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VKORC1-based resistance to anticoagulant rodenticides widespread in Finnish house mice but not in brown rats

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ABSTRACT

Anticoagulant rodenticide (AR) resistance is widespread across Europe, but its prevalence has not been previously studied in Finland. We sampled 48 house mice (*Mus musculus*) and 48 brown rat (*Rattus norvegicus*) in farms and urban locations from southern Finland and here we report the identification of *Vkorc1* polymorphisms linked to AR resistance.

Two thirds of mice carried *Vkorc1* polymorphisms. The most common was the type Y139C (resistance to first-generation ARs, bromadiolone and difenacoum), whereas the type L128S (resistance to FGARs and bromadiolone) was much less abundant. Only two rats tested positive, both with a rare type R33P (resistance probably to warfarin). No clear patterns based on subregion, sex and urban/rural division were seen.

We suggest that the type Y139C polymorphism should be taken into account when planning control actions targeting house mice in Finland. The low number of AR resistant rats found suggests a low prevalence of resistance in rats. To fully understand the state of AR resistance in Finland, additional studies on yellow-necked mouse (*Apodemus flavicollis*) and bank voles (*Myodes glareolus*) are needed.

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Commensal rodents; evolution; integrated pest management; urban; rural

Introduction

The most common chemical method for rodent control worldwide is the use of anticoagulant rodenticides (ARs), which block the vitamin K cycle and cause death by hemorrhage (Laakso, Suomalainen, and Koivisto 2010; Murphy 2018). Although ARs are perceived as an easy and cost-effective way to control rodents, there are several problems related to their use (Berny 2011). First, both non-target wildlife and domestic animals are exposed unintentionally to ARs either through consumption of baits meant for rodents or by consumption of poisoned rodents (Lefebvre et al. 2017; Koivisto et al. 2018). Second, ARs cause suffering in target rodents which raise animal welfare issues (Baker et al. 2022). Third, sublethal dosing of rodenticides could increase zoonotic pathogen prevalence in rodents, thus increasing human risks for infectious diseases (Murray and Sánchez 2021). Fourth, because chemical control of rodents relies on ARs, many distinct resistant strains, especially in the brown rat (*Rattus norvegicus*) and the house mouse (*Mus musculus*) have emerged, e.g., in France, Germany, and the UK (Pelz et al. 2005; Berny 2011; McGee, Borstein, et al. 2020).

Anticoagulants can be divided into first- and second-generation substances (McGee, Borstein, et al. 2020). The first-generation rodenticides (FGARs) were introduced for pest control in the 1940s and some of them, like warfarin, are still in use. First generation rodenticides are less persistent, require multiple doses to be fatal and are physiologially eliminated within few days. Second-generation anticoagulant rodenticides (SGARs), which are toxic at a much lower dose than FGARs (IPCS 1995), were developed after rodents started to exhibit resistance to FGARs. The SGAR includes bromadiolone, difenacoum, and the stronger substances brodifacoum, difethialone and flocoumafen.

The origin of AR resistance has been identified to specific genetic mutations, namely polymorphism in the *Vkorc1* gene, which codes an enzyme involved in the catalytic recycling of vitamin K (Li et al. 2004; Rost et al. 2004). Mutations within the gene render rodenticides ineffective. Anticoagulant resistance in brown rats and house mice has been found to be linked to single nucleotide polymorphisms (SNPs) in the coding region of *Vkorc1*. *Vkorc1* resistance is co-dominant, meaning that heterozygous

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individuals are more susceptible to rodenticides than homozygous individuals, but less susceptible than wild type rodents without *Vkorc1* polymorphisms (Grandemange et al. 2009; Berny et al. 2018; Baxter et al. 2022). As resistance to ARs developed so quickly after the introduction of rodenticides, it has been suggested that the resistance mediating *Vkorc1* polymorphisms arise from standing genetic variation (Hermisson and Pennings 2005; Hawkins et al. 2019; McGee, Borstein, et al. 2020). Whereas AR resistance is costly to the animals in terms of lower reproductive success, it is beneficial under the use of anticoagulants. Thus, AR resistance have been selected for and its prevalence has increased (Lefebvre et al. 2016).

Indeed, during the same decade that FGARs were introduced for rodent control, the first warfarin resistant rodent strains were discovered (Berny et al. 2018). Following this discovery, new and stronger SGARs were introduced to overcome the resistance problem. SGARs eliminate considerably slower than FGARs and require only a single dose to be lethal (IPCS 1995). Since all ARs have similar chemical structures and a similar mode of action, resistance to the first generation anticoagulants brought with it a measure of cross-resistance to the second-generation compounds and soon also populations with reduced susceptibility to the more potent SGARs began to appear (Greaves et al. 1982). Nowadays, AR resistant strains of commensal rodents such as brown rats and house mice occur throughout the world (Boyle 1960; Berny et al. 2018). In many European countries, a high prevalence of *Vkorc1* polymorphism has been observed in wild-caught house mice (e.g. Pelz et al. 2012; Goulois et al. 2017; McGee, Borstein, et al. 2020). For example, in Germany over 90% of the house mice tested carried genetic resistance types (RRAG 2012). At least 10 and 15 different types of polymorphism have been found in the brown rat and the house mouse in Europe, respectively.

Resistant rodent populations can only be controlled with the most persistent and toxic ARs but this leads to a higher risk for non-target species and the environment. In order to have the least impact on the environment, these most toxic agents should only be used in cases of resistance. Thus, knowing if resistant strains exist and if they do, which type(s) they are, is useful knowledge not only for effective rodent control in practice but also for reducing environmental risk.

In Finland, previous sporadic evidence on the occurrence of AR resistant house mouse strains has been found with questionnaires (Myllymäki 1995), but no systematic screening of rodents has been conducted. As the use of ARs is common in Finland and resistance occurs in many European countries there was a need to examine the resistance situation also in Finland. Here we report on results of a prevalence study of *Vkorc1* gene polymorphism on exons 1-3 which have been linked to AR resistance in populations of brown rat and house mouse in Finland.

Material and methods

Sample collection

We assessed the prevalence of AR resistance (*Vkorc1* gene polymorphisms) in populations of two species of commensal rodents (house mouse and brown rat) in Finland. We collected tissue samples from 96 animals (48 mice and 48 rats) in both rural environment (farming areas; 45 mice and 20 rats) and urban environment (i.e. in the cities of Helsinki, Turku and Pori; 3 mice and 28 rats) in south western Finland (Figure 1) from December 2016 to January 2019. The brown rat is a commonly controlled species of commensal rodents in Finland in urban and semi-urban areas and on farms. The house mouse is a less frequent pest rodent and its current distribution is limited to South-Western Finland where it occurs in both old buildings in urban areas and in farmhouses. We did not include the yellow-necked mouse (*Apodemus flavicollis*) in this study, although it is a common pest in both urban and rural areas of southern Finland, as pilot sequencing was unable

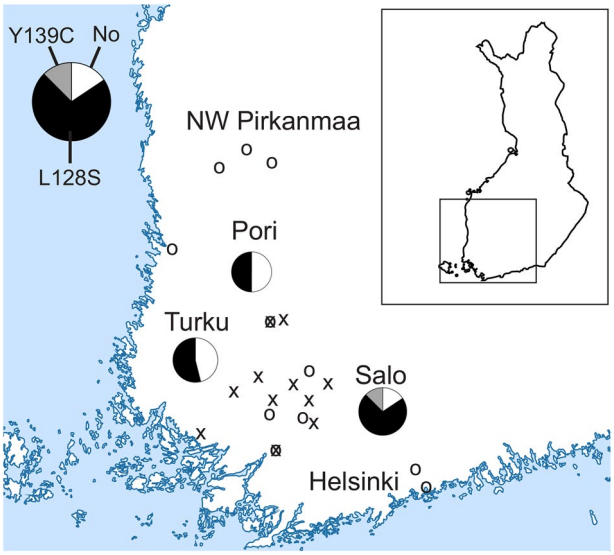

Figure 1. The map of Southern Finland with five subregions highlighted and sampling sites marked with X (mice) and O (rats) (See also Table 2). The pie charts represent the prevalence of AR resistance related polymorphisms in three subregions with sampled mice: Pori and Turku has a prevalence of 50% of L128S, whereas Salo had a prevalence of 73% of Y139C and L128S. Subregions were formed by combining neighboring sub-regional units (based on EU LAU1 statistical areas).

to identify relevant *Vkorc1*-polymorphism from samples of this species.

We collected samples from research projects on urban rats (Helsinki Urban Rat Project) and on the role of rodents as pests in cattle and pig farms and got additional samples from pest control operators (PCOs). We used standard lethal snap traps and PCOs used electronic traps in line with Finnish animal welfare legislation. Trapped individuals were frozen whole immediately at −20 °C for dissection and analysis. Most sampling was carried out in autumn, winter and spring months when ambient temperatures were < 10 °C, which minimized potential degradation of DNA material. In the laboratory, we removed tail snippets from rodents as tissue samples while still frozen, stored them in 70% (v/v) ethanol in microtubes and shipped on dry ice to Germany (Julius-Kühn-Institute, Münster) for sequencing. PCOs followed all occupational health regulations related to rodents and to rodenticide use. The tail snippet removal was done under laminary flow in BSL-2 laboratory in compliance to laboratory safety procedures.

No detailed knowledge is available regarding the type of anticoagulant used, the longevity of application or the exposure history of rodents to them in these sites. Nonetheless, all farm sites (according to PCOs and farmers) have applied anticoagulant rodenticides. Typically, continuous baiting is used on farms, while PCOs operating in urban sites more often employ pulsed baiting protocols, adhering to the instructions of use of the rodenticides and the code of good practice (https://tukes.fi/tietoa-tukesista/materiaalit/biosidit/jyrsijatorjunnan-hyvan-kaytannon-ohje). A questionnaire from 2014 on PCOs indicated that approximately 50 per cent of the rodenticide used was bromadiolone while difenacoum and coumatetralyl were approximately 25 per cent of the total use each (Koivisto et al. 2016).

The raw data from our study is deposited in Figshare (doi: 10.6084/m9.figshare.22347271).

Genetic sequencing

We took subsamples of tail tissue and extracted DNA using Gentra Puregene Mouse Tail Kit (Qiagen) at the Julius Kühn Institute. The three exons of the *Vkorc1* were amplified by PCR using established inner and outer primers (Rost et al. 2004, Pelz et al. 2005, 2012). Amplifications were carried out in about 26 µl reaction volumes including DNA, 10× Buffer, 2,22 ul dNTPs, 1 µl of each primer, and 0,3 µl Taq polymerase. The amplification was performed at 95 °C, for 3 min, followed by 32 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 3 min. Subsequent molecular work was performed at Eurofins Genomics Europe Sequencing GmbH. All sequences were generated using BigDye terminator chemistry (version 3.1) (Thermo Fisher Scientific, Waltham, MA USA). For sequencing reactions peqStar 96 HPL (PEQLAB Biotechnologie GMBH, Erlangen, Germany) and/or GeneTouch (Biozym Scientific GmbH, Oldendorf, Germany), and/or Biometra Tadvanced (Biometra GmbH, Göttingen, Germany) thermal cyclers were used. Sequencing reaction cleanup was done on a Hamilton Starlet robotic workstation (Hamilton Robotics GmbH, Martinsried, Germany) by gel-filtration through a hydrated Sephadex matrix filled into appropriate 96-well filter plates followed by a subsequent centrifugation step. Finally, all reactions were run on ABI3730xl capillary sequencers equipped with 50 cm capillaries and POP7 polymer (Thermo Fisher Scientific, Waltham, MA USA).

Sequencing data were called using the original Sequencing Analysis Software 6 (Applied Biosystems) including the KB-basecaller (Thermo Fisher Scientific, Waltham, MA USA), which assigns quality values to all called bases similar to PHRED quality score (Ewing and Green 1998). Additional basecalling was performed using the PeakTrace basecaller from Nucleics Pty Ltd (Woollahra, AUS) to improve the single peak resolution and quality values and therefore increase the reading lengths. Assembly was built using the Staden Software Package (Roger Staden, LMB, Cambridge, UK/Pregap4 version 1.6, Gap4 version 4.11.2). For quality, a sliding window of 10 nucleotides was clipped to average the confidence.

The assembly, i.e. generation of a consensus sequence from aligning single sequences (forward/reverse) and clipping of low-quality areas as well as clipping of intronic parts of the amplicon, was performed as a normal shotgun assembly using the quality clipped reads with minimum initial match 20, maximum pads (gaps arising in one read due to the alignment with others) per reads 25, maximum percent mismatch 5.00. Potential joins were searched with the find internal joins function using minimum overlap 20 and maximum percent mismatch 30.00. Plausibility was checked manually on these additional joins. Manual editing steps were necessary to resolve base caller errors. The comparison was performed to the reference sequences (Genbank number NM 178 600 for house mice and *Vkorc1* GenBank accession no. NM 203 335 for brown rat) as an assembly, using the same parameters as for the single read assembly.

Table 1. The prevalence of identified anticoagulant resistance related *Vkorc1* polymorphisms by species and sex of samples. +/+ refers to homozygous and +/- to heterozygous individuals.

				Y139C		L128S		R33P
Species	Sex	Sample size	Any polymorphism	+/+	+/-	+/+	+/-	+/-
Mus musculus	Females	16	50 % (8)	13 % (2)	38 % (6)	0 % (0)	6 % (1)	0 % (0)
	Males	28	71 % (20)	21 % (6)	39 % (11)	7 % (2)	4 % (1)	0 % (0)
	NA	4	75 % (3)	unknown zygosity 75% (3)		0 % (0)	0 % (0)	0 % (0)
	Total	48	65 % (31)	18 % (8)	35 % (17)	4 % (2)	4 % (2)	0 % (0)
Rattus norvegicus	Females	20	10 % (2)	0 % (0)	0 % (0)	0 % (0)	0 % (0)	10 % (2)
	Males	24	0 % (0)	0 % (0)	0 % (0)	0 % (0)	0 % (0)	0 % (0)
	NA	4	0 % (0)	0 % (0)	0 % (0)	0 % (0)	0 % (0)	0 % (0)
	Total	48	4 % (2)	0 % (0)	0 % (0)	0 % (0)	0 % (0)	4 % (2)

Table 2. Spatial variation in sample collection and assessed prevalence of anticoagulant resistance based on *Vkorc1* polymorphisms.

Subregion	Population	Type	Rats	Mice	AR Prevalence
Helsinki	Helsinki 1-7	Urban	24	0	0 %
	Helsinki 8	Rural	4	0	0 %
	Vantaa	Urban	2	0	0 %
Salo	Salo	Rural	1	6	100 % in mice 0% in rats
	Somero 1	Rural	0	6	50 %
	Somero 2	Rural	0	5	60 %
	Somero 3	Rural	4	0	0 %
	Forssa	Rural	2	0	0 %
	Jokioinen	Rural	0	6	100 %
	Ypäjä	Rural	1	0	0 %
Turku	Turku	Urban	0	3	100 %
	Loimaa	Rural	0	4	25 %
	Koski Tl 1	Rural	3	0	66 %
	Koski Tl 2	Rural	0	6	50 %
	Pöytyä	Rural	1	0	0 %
Pori	Pori	Urban	1	0	0 %
	Punkalaidun 1	Rural	0	6	33 %
	Punkalaidun 2	Rural	1	6	66 % in mice 0% in rats
NW Pirkanmaa	Parkano	Rural	2	0	0 %
	Honkajoki	Rural	1	0	0 %
	Karvia	Rural	1	0	0 %

Notes: Populations are considered as distinct sampling sites within mentioned municipalities. Subregions were formed by combining neighboring sub-regional units (based on EU LAU1 statistical areas).

Results

A *Vkorc1* polymorphism was found in 31 (65%) house mouse samples (Table 1). 90% of positive house mice had a type Y139C while 13% had a type L128S polymorphism. One individual had both polymorphisms. In rats, on the other hand, only two samples out of 48 (4%) were positive, both with a type R33P polymorphism (Table 1).

Prevalence of the *Vkorc1* polymorphisms varied between locations (between 0% and 100% in *Mus musculus*): for example, in a farm in Jokioinen and other one in Salo, both had 6 sampled mice and all where positive for Y139C polymorphism (Table 2). Both rats positive for R33P polymorphism were sampled in a farm in Koski TL and all four mice positive for L128S polymorphism were from a farm in Salo. In general, there was a lack of overlap in house mice and rat populations: only two locations, farms in Punkalaidun and Salo had both. In each of the subregions where mice were sampled, there were Y139C polymorphisms ranging from 40 to 100%. The majority (62%) of rural mice were positive for any polymorphism whereas only a small minority (12%) of rats were.

The type Y139C polymorphism was heterozygous in 17 and homozygous in 8 mice (and for 3 individuals zygosity could not be determined), whereas the type L128S was heterozygous in 2 and homozygous in 2 mice. Both rats with type R33P polymorphism were heterozygous.

Discussion

We found evidence of AR resistance (*Vkorc1* gene polymorphism) in populations of two commensal rodent species, house mouse and brown rat, in Finland. In mice, type Y139C was quite common, but a few individuals with type L128S were also found. In rats, however, only two sampled individuals tested positive for a *Vkorc1* polymorphism and none of the types common in other parts of Europe were found. The type found in our rat samples was of a rare type, R33P, reported earlier from the UK (Rost et al. 2009) and Japan (Tanaka et al. 2013).

The types and total prevalence of *Vkorc1* polymorphism in house mice exon 3 where similar to other studies conducted in Europe. For example, our 65% compares to 73% prevalence in France (Goulois et al. 2017), 94% in UK (Buckle et al. 2022), 84% in Ireland (Mooney et al. 2018), 89% in Germany (Pelz et al. 2012) and 94% in the Moscow region (Maltsev et al. 2022). In contrast, the prevalence of exon 3 mutations have been low for example in Pontine Island in Italy (3.3%; Iannucci et al. 2019) and in Barcelona (8% for Y139C and 12% for L128S, though mutations were numerous in exons 1 and 2; Ruiz-López et al. 2022).

Type Y139C causes resistance against FGARs and the SGARs bromadiolone and difenacoum (Buckle and Prescott 2012). Brodifacoum has been found to be effective against house mice with this mutation (RRAG 2012). Most individuals in our study were heterozygous and as homozygosity indicates a high degree of selection for anticoagulant resistance, our results suggest that the use of rodenticides has not introduced a strong selection. Type L128S was found in a few individuals, but in only one of the study sites. One individual tested positive for both types. Double polymorphism, i.e. mice carrying two resistance types of the *Vkorc1* gene, are associated with decreased susceptibility to all anticoagulant rodenticides based on measures of enzyme activity (Goulois et al. 2017), although the most toxic agents can be effective in practice following feeding tests (Blažić et al. 2023).

The observed AR prevalence in rats (4%) is very low compared to what has been observed in many other countries, though low prevalence is not a unique situation. For example, the prevalence in France was 56% (Desvars-Larrive et al. 2017), 72% in Belgium, 13% in Italy, whereas it was 0% in Ireland (Mooney et al. 2018) Africa, South America and USA (Iacucci et al. 2018). It should be noted that not all AR resistance studies look at all three exons as some concentrate on the exon 3, where most widespread mutations are situated. There is a distinct lack of longitudinal studies, but a study from Netherlands suggests that rat AR prevalence has decreased from 2013 to 2021 (Krijger et al. 2023). The Y139C and Y139F types prevail in Europe (Iacucci et al. 2018). In our samples, however, no Y139C, nor any of the other commonly observed types, were detected. Instead, we found two individuals with the R33P polymorphism of *Vkorc1* exon 1, which has been so far found to confer resistance to warfarin in the laboratory (Tanaka et al. 2013). In New Zealand (Cowan et al. 2017) and in Martinique (Marquez et al. 2019) *Rattus* species had novel polymorphism types but none of the most common ones. Iacucci et al. (2018) did not find the common types in their study conducted on rats in Italy but instead they found a new type of *Vkorc1* gene polymorphism, I123S. It is not known if this type confers anticoagulant resistance in rats, but since it is located in the same position as type I123N, responsible for anticoagulant resistance in humans (Oldenburg et al. 2014), Iacucci et al. (2018) suggest that it could be involved in resistance development in rats too. Iacucci et al. (2018) propose that the total absence in the Italian samples of known polymorphisms associated with resistance may be attributable to the different pattern of use of anticoagulant rodenticides compared to that of many other European countries. In United Kingdom, for example, a much stricter regulation does not allow the use of more potent SGARs (i.e. brodifacoum and flocoumafen) in outdoor areas. According to Iacucci et al. (2018) in Italy, there is often routine use of the most potent anticoagulants.

Limitations of the study

The sample size per population is limited in our study which precludes the possibility of carrying out robust statistical analysis. Similarly, the comparison between house mice and rats is made more difficult as there was a lack of sympatric populations. This is partly due to short trapping periods, which are not optimal for catching neophobic species, such as brown rats. Runge et al. (2013) studied the distribution of rodenticide resistance in urban and rural rat populations in Germany and found homozygous polymorphism in urban areas only. They hypothesized that these results could reflect differing selection pressures due to differences in rodent control practices between urban and rural areas, in that AR use might be more intensive in the urban sites. In Finland, rodenticides are advised to be used for a maximum of six weeks, whereas at the sites where food is handled, including farms, some deviation is allowed. Comparison between urban and rural areas is difficult, though: in our study, all urban mice were positive for Y139C polymorphism whereas the only two rats positive for R33P polymorphism were rural. Due to low prevalence of AR resistance and uneven distributions of rat and mouse samples between urban and rural areas, we are unable to address this topic in this study. We are unsure of the origin of the R33P type present in our samples. The single location in which the resistant rats were trapped was a farm in southwestern Finland, which largely rules out the possibility of an incidental introduction of individuals (*via,* e.g., ships) carrying an exotic resistance type. The observed R33P polymorphism found in our samples could be a result of

a spontaneous polymorphism occurring in our study population. It might also be possible that exon 1 situated type R33P could be globally more common than currently observed as the testing of *Vkorc1* polymorphism has been primarily focused on searching for the most common polymorphisms on exon 3, as their presence plays a more important role in terms of pest management.

Longitudinal sampling on the same sites would allow an understanding of how persistent AR resistance polymorphism are in mice and rat populations. Indeed, AR resistance is costly to the animals (Smith et al. 1991; Kohn et al. 2008; Jacob et al. 2012) and thus, it seems likely that when rodenticide use ceases, the selection pressure should turn against AR resistance. However, this did not occur in every context (Smith et al. 1993; Heiberg et al. 2006). In a large-scale study in the UK (Haniza et al. 2015), the rat population does not seem to be in equilibrium, but rather under constant gene flux probably due to drift caused by small population sizes. This poses the question of how strong the selective pressures can be in either direction in isolated small populations, such as Finnish farm populations. More research is needed specifically to address factors leading to AR resistance in house mice under varying environmental conditions and regimes of AR application. In addition, to fully understand the state of resistance in Finland, studies on effectiveness and resistance ought to be performed on two widely occurring and sporadically commensal rodent species, the yellow-necked mouse (*Apodemus flavicollis*) and the bank vole (*Myodes glareolus*).

Recommendations for commensal rodent management

Our results suggest that the type Y139C polymorphism of *Vkorc1* is common in Finnish house mouse populations and this knowledge should be considered when planning professional control actions against the species. Mechanical and/or electronic traps should be favoured for the professional control of house mouse and if anticoagulant rodenticides are used, it would be preferable to use pulsed baiting only with the most potent active substances (brodifacoum, difethialone, flocoumafen; Blažić et al. 2018). These recommendations are similar to guidelines in the UK (RRAG 2012). Private use is not considered as a major risk for the development and spread of resistance in Finland as the use of ARs against rats has been disallowed since 2018. Private use is only allowed against mice indoors with prefilled bait boxes, and the public can only use the anticoagulants for which no resistance has been observed yet. In addition, Finnish Safety and Chemicals Agency Tukes recommends the use of mechanical traps as a priority for the control of mice in private houses and cottages.

We sampled rats in the areas with presumed highest rat populations (i.e., the southern intensive farming area and the Helsinki capital region which is the biggest metropolitan area) and found a low number of AR resistant rats and the absence of the most common *Vkorc1* polymorphism. This suggests a low prevalence of resistance in rats in Finland. This seems to indicate that current pest management practices work well in managing AR resistance occurrence in rat populations and FGAR use does not pose a specific threat for the evolution of AR resistance.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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