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Emergence of the L phenotype in Group B Streptococci in the South of Ireland

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SUMMARY

Group B Streptococcal isolates ($n = 235$) from the South of Ireland were characterised by serotyping, antimicrobial susceptibility and determination of the phenotypic and genotypic mechanisms of resistance. Resistance to erythromycin and clindamycin was observed in 21·3% and 20·4% of the total population, respectively. The c-MLSB phenotype was the most common phenotype detected (62%), with *ermB* being the predominant genetic determinant, present in 84% of resistant isolates. The rare L phenotype was observed in 2·9% ($n = 7$) of isolates, four of which harboured the *lsaC* gene responsible for clindamycin resistance. Serotypes Ia, III and II were the most common amongst the entire study population (28·1%, 24·7% and 14%, respectively). Four of the seven L phenotype isolates were serotype III and two of these strains were confirmed as the hypervirulent clone, ST-17 and harboured the *hvgA* gene. This is the first documented case of the L phenotype in Ireland to date and the study findings emphasise the need for continued monitoring of antibiotic resistance and serotype distribution in GBS isolates from Ireland.

Q3 **Key words:** Microbiology, ~~*Staphylococcus aureus*~~, Streptococcal infections, surveillance.

INTRODUCTION

Group B Streptococcus (GBS), or *Streptococcus agalactiae*, frequently colonise the genitourinary tract in pregnant women and can cause invasive disease in neonates, with fatality rates as high as 10% [1] and up to 25% of neonates that survive infection may suffer severe neurological impairment [2]. In recent years, GBS has also emerged as an important pathogen in non-pregnant adults, particularly the elderly and those with underlying diseases such as diabetes [3].

Ten serotypes of GBS exist but studies have shown that serotypes Ia, Ib, II, III and V occur more

frequently and are largely responsible for invasive disease [1, 3]. GBS are further classified by sequence types (ST) based on multilocus sequence typing (MLST), with ST-1, ST-10, ST-17, ST-19 and ST-23 being more frequently associated with invasive infection [1, 4, 5]. The 'hypervirulent' clone, ST-17 is often linked with serotype III strains in invasive neonatal disease [6] but recent studies have also shown an association between serotype V, ST-1 strains and invasive disease in non-pregnant adults [7].

Current practice in Ireland for prevention and control of neonatal GBS disease involves a risk-based assessment, as per the Royal College of Obstetricians and Gynaecologists guidelines, with intrapartum antibiotic prophylaxis (IAP) administered only to those considered at significant risk [8]. However, the use of IAP has raised concerns regarding the potential for

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development of antibiotic resistance in GBS isolates [9]. Penicillin remains the first line agent for IAP and treatment of GBS infections [10], however, isolates with reduced susceptibility have been confirmed in some countries [11]. When a patient presents with beta-lactam allergies alternative treatment options are required, with lincosamides (clindamycin) and macrolides (erythromycin) considered the next choice of treatment, but resistance levels to these antibiotics continue to rise [3, 10].

Macrolide resistance is mediated by two mechanisms, namely ribosomal modification by methylation, encoded by *erm* genes, being the most common, and efflux pumps. In streptococci, *ermB* and *ermTR* genes confer resistance to erythromycin, and constitutive or inducible resistance to clindamycin and streptogramin B antibiotics (c- or i-MLS_B phenotypes respectively). Efflux pumps are encoded by *mef* genes and restrict resistance to 14- and 15-membered macrolides only (e.g. erythromycin), which is associated with the M phenotype [12, 13].

Resistance to clindamycin occurs primarily through *erm* genes, however, isolates displaying resistance to clindamycin but susceptibility to erythromycin (L phenotype) also occur. This unusual mechanism of lincosamide-specific resistance occurs by enzymatic inactivation of the drug by nucleotidyl-transferases and is mediated by *lnu* genes [14]. Furthermore, this type of lincosamide resistance may also occur with cross resistance to streptogramins A and pleuromutins, designated LSA and LSAP phenotypes, which are mediated by the ABC transporter, *lsaC* [15]. A retrospective study conducted at the Centers for Disease Control and Prevention in 2016 confirmed that while the incidence of these rare phenotypes is low, their rates are increasing and thus should be actively monitored [16].

This study aimed to provide current epidemiological data on serotype distribution and antibiotic resistance rates among colonising and invasive GBS isolates in the South of Ireland, and to investigate the genetic basis for antibiotic resistance and the emergence of new strain phenotypes.

MATERIALS AND METHODS

Bacterial isolates

A total of 235 GBS isolates collected over a 6-year period (2010–2016) was included in this study. Colonising isolates ($n = 201$) were collected from the

Microbiology Department at Cork University Hospital, obtained from high vaginal swabs (HVS) of women between the ages of 16 and 45 years; one colonising GBS isolate was recovered from a placental swab (PS) was provided by the National Maternity Hospital, Holles Street, Dublin. Invasive GBS isolates ($n = 34$) were collected from blood culture samples from Cork University Hospital and University Hospital Limerick. Isolates were cultured on Columbia Blood Agar (Fannin Ltd, Dublin, Ireland) and incubated at 37 °C for 24 h. Isolates were identified as GBS by beta-haemolysis on blood agar, a positive Gram stain and a negative catalase test and identified to species level by MALDI-TOF MS (Bruker Daltonics, Leipzig, Germany).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates was assessed by a disk diffusion method, according to EUCAST guidelines (http://www.eucast.org/ast_of_bacteria/) and all were tested for susceptibility to penicillin, erythromycin, clindamycin, rifampicin and tetracycline. Inducible resistance to clindamycin was determined using the double-disk diffusion method (D test) (EUCAST) and resistance phenotypes were characterised as constitutive (cMLS_B), inducible (iMLS_B), M phenotype (erythromycin resistant, clindamycin sensitive) or L phenotype (erythromycin sensitive, clindamycin resistant). Minimum inhibitory concentration (MIC) testing of erythromycin and clindamycin was performed using MIC Evaluator strips (Oxoid, Basingstoke, UK) and interpreted according to EUCAST breakpoints (http://www.eucast.org/clinical_breakpoints/).

DNA extraction from bacterial cultures

DNA was extracted using the PureLink Genomic DNA Kit (Invitrogen, ThermoFisher Scientific, California, USA) with minor modifications, which included a mechanical lysis of GBS by bead-beating using the MagNA DNA lyser (Roche, Indiana, USA). Briefly, an overnight culture of GBS in Brain Heart Infusion broth (BHI, Cruinn Diagnostics, Dublin, Ireland) was centrifuged at 6500 rpm for 10 min. The supernatant was removed and the cell pellet was re-suspended in 1 ml TE buffer. To the washed cells, 470 mg of 150–212 µm glass beads (Sigma-Aldrich, Missouri, USA) were added and the samples were bead-beated at 6500 rpm for 60 s. Samples were then

centrifuged at 13 000 rpm for 10 min. From each lysed sample, 240 µl of supernatant was removed and processed using the extraction and purification protocol described by the manufacturer, beginning with the proteinase K digestion step.

Molecular characterisation of antimicrobial resistance genes

A multiplex PCR assay was used to detect the presence of the erythromycin resistance genes *ermB* and *mefA/E*, with inclusion of an *S. agalactiae*-specific internal control targeting the *cfb* gene [17, 18]. Other genes involved in promoting erythromycin resistance (*ermTR* and *ermT*) were detected by PCR as previously described [19, 20]. A multiplex assay was performed to detect the clindamycin resistance genes *lsaC* and *lnuB* [14, 15]. Additional clindamycin resistance genetic determinants (*lnuA*, *lsaB*, *lsaE* and *vgaC*) were investigated by PCR according to previous studies [21, 22].

Capsular typing and ST-17 detection

All isolates were assigned serotypes according to the recommended polymerase chain reaction (PCR)-based capsular genotype algorithm [23]. Briefly, isolates were initially serotyped using two multiplex PCR reactions [24] and any serotype VII or non-typeable isolates were further identified by PCR [25]. Detection of the hypervirulent ST-17 clone was performed using a modified version of a multiplex PCR assay targeting a region of the *hvgA* gene [26].

Statistical analysis

The χ^2 test and Fisher's exact test were used to analyse associations using SPSS v. 24.0 (IBM, UK). A *P*-value ≤ 0.05 was considered statistically significant.

RESULTS

Antimicrobial susceptibility

All isolates ($n = 235$) were susceptible to penicillin and rifampicin. Resistance to tetracycline, erythromycin and clindamycin (both constitutive and inducible) was observed in 87.2% ($n = 205$), 21.3% ($n = 50$) and 20.4% ($n = 48$) of isolates, respectively. Differences in overall resistance rates were evident between invasive ($n = 34$) and colonising ($n = 201$) isolates, with all of the former group being resistant to tetracycline

compared with 85.1% ($n = 171$) of colonists. Similarly, erythromycin resistance was found in 35.3% ($n = 12$) of invasive vs. 18.9% ($n = 38$) of colonising isolates ($P = 0.041$), and clindamycin resistance in 32.4% ($n = 11$) and 18.4% ($n = 37$) of invasive and colonising isolates, respectively ($P = 0.069$).

Macrolide resistance phenotypes and genotypes

In total, 50 isolates were resistant to erythromycin ($n = 12$ invasive; $n = 38$ colonising) and of these, 62% ($n = 31$) exhibited the c-MLS_B phenotype ($n = 9$ invasive; $n = 22$ colonising), while 20% ($n = 10$) were of the i-MLS_B phenotype ($n = 2$ invasive; $n = 8$ colonising) and 18% ($n = 9$) the M phenotype ($n = 1$ invasive, $n = 8$ colonising) (Table 1). The *ermB* gene was the most frequent (84%, $n = 42$) among macrolide resistant isolates while *mefA/E* and *ermTR* genes were each detected in 18% ($n = 9$) of isolates; *ermT* was restricted to a single, which was also positive for the *ermB* gene. One isolate harboured a combination of *ermB* and *mefA/E* genes, and another was positive for both *ermTR* and *mefA/E*. All 31 isolates exhibiting the c-MLS_B phenotype were *ermB* positive and three of these (9.7%) also harboured the *ermTR* gene. The *ermB* gene was detected in all 10 isolates displaying the i-MLS_B phenotype and five of these isolates were positive for the *ermTR* gene; another single isolate harboured the *ermT* gene. All M phenotype ($n = 9$) representatives contained the *mefA/E* resistance determinant, with only one isolate being additionally positive for *ermB* (Table 1).

Characterisation of the L phenotype

The L phenotype of erythromycin sensitivity and clindamycin resistance was identified in 2.9% ($n = 7$) of all GBS isolates and confirmed by MIC susceptibility testing (Table 2). Four isolates were positive for the *lsaC* gene and the remaining three were negative for all genetic determinants investigated. The L phenotype was not detected among invasive isolates.

Serotype distribution amongst GBS populations

Nine serotypes were identified among the total study collection; serotype IX was not found and one isolate was non-typeable by both PCR methods. The most common serotypes were Ia (28.1%), III (24.7%), II (14%), V (12.8%) and Ib (11.9%), and serotypes IV, VI, VII and VIII accounted for 4.3%, 1.3%, 1.7%,

Table 1. Genotypic and phenotypic characterisation of group B streptococcal resistant to erythromycin

| Genotype | Phenotype | | | | | |
|--|---------------------|-------------------|--------------|---------------------|-------------------|--------------|
| | Invasive (n = 12) | | | Colonising (n = 38) | | |
| | Macrolide-resistant | | | Macrolide-resistant | | |
| | c-MLSB (n = 9) | i-MLSB (n = 2) | M (n = 1) | c-MLSB (n = 22) | i-MLSB (n = 8) | M (n = 8) |
| <i>ermB</i> ⁺ , <i>mefA/E</i> ⁻ , <i>ermTR</i> ⁻ , <i>ermT</i> ⁻ | 8 | 1 | | 20 | 3 | |
| <i>ermB</i> ⁺ , <i>mefA/E</i> ⁻ , <i>ermTR</i> ⁺ , <i>ermT</i> ⁻ | 1 | 1 | | 2 | 4 | |
| <i>ermB</i> ⁺ , <i>mefA/E</i> ⁻ , <i>ermTR</i> ⁻ , <i>ermT</i> ⁺ | | | | | 1 | |
| <i>ermB</i> ⁺ , <i>mefA/E</i> ⁺ , <i>ermTR</i> ⁻ , <i>ermT</i> ⁻ | | | | | | 1 |
| <i>ermB</i> ⁻ , <i>mefA/E</i> ⁺ , <i>ermTR</i> ⁻ , <i>ermT</i> ⁻ | | | 1 | | | 6 |
| <i>ermB</i> ⁻ , <i>mefA/E</i> ⁺ , <i>ermTR</i> ⁺ , <i>ermT</i> ⁻ | | | | | | 1 |

c-MLSB, constitutive macrolide and lincosamide resistance; i-MLSB, inducible clindamycin resistance in the presence of erythromycin; M, resistance to erythromycin only.

Table 2. Characterisation of *L* phenotype isolates of group B streptococci

| Isolate no. | MIC (µg/ml) | | Genetic determinant | Serotype |
|-------------|-------------|--------------|---------------------|------------------|
| | Clindamycin | Erythromycin | | |
| 1 | ≥ 256 | 0.25 | – | V |
| 2 | ≥ 256 | 0.25 | – | V |
| 3 | ≥ 256 | 0.5 | – | II |
| 4 | 2 | 0.03 | <i>lsaC</i> | III |
| 5 | 1 | 0.03 | <i>lsaC</i> | III ^a |
| 6 | 1 | 0.03 | <i>lsaC</i> | III |
| 7 | 1 | 0.03 | <i>lsaC</i> | III ^a |

All isolates were screened for the presence of *lnuA*, *lnuB*, *lsaB*, *lsaC*, *lsaE* and *vgaC* determinants. Isolates 1–6 were of high vaginal swab origin, isolate 7 was from a placental swab.

^a Isolates of the ST-17 clone.

and 0.85% of isolates, respectively. Among colonising isolates the predominant serotypes were serotypes Ia (29.4%), III (25.9%), II (15.4%), V (11.4%) and Ib (9.95%). However, the serotype distribution for invasive isolates differed, with Ib (23.5%), Ia (20.6%), V (20.6%) and III (17.6%) being the most prevalent, but the difference between the two isolate groups did not reach statistical significance ($P = 0.081$).

The hyper-virulent clone, ST-17, was identified in 12.3% ($n = 29$) of all isolates, with 20.6% ($n = 7$) and 10.9% ($n = 22$) accounting for invasive and colonising populations. All, but one, of the ST-17 isolates were serotype III ($P < 0.0005$), the other isolate being confirmed by both PCR methods as serotype IV.

Table 3 shows that there was no significant difference in serotype distribution between neonatal and adult invasive isolates ($P = 0.378$). Two invasive

adult isolates were serotype III and ST-17, and another strain of serotype IV/ST-17 isolate was also confirmed from an adult sample. Of the 52 serotype III colonising isolates, 42.3% ($n = 22$) were ST-17.

Within the 50 isolates resistant to erythromycin, serotype III predominated ($n = 15$) followed by serotype V ($n = 12$), Ia ($n = 8$), II ($n = 6$) and Ib ($n = 4$). Both serotypes IV and VII were found in two isolates each and serotype VI was identified in a single isolate only. The majority of the 15 serotype III erythromycin resistant isolates expressed the c-MLSB phenotype (86.7%) and all harboured the *ermB* gene; the c-MLSB phenotype was also found in 9 of 12 serotype V isolates but these also harboured varied erythromycin resistance determinants (Table 4). Significant associations were found between serotype Ia and the M phenotype ($P = 0.0001$) and serotype Ia and the

Table 3. Correlation of sample origin and serotype distribution amongst invasive and colonising isolates of group B streptococci

| Serotype | Invasive | | | Colonising | | Total (n = 235) |
|----------|---------------------|-------------------|---------------------------------|-------------------------------|----------------------------|---------------------|
| | Neonatal (n = 7) | Adult (n = 20) | Unknown ^a (n = 7) | HVS ^b (n = 200) | PS ^c (n = 1) | |
| Ia | 1 | 4 | 2 | 59 | | 66 |
| Ib | | 6 | 2 | 20 | | 28 |
| II | 1 | 1 | | 31 | | 33 |
| III | 3 ^{d100} | 2 ^{d100} | 1 ^{d100} | 51 ^{d41-2} | 1 ^{d100} | 58 ^{d48-3} |
| IV | | 2 ^{d50} | | 8 | | 10 ^{d10} |
| V | 1 | 5 | 1 | 23 | | 30 |
| VI | 1 | | | 2 | | 3 |
| VII | | | | 4 | | 4 |
| VIII | | | 1 | 1 | | 2 |
| IX | | | | | | |
| NT | | | | 1 | | 1 |

^a Lack of clinical information.

^b High vaginal swab.

^c Placental swab.

^d Percentage of ST-17 isolates within group.

Table 4. Correlation between serotype, phenotype and genotype of group B streptococcal isolates

| CPS Serotype (No. of isolates) | E-resistant ^a | DA-resistant ^b | Phenotype | Genotype |
|--------------------------------|--------------------------|---------------------------|--|---|
| Ia (8) | 8 | | c-MLSB (3) M (5) | <i>ermB</i> (3), <i>ermTR</i> (1) <i>mefA/E</i> (5), <i>ermB</i> (1) |
| Ib (4) | 4 | | c-MLSB (2) i-MLSB (2) | <i>ermB</i> (2) <i>ermB</i> (2), <i>ermTR</i> (1) |
| II (7) | 6 | 1 | c-MLSB (3) i-MLSB (3) L (1) | <i>ermB</i> (3) <i>ermB</i> (2), <i>ermTR</i> (2), <i>ermT</i> (1) |
| III (19) | 15 | 4 | c-MLSB (13) i-MLSB (2) L (4) | <i>ermB</i> (13) <i>ermB</i> (2) <i>lsaC</i> (4) |
| IV (2) | 2 | | c-MLSB (1) i-MLSB (1) | <i>ermB</i> (1) <i>ermB</i> (1), <i>ermTR</i> (1) |
| V (14) | 12 | 2 | c-MLSB (8) i-MLSB (2) M (2) L (2) | <i>ermB</i> (8), <i>ermTR</i> (1) <i>ermB</i> (2), <i>ermTR</i> (2) <i>mefA/E</i> (2) |
| VI (1) | 1 | | c-MLSB (1) | <i>ermB</i> (1) |
| VII (2) | 2 | | M (2) | <i>mefA/E</i> (2), <i>ermTR</i> (1) |

^a E-resistant = all erythromycin-resistant isolates resistant or sensitive to clindamycin.

^b DA-resistant = isolates resistant to clindamycin but susceptible to erythromycin.

mefA/E gene ($P = 0.00046$). Serotype V was also frequent among invasive erythromycin-resistant population (41.7%), while serotype III predominated among colonising isolates resistant to erythromycin (31.6%) but there was no significant difference in serotype distribution within the two populations ($P = 0.317$). Three of the 12 erythromycin resistant

invasive isolates were ST-17 and were also resistant to clindamycin; correspondingly, three colonising isolates of the same resistance phenotype were members of the same ST-17 clone.

Four of the seven isolates exhibiting the L phenotype were serotype III, two were of serotype V and one was serotype II (Table 2). The four L phenotype

serotype III isolates harboured the *lsaC* gene and two of these were ST-17.

DISCUSSION

Despite recent reports of reduced beta-lactam susceptibility among GBS isolates [11], data from this study indicate that susceptibility to penicillin is universal amongst GBS strain populations in Ireland. This reaffirms the appropriateness of penicillin as the antibiotic of choice for first line IAP and for the treatment of GBS infections. The incidence of erythromycin resistance found (21.3%) is similar to previous studies conducted in Ireland (18.6%), Poland (18.4%) and Italy (20%) [5, 27, 28], but lower than rates in France (34.7%) and Taiwan (46%) [29, 30]. Interestingly the rate of clindamycin resistance in the current study indicates that resistance has increased in the Irish population from 15.25% [5] to 20.4% and is higher than reports from other countries, including Poland and Africa [27, 31]. As with other countries, a high rate of tetracycline resistance (87.2%) was evident for the isolates reported here [28, 29].

Colonising GBS isolates can be reservoirs of virulence and antibiotic resistance markers and vaginal colonisation may pose a risk for subsequent invasive disease in neonates. Both erythromycin and clindamycin resistance are higher among invasive (35.3% and 32.4% respectively) than colonising isolates (18.9% and 18.4% respectively), although this only reached statistical significance for erythromycin resistance ($P = 0.041$).

Regarding antibiotic resistance mechanisms, the c-MLSB phenotype predominated overall (62%) and was more frequent among invasive (75%) than colonising isolates (57.9%). The *ermB* gene was the most common resistance determinant identified (84%) and this finding corroborates the results of the most recent Irish study [5]. The presence of the *ermT* gene, although only in a single isolate, is notable as it was associated with the i-MLSB phenotype. This determinant may be of importance as it is plasmid-borne and has been shown to be readily transferable between GBS and *Enterococcus faecalis*, which could promote its wider dissemination [20]. It is interesting to note the co-occurrence of *ermB* and *ermTR* genes in eight isolates and *mefA/E* and *ermTR* genes in another isolate. Co-occurrence of *ermB* and *ermTR* has also been noted in other studies, although in a lower number of isolates [28, 29, 32, 33].

The L phenotype was first documented in *E. faecium* in 1999 [14] and shortly after by the first report in a GBS strain in Canada in 2001 [19]. Since then there have been reports of the L phenotype in Spain [21], Korea [32], the USA [34], Argentina [35] and South Africa [36] with incidence rates varying from 0.26% in Italy [28] to 15.9% in Korea [32]. To date, the phenotype had not been confirmed in GBS isolates from Ireland but was found here to represent almost 3% of isolates which, interestingly, were collected in the latter part of the study period (2015–2016).

Four of the seven L phenotype isolates harboured the *lsaC* gene and were serotype III. This observation concurs with reports from previous studies [15, 16, 21, 32, 37]. Moreover, the three other isolates of this phenotype with a MIC for clindamycin >256 µg/ml were negative for all other determinants tested including the *lnu*, *lsaB*, *lsaE* and *vgaC* genes. Two of these isolates were confirmed as serotype V and the other was serotype II. To the best of our knowledge, this is the first documented report of GBS isolates with the L phenotype and characterised as containing the *lsaC* gene in Ireland. Although the L phenotype was exclusively identified in colonising isolates in this study, it has been found by others in patients with invasive GBS disease [16, 37]. Also of interest, two of the seven isolates were confirmed as belonging to the hypervirulent ST-17 clone. The emergence of this uncommon phenotype in Irish GBS strains corroborates the need for continued surveillance of emerging antibiotic resistance, as recently reported in the USA [16]. This USA study also revealed the presence of L phenotypes with cross-resistance to streptogramins A and pleuromutilins, the LSA/LSAP phenotypes, which have been reported elsewhere [15, 37]. Another recent study using whole genome sequencing to identify resistance mechanisms in GBS, identified the LSA/LSAP phenotypes using this approach and highlighted the co-occurrence of certain genes, particularly *lsaC* and *ermB*, amongst isolates displaying this phenotype [33]. While we did not specifically investigate the LSA/LSAP phenotypes, they may be clinically significant going forward and warrant further study into the development of alternative antimicrobials.

Here, nine serotypes were confirmed among the isolate collection and only one proved non-typeable by both PCR methods used. Overall, serotype Ia predominated (28.1%), followed by types III, II and V. This distribution is comparable with a previous Irish study [5] and indicates a level of stability within

the GBS population in this country. Serotype III was also predominant among erythromycin-resistant isolates in contrast to a previous study in the South of Ireland where serotype V was more frequently associated with erythromycin-resistant strains [38].

The ST-17 lineage accounted for 12.3% of all isolates studied with rates of 20.6% and 10.9% in the invasive and colonising populations respectively. The high prevalence of this clone in the latter group is of concern because of its potential to progress to invasive disease, thus highlighting the importance of monitoring reservoirs of colonising strains. Three of the ST-17 invasive isolates were of neonatal origin and of serotype III, and three were from invasive adult infections, but of different serotypes (III and IV) which may suggest the occurrence of capsular antigen switching as reported in other studies [5, 39]. Together, these findings serve to emphasise the need for continuous surveillance of serotypes which is highly relevant to the constitution and development of vaccines [40].

In conclusion, the increase in resistance to erythromycin and clindamycin in GBS strains is of significance particularly for individuals with penicillin allergies. Emergence of the L phenotype in the Irish GBS population is a concern and warrants continued surveillance of resistance rates and further elucidation of the underlying genetic mechanisms.

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DECLARATION OF INTEREST

The authors declare no conflict of interest.

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