316	–	
1572		1572	–	
11444		negative	negative	
11447		negative	negative	
negative	negative	35	ST-35 complex	
negative	negative	11436	ST-364 complex	
negative	negative	36	–	
negative	negative	12187	–	
negative	negative	9157	–	
negative	negative	12186	–	
negative	negative	120	–	
negative	negative	undetermined <sup>1</sup>	undetermined <sup>1</sup>	
Genogrup E29				
11434	ST-254 complex	11434	ST-254 complex	
11438		11438	–	
1138	ST-60 complex	1138	ST-60 complex	
11439		11439	–	
11439		11439	–	

Table I continued

ST 2015	Clonal complex 2015	ST 2016	Clonal complex 2016
11445	ST-60 complex	negative	negative
negative	negative	1157	ST-1157 complex
negative	negative	60	ST-60 complex
negative	negative	11438	ST-11438 complex
Genogrup C			
2433	ST-41/44 complex/Lineage 3	2433	ST-41/44 complex/Lineage 3
2003	–	2003	–
5238	–	negative	negative
5133	ST-103 complex	negative	negative
negative	negative	3346	ST-41/44 complex/Lineage 3
negative	negative	3346	
negative	negative	2433	
negative	negative	8108	ST-174 complex
Genogrup Y			
767	ST-167 complex	767	ST-167 complex
9316	–	9316	–
negative	negative	767	ST-167 complex
negative	negative	767	
negative	negative	3342	ST-865 complex
Genogrup W			
112	ST-41/44 complex/Lineage 3	112	ST-41/44 complex/Lineage 3
negative	negative	22	ST-22 complex
NG			
9268	ST-53 complex	9268	ST-53 complex
10159		53	
53		negative	negative
1136	ST-1136 complex	1136	ST-1136 complex
1136		negative	negative
198	ST-198 complex	negative	negative
198		negative	negative
11441	ST-364 complex	11441	ST-364 complex
negative	negative	4431	ST-41/44 complex/Lineage 3
negative	negative	3461	
negative	negative	9466	
Genogrup B / Genogrup Y <sup>2</sup>			
11437	ST-41/44 complex/Lineage 3	92	ST-92 complex

<sup>1</sup> Sequence type and clonal complex could not be determined

<sup>2</sup> Genogrup B was identified in the first collection and genogrup Y was detected in the second collection

Stephens 2000). In Poland, there have been reports of IMD cases in recent years; however, the actual carriage incidence of *N. meningitidis* among Polish residents has not been determined (Jafri et al. 2013). Many of Polish and European publications on the *N. meningitidis* carriage incidence are limited to military environment (Chapalain et al. 1992; Tyski et al. 2000; Korzeniewski et al. 2015). According to the research findings, meningococcal carriage is high among conscripted soldiers serving in European armies, whereas among profes-

sional soldiers it was found to be like the carriage rates observed in the general population (Korzeniewski et al. 2017). Meningococcal carriage studies conducted among military personnel also revealed that serogroup B was the most prevalent (Taha 2000). In Denmark, 43% of newly drafted recruits were found to be carriers of *N. meningitidis*, of whom 34% were transient carriers (this was associated with the colonization variability in the nasopharyngeal mucosa); 34% of the strains isolated from carriers belonged to serogroup B (Andersen et al.

Table II  
Socio-demographic variables between control group (non-carriers) and carriers of *N. meningitidis* in years 2015–2016.

Socio-demographic variables	Control group (non-carriers n = 807)	Carriers (n = 76)	p-value
<b>Age</b>			0.0083
Mean (SD)	32.1 (5.3)	30.4 (4.7)	
Range	21.0–59.0	23.0–45.0	
Median	31.0	30.0	
<b>Sex</b>			0.6490
Women	65 (8.1%)	5 (6.6%)	
Men	742 (91.9%)	71 (93.4%)	
<b>Place of residence</b>			0.3318
Rural area	322 (39.9%)	26 (34.2%)	
Urban area	485 (60.1%)	50 (65.8%)	
<b>Smoking cigarettes</b>			0.0012
Yes	265 (32.8%)	39 (51.3%)	
No	542 (67.2%)	37 (48.7%)	
<b>Respiratory tract infection</b>			0.1106
Yes	61 (7.6%)	2 (2.6%)	
No	746 (92.4%)	74 (97.4%)	
<b>Military rank</b>			0.0088
Private	479 (59.4%)	57 (75.0%)	
Non-commissioned officer	276 (34.2%)	19 (25.0%)	
Officer	52 (6.4%)	0 (0.0%)	
<b>Vaccination</b>			0.1567
Yes	148 (18.3%)	9 (11.8%)	
No	659 (81.7%)	67 (88.2%)	

1998). The study, which was conducted in six military camps in Bavaria, found that 32% of the soldiers tested were carriers of *N. meningitidis*, and serogroup B was identified in 42% of them (Claus et al. 2005). Exceptionally high rates of meningococcal carriage, over 70%, were observed among British and Norwegian recruits, in whom serogroup B was also the most commonly found (Fraser et al. 1973; Caugant et al. 1992). There have been very few meningococcal carriage surveillance studies outside Europe. One of such studies, with the participation of Iranian soldiers, demonstrated that the incidence of *N. meningitidis* carriage among newly drafted soldiers was 11%, but after 2 months of service it increased to 33%. The study also revealed that serogroup B was predominant among the recruits tested (Eslami-Nejad et al. 2005). A study involving Iranian recruits which was performed a decade later demonstrated a lower carriage rate (8%), with a dominance of serogroup C (Ataee et al. 2016). A research study into Polish recruits carried out between 1998 and 1999 demonstrated meningococcal carriage rate at 31%, with the predominance of serogroup B (Tyski et al. 1998; Tyski et al. 2000). The first meningococcal

carriage study in Polish professional soldiers (n = 559), which was conducted in 2013, showed the incidence of *N. meningitidis* carriage at 5.7% (Korzeniewski et al. 2015). A significant factor contributing to a reduction in meningococcal carriage incidence in the military environment was a change in the character of military service after the Polish Armed Forces have been transformed into a professional organization. Recruits used to serve on a 24/7 basis, they were permanently accommodated in the barracks and had all their meals in military dining facilities. Professional soldiers, on the other hand, typically work 8 hours a day and they are accommodated outside the military facilities (professional military service has changed to a regular job, with risk factors similar to those observed in the civilian environment). Another factor contributing to decreasing of meningococcal carriage prevalence was raise of the age of soldiers (19–20 year old recruits vs. professional privates who usually begin their military career at the age of 25–30 years old) (Korzeniewski et al. 2017). Meningococcal vaccination may also have a positive impact on reducing carriage prevalence of *N. meningitidis* and lowering the number of new IMD

cases; however, its effectiveness varies depending on the type of vaccine used, with conjugate vaccines being the most effective (Decker 2016). It needs to be pointed out that meningococcal vaccination does not completely prevent individuals from acquiring an infection, e.g. cases of the disease were reported among French soldiers, despite the use of immunoprophylaxis (Duron et al. 2016). The studies with the participation of Polish military personnel demonstrated that meningococcal carriage incidence was lower in older soldiers; the results are consistent with the available research findings (Taha 2000; Tzeng and Stephens 2000; Caugant et al. 2009; Soriano-Gabarro et al. 2011). Another factor associated with an increased risk for meningococcal carriage was found to be tobacco smoking (Blackwell et al. 1990; Caugant et al. 1992; Korzeniewski et al. 2017). The studies by Caugant et al. (1988) demonstrated that hypervirulent *N. meningitidis* strains rarely colonize the nasopharyngeal mucosa of carriers. The present study, and especially the application of sequencing methods, has proved to be useful in determining the genetic relationship among different strains and estimating the risk for IMD among carriers. The study involving Polish soldiers found virulent strains in 15.8% of carriers (n = 76) and 1.4% of the whole study group (n = 883). Stephens (1999) estimated that the risk of developing IMD by people living in closed environments is 1–1.5%. The incidence of specific clonal complexes is associated with the dominance of individual serogroups (Trotter et al. 2007; Jandova et al. 2016). In Poland serogroup B, comprising ST-32CC, ST-18CC, ST-41/44CC, ST-213CC and ST-269, was found to be predominant. Serogroup C covers ST-103CC, ST-41/44CC and ST-11CC. Serogroup W is represented by ST-22CC, and Y by ST-22CC, ST-23CC, ST-92CC, ST-167CC (Skoczyńska et al. 2013; Waśko et al. 2015). The present study confirmed that serogroup B was predominant among carriers; it was mostly represented by ST-41/44CC. Similar sequencing results were observed in Greece in a population of military recruits (Tryfinopoulou et al. 2016). Hypervirulent clonal complexes ST-213CC, ST-213CC, which have been observed in Poland, were also found in the study participants. Among serogroup C isolates the most common were clonal complexes ST-41/44CC and ST-103CC. Serogroup C was the third most common, after serogroup B and E29. Serogroup E29 was represented by the clonal complex ST-254CC and ST-60CC. At present, there is no data available concerning IMD morbidity from this particular serogroup (PubMLST, 2018). Serogroup Y covered the virulent clonal complex ST-167CC and once – ST-92CC. In this study, among carriers of serogroup W the clonal complex ST-22CC was identified, which was listed by the National Reference Center for the Diagnostics of Bacterial Infections of Central

Nervous System in Warsaw as the strain responsible for causing IMD cases in Poland (Skoczyńska et al. 2013). In this current study, the hyperepidemic and hypervirulent strain ST-11 has not been identified. This strain is rarely found in carriers, its carriage is usually transient as the strain is rapidly transmitted to other hosts (Moxon and Jansen 2005). In general, there are significant differences in the incidence of the clonal complexes causing IMD and those responsible for meningococcal carriage (Caugant et al. 2007). Studies aimed at detecting pathogenic strains have been found to be valuable in determination of transmission routes, the distribution of individual genogroups and the epidemiological variability of *N. meningitidis*. While the surveillance of circulating strains is useful in selecting the best immunoprophylaxis (Millar et al. 2016).

## Conclusions

The overall carriage rates and types of serogroups of *N. meningitidis* among Polish professional soldiers were similar to the carriage reported in the general population. Clonal complexes responsible for IMD were detected in 15.8% of carriers and 1.4% of the whole study participants. Meningococcal carriage in professional soldiers was largely associated with a younger age, low military rank and frequent tobacco smoking.

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## Conflict of interest

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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