Sustained transmission of high-level azithromycin resistant *Neisseria gonorrhoeae* in England; an observational study

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Summary

Background

Between November 2014 and February 2017, 70 cases of high-level azithromycin-resistant (HL-AziR; minimum inhibitory concentration (MIC) ≥256 mg/L) *Neisseria gonorrhoeae* were reported from across England. Whole-genome sequencing (WGS) was performed to investigate this outbreak.

Methods

WGS was performed on 60 of the HL-AziR *N. gonorrhoeae* isolates from England. As comparators, 110 isolates with a range of azithromycin MICs were also sequenced, including eight isolates from Scotland with azithromycin MICs ranging from 0·12 to 1·0 mg/L which were NG-MAST ST9768, which was the ST initially responsible for the outbreak. The presence of mutations or genes associated with azithromycin resistance was also investigated.

Findings

The majority of HL-AziR isolates from England (37/60) belonged to ST9768, and were genetically similar (mean 4·3 SNPs). An A2059G mutation was detected in 3-4 alleles of the 23S rRNA gene. Five susceptible ST9768 isolates had one mutated 23S rRNA allele and one low-level resistant ST9768 isolate had two mutated alleles. The phylogeny suggested that the HL-AziR isolates were descendants of the low-level azithromycin resistant isolates.

Interpretation

Sustained transmission of a successful HL-AziR clone was seen across England. Mutation A2059G was found in isolates with lower azithromycin MICs. Azithromycin exposure may have provided the selection pressure for one or two mutated copies of the 23S rRNA gene to recombine with wild-type copies, leading to three or four mutated copies and the HL-AziR phenotype. HL-AziR could emerge in isolates with low azithromycin MICs and eliminate the effectiveness of azithromycin as part of dual therapy for the treatment of gonorrhoea.

Funding

This work was performed using internal Public Health England funding.

Research in context

Evidence before this study

We searched PubMed for articles published in English on or before June 30, 2017, with the terms "*Neisseria gonorrhoeae"* or "gonorrhoea" with "azithromycin", and "*Neisseria gonorrhoeae*" or "gonorrhoea" with "sequencing" or "molecular epidemiology". HL-AziR *N. gonorrhoeae* has been observed sporadically in the UK and elsewhere, with occasional small clusters reported. There have been no reports of sustained transmission on the scale described in this paper. Additionally, previous studies describe the national molecular epidemiology of *N. gonorrhoeae*, but do not describe the real-time use of WGS as part of an outbreak investigation.

Added value of this study

This report provides evidence of sustained transmission of *N. gonorrhoeae* with the HL-AziR phenotype on a national scale. WGS was a valuable tool for understanding the spread of resistance; the SNP phylogeny provided a level of discrimination beyond that of NG-MAST in terms of determining which samples were likely to be linked by recent transmission. Mutation A2059G was detected in the 23S rRNA genes of isolates with low azithromycin MICs, including isolates that were susceptible; the phylogeny suggested that the HL-AziR isolates emerged from susceptible isolates.

Implications of all the available evidence

WGS analysis of *N. gonorrhoeae* provides a discriminatory typing method, which can be used to investigate and inform outbreak control strategy in real-time. Widespread, sustained transmission of a successful HL-AziR clone and the finding that high-level azithromycin resistance may emerge from low-level resistance is of great concern, and has implications for the long-term viability of azithromycin as part of dual therapy for the treatment of gonorrhoea.

Introduction

In 2012 the World Health Organisation estimated that there were 78.3 million new gonorrhoea cases worldwide.[1] Untreated infection can result in pelvic inflammatory disease, ectopic pregnancy and infertility, and may increase the risk of transmission and acquisition of HIV. *Neisseria gonorrhoeae*, the causative pathogen of gonorrhoea, has developed resistance to successive classes of antibiotics over decades,[2] and few antimicrobials remain effective in its treatment. Extended-spectrum cephalosporins (ESC) such as ceftriaxone are last-line treatment options for *N. gonorrhoeae*, as there are currently no new antimicrobials available. In an attempt to delay the accumulation of resistance, many countries introduced dual antimicrobial therapy with an ESC plus azithromycin as first-line treatment. In 2011, the British Association of Sexual Health and HIV (BASHH) gonorrhoea treatment guidelines changed to recommend dual therapy with single dose ceftriaxone (500 mg intramuscularly) in combination with a single oral dose of azithromycin (1 g).[3]

Public Health England (PHE) detected an outbreak of high-level azithromycin resistant *N. gonorrhoeae* (HL-AziR; minimum inhibitory concentration (MIC) ≥256 mg/L) in Leeds, northern England, in March 2015.[4] Prior to this, HL-AziR had been a rare phenotype observed only sporadically in England. Since then there have been ongoing reports of HL-AziR *N. gonorrhoeae* cases from across England. This presents a significant threat to front-line dual therapy for gonorrhoea, as it renders the azithromycin component ineffective.

We previously described the use of whole genome sequencing (WGS) in the investigation of seven cases from the beginning of this outbreak in Leeds.[4] All seven isolates characterised by WGS were *Neisseria gonorrhoeae* multi-antigen sequence typing (NG-MAST)[5] sequence type (ST) 9768 and were virtually identical on WGS core genome comparison (zero or one single nucleotide polymorphisms (SNPs)). All of the isolates showed mutation A2059G (*Escherichia coli* numbering) in all four alleles of the 23S rRNA gene, conferring high-level resistance to azithromycin.

Here we present an analysis of WGS data for isolates from this national outbreak, plus additional isolates from across the United Kingdom (UK) with a wide range of azithromycin MICs, to determine whether the ongoing outbreak represented clonal spread of the HL-AziR *N. gonorrhoeae* strain identified in Leeds. We also wanted to elucidate the molecular mechanisms of azithromycin resistance in *N. gonorrhoeae* in the UK.

Methods

Description of the outbreak

The outbreak first emerged with 16 cases diagnosed in residents of Leeds between November 2014 and October 2015. A local incident control team in Leeds was formed to coordinate the response. An alert to clinicians was issued through BASHH; this highlighted the need to ensure that patients were followed up, received a test-of-cure, and that every effort was made to contact partners. A National Resistance Alert[6] was also disseminated to all microbiology laboratories in England, advising that isolates found to be resistant to azithromycin should be sent to PHE's national reference laboratory. Between November 2014 and February 2017, a total of 70 confirmed cases were reported to PHE from across England. A nationally-led outbreak control team was convened during this time; measures included the establishment of an enhanced surveillance system and active case follow-up.

Since February 2017, cases have continued to be reported from across England, but have not been included in the study.[7]

Neisseria gonorrhoeae isolates

Primary diagnostic laboratories were requested to send all *N. gonorrhoeae* isolates found to be resistant to azithromycin (either by disc diffusion or gradient strip methodology) to PHE for confirmation. Isolates were confirmed as *N. gonorrhoeae* by MALDI-TOF (Bruker, Coventry, UK) and azithromycin MICs (mg/L) were confirmed by Etest (bioMérieux, Basingstoke, UK) on GC agar (BD, Oxford, UK) supplemented with 1% Vitox (Oxoid, Basingstoke, UK), as stated in the manufacturer's instructions. EUCAST clinical breakpoints[8] were used to report azithromycin resistance; MIC >0·5 mg/L. High-level azithromycin resistance was defined as MIC ≥256 mg/L. Ceftriaxone MICs were also determined using the same methodology, with the EUCAST clinical breakpoint used to define resistance as MIC >0·125 mg/L.

Study samples

Between November 2014 and February 2017, there were 70 confirmed cases of HL-AziR *N. gonorrhoeae* from across England. Seven of these were previously sequenced[4] and were included in the analysis. WGS was attempted on the remaining 63 isolates.

To provide context to the WGS data, the genomes of 110 additional isolates were sequenced: ten HL-AziR isolates identified between 2015 and February 2017 from outside of England (one from Wales, four from Scotland, four from Northern Ireland and one from the Republic of Ireland); five and 14 HL-AziR isolates from previous clusters from 2004 - 2007 in England and Scotland, respectively;[9,10] 16 HL-AziR isolates from the reference service or the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) from 2009 – 2016; 28 isolates of NG-MAST genogroup-1407 (azithromycin MICs 0·03 – 1·0 mg/L); 27 isolates with a range of azithromycin MICs (1·0 – 36 mg/L); two intermediate azithromycin isolates (0·5 mg/L) from Leeds; and eight isolates from Scotland of NG-MAST ST9768 with azithromycin MICs ranging from 0·12 to 1·0 mg/L, seven isolated between April and October 2014 and one from February 2016.

Whole genome sequencing and antimicrobial genotyping

Short read data from the isolates were mapped to a close reference sequence (WHO P)[11] as determined using the mash algorithm,[12] and variant call files were produced.[13] The vcf files were filtered and processed to produce an alignment from which recombination was removed using the gubbins algorithm (version 2·0·0)[14] and a maximum likelihood tree produced including rapid bootstrap analysis.[15] Bayesian time-measured phylogenetic analysis was performed using BEAST (https://www.beast2.org/), NG-MAST STs were determined(http://www.ng-mast.net) and the presence of mutations or genes that have previously been associated with azithromycin resistance was analysed (supplementary methods). The sequences were also compared with international HL-AziR *N. gonorrhoeae* whole genome sequences from published studies[16-18] available within the short read archive (https://www.ncbi.nlm.nih.gov/sra) or acquired directly from the corresponding author.[19]

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Outbreak cases

Most of the initial cases identified in Leeds were young (16-20 years) and all were heterosexual. Over time, the majority of cases were reported from London, including 20 cases in men who have sex with men (MSM), and four of these men were known to be bisexual. 61% of cases were known to be symptomatic at presentation. 73% were positive from a single site (predominantly genital) and 27% were positive from more than one site (33% were positive from pharyngeal specimens; seven females and 14 males). 26% of cases had co-infection with chlamydia at the time of first attendance. The isolates were all susceptible to ceftriaxone (MICs 0·008 – 0·032 mg/L). The majority of the patients were treated empirically with dual therapy before susceptibility testing results were available and there were no confirmed treatment failures.

Partner notification had limited success: out of a total of 139 partners reported, only 28 (20%) were known to have been successfully contacted. Of those partners with a confirmed test result, 77% (20/26) tested positive for *N. gonorrhoeae*. Particularly in the MSM population in London, patients reported multiple anonymous partners and it was not possible to verify if any were successfully contacted and tested (n=63). The patients did not provide information to allow clear links to be established between them with the exception of nine couples and two sets of four linked individuals. It was not possible to describe a clearly linked sexual network which gives an indication of the number of undiagnosed cases involved in transmission.

Whole-genome sequencing

Of the 70 HL-AziR *N. gonorrhoeae* isolates referred from England between November 2014 and February 2017, ten isolates could not be retrieved or failed sequencing. Seven isolates had been sequenced previously and were all ST9768.[4] In-silico NG-MAST for the remaining isolates revealed ten STs: 649 (7 cases), 2475 (4 cases), 5543 (1 case), 7573 (1 case), 9768 (30 cases), 13124 (2 cases), 13125 (1 case), 13377 (3 cases), 14110 (1 case), and 14308 (3 cases). The majority of isolates from England (37 of the 60 cases for which WGS was successful) were ST9768.

A phylogeny based on whole genome SNPs was produced as described in the supplementary methods and results. Within the phylogeny three clades were defined to describe the isolates from the 2014 -2017 period, which included the additional non-outbreak isolates as described in Methods (Figure 1). Clade 1 consisted of the ST9768 isolates and the ST14308 isolates. Clade 3 consisted of isolates closely related to clade 1, and all were ST649. Clade 2 consisted of isolates with a more distant common ancestor to that of clades 1 and 3 and had a mixture of STs including some (5/16) of ST649. Analysis using BEAST (Figure 2, supplementary results) suggested that the time to the most recent common ancestor (TMRCA) for the three clades was around 6.4 years (95% confidence interval 5 to 8 years). The TMRCA for clades 1 and 3 was 4.3 years (95% confidence interval 3.5 to 5.3 years). A description of the genetic differences of the isolates within clades and between clades is shown in Table 1. Four of the HL-AziR *N. gonorrhoeae* isolates referred during the outbreak (STs 7573, 13124 and 14110) were unrelated to the rest of the isolates and are not shown in the large

phylogeny. The number of SNPs observed between any two isolates from the epidemiologically linked couples or the two sets of linked individuals ranged from zero to three.

Isolates of ST9768 were all genetically similar, with a mean of 4·3 SNPs. When one outlying sample (Sample 2016-02_73, azithromycin susceptible) was excluded, the mean SNP difference between clade 1 isolates was 3·9 SNPs, with a maximum SNP difference between any two isolates of 14 SNPs. All of these ST9768 isolates shared a recent common ancestor indicative of recent transmission. The three ST14308 isolates were of a lineage directly derived from the ST9768 lineage (bootstrap value of 100). They were all from Leeds and were isolated at the end of 2016, a year after the previous Leeds isolates. Three HL-AziR isolates from Scotland and the single Welsh isolate were also ST9768 and fell within clade 1, but those from Northern Ireland and the Republic of Ireland, and three from Scotland were different STs and were in the phylogenetically distinct clade 2. The minimum difference between any clade 2 isolate and any isolate within clade 1 and clade 3 was 14 and 18 SNPs, respectively, whilst the minimum difference between any clade 1 and any clade 3 isolate was seven SNPs. There were ten unique SNPs that separated clade 2 and clades 1 and 3, and six unique SNPs that separated clade 1 and clade 3.

Almost all of the HL-AziR isolates had the A2059G mutation (*Escherichia coli* numbering) either in three or all four alleles of the 23S rRNA gene, apart from one isolate (2015-07_101) which had two mutated alleles. Unfortunately, sequencing could not be repeated to confirm this because the sample was lost due to an archiving error. The eight isolates from Scotland (2014-04_61, 2014-04_62, 2014-04_63, 2014-06_64, 2014-06_65, 2014-08_66, 2014-10_67, 2016-02_73) that were NG-MAST ST9768 with azithromycin MICs ranging from 0.12 to 1.0 mg/L were in some cases within zero SNPs of the ST9768 HL-AziR isolates, suggesting 23S rRNA copy number evolution occurred more rapidly than the accumulation of neutral SNPs. Five susceptible isolates (MIC 0·25 mg/L) had one mutated 23S rRNA allele and one low-level resistant isolate (MIC 1·0 mg/L) had two mutated alleles. Two susceptible isolates did not have any mutated alleles. The phylogeny provided evidence that the HL-AziR isolates were descendants of the low-level azithromycin resistant isolates, which were in turn, descendants of the susceptible isolates. The position of sample 2016-02_73 within the phylogeny is the only sample not congruent with this suggested scenario, but the bootstrap value for this node is low. The BEAST phylogeny generated using the same sequence data (Figure 2, supplementary results) grouped this isolate with the other susceptible isolates, although the posterior probabilities for this section of the tree were low.All of the HL-AziR isolates also carried a G45D mutation in *mtrR*, but none had the C2611T mutation in the 23S rRNA gene or any mutations in other genes associated with macrolide resistance.

Comparison with the few international HL-AziR *N. gonorrhoeae* whole genome sequences available within the short read archive showed that the UK HL-AziR *N. gonorrhoeae* from 2014 onwards were distinct from those reported elsewhere (Figure 3A and 3B, supplementary results).

Discussion

This is the first report of sustained transmission of a clonal outbreak of HL-AziR *N. gonorrhoeae* over several years. Previously the HL-AziR phenotype has been observed sporadically and in small clusters in the UK and elsewhere.[9,10,16,20-23] Our study included the largest number of HL-AziR isolates sequenced to date; we found that they clustered into three phylogenetic clades and were distinct from the majority of the samples with low-level azithromycin resistance. We have also shown that

high-level resistance may emerge from susceptible strains or those with low-level resistance, over a relatively short time period.

The molecular clock of *N. gonorrhoeae* needs to be understood in order to predict the likelihood of transmission. However it was difficult to interpret the SNP phylogeny due to the lack of context to other English samples since *N. gonorrhoeae* is not routinely sequenced as part of PHE's surveillance programme. Data from Silva et al.[24] suggest that within a 12-month period the mean number of expected substitutions per genome is four, with an upper 95% confidence limit of 14. Within clade 1 the earliest sample was from April 2014 and the latest sample was February 2017; a period of two years ten months. The isolates of ST9768 differed by less than 14 SNPs, which was close to the mean of four SNPs per genome per year. Together with our BEAST analysis, this supports the conclusion that these isolates shared a common ancestor indicative of very recent transmission.

In contrast, the isolates from clade 2 differed from all isolates in clade 1 by at least 14 SNPs. There are isolates within clade 2 with similar isolation dates to those in clade 1 from the early part of the outbreak period. This, together with the topology of the phylogeny and BEAST analysis, suggested that clades 1 and clades 2 were unlikely to be part of the same recent transmission chain but shared a common ancestor 6.5 years ago. These lineages may have evolved from a UK-derived common ancestor and have been circulating in the UK population, or may be due to separate introductions of an internationally successful clone that diversified to produce the extant variation observed in clades 1 and 2. The minimum distance between any two isolates in clades 1 and 3 was seven SNPs. Given the earliest sample in clade 3 was from April 2016 and the most ancestral part of clade 1 was from late 2014, it seems likely that the common ancestor diverged into two clonal expansions; one of which manifested as the successful ST9768 clade and the other which manifested as clade 3, but that we have failed to detect cases in the intervening period.

The SNP phylogeny gave a level of discrimination beyond that of NG-MAST in terms of determining which isolates were likely to be linked by recent transmission; for example, NG-MAST ST649 was found throughout the phylogeny. A ST649 HL-AziR *N. gonorrhoeae* cluster was identified in the UK in 2007,[9,10] and this ST has been associated with HL-AziR *N. gonorrhoeae* internationally.[20-22] It is possible that the ST9768 clone is a descendant of one of the lineages of the globally successful HL-AziR ST649. Interestingly, within the HL-AziR ST9768 clade were the Scottish ST9768 isolates which were susceptible or demonstrated low-level resistance to azithromycin. The phylogeny showed that the HL-AziR ST9768 isolates were descendants of the low-level azithromycin resistant isolates, which were in turn, descendants of the susceptible isolates. However, the maximum likelihood and BEAST phylogenies both indicate that the susceptible ST9768 Scottish isolates originated from an azithromycin resistant parent but became susceptible through reversion of mutant alleles of the 23S rRNA gene to wild-type, either through 'back mutation' or recombination. This may perhaps occur if HL-AziR is associated with a fitness cost. We hypothesise that azithromycin exposure may then have provided selection pressure for the one or two mutated alleles to recombine with wild-type copies without the mutation. Alternatively, azithromycin exposure may have induced additional A2059G mutations in the wild-type alleles. This would lead to three or four mutated copies, which would confer the HL-AziR phenotype, and has been demonstrated to occur in the laboratory.[25] However without more comprehensive sampling, particularly of isolates from England, we cannot know this for certain. Azithromycin is used in the treatment of other sexually transmitted infections, particularly chlamydia, and it is possible that concurrent undiagnosed *N. gonorrhoeae* could be exposed to sub-therapeutic levels of azithromycin and select for resistance. Additionally,

inappropriate azithromycin monotherapy for *N. gonorrhoeae* may lead to treatment failure by providing selection pressure for the development of resistance.

We found that the majority of HL-AziR *N. gonorrhoeae* isolates had the A2059G mutation in all four copies of the 23S rRNA genes. Low-level azithromycin resistance (MICs 1·0 – 32 mg/L) is commonly associated with a C2611T 23S rRNA gene mutation. WGS of 75 azithromycin-resistant isolates from Europe[19] identified the C2611T mutation in two to four alleles of the 23S rRNA gene in isolates with MICs ranging from 4-8 mg/L, and in all four alleles of isolates with MICs 16-32 mg/L. The A2059G mutation was detected in all four alleles of isolates with MICs ≥256 mg/L (n=4), but not in isolates with lower MICs. In addition, mutations in *mtrR* and its promoter, leading to overexpression of the MtrCDE efflux pump, occurred in isolates across the whole MIC range. A Canadian study,[17] which included five isolates with HL-AziR, had similar findings. In both studies the resistant isolates clustered clonally into distinct lineages, but the HL-AziR isolates were found across the phylogenetic tree. In contrast, a study in the United States[18] found that isolates with azithromycin resistance were more diverse and showed less clonal expansion. Only two isolates had HL-AziR, both with four alleles with the A2059G mutation.

The UK HL-AziR isolates from 2014 onwards were distinct from those seen previously in the UK, from the international HL-AziR *N. gonorrhoeae* sequences available from the studies described above[17-19] and also from a cluster of seven cases of HL-AziR *N. gonorrhoeae* reported in Hawaii in 2016.[16] A retrospective prevalence study from Hangzhou, China in 2012 found HL-AziR in 21/118 isolates tested, suggesting that HL-AziR may be more widespread in this region. These isolates belonged to seven different NG-MAST STs, although some of the STs were >99% similar;[26] these were different STs to those found in our study. It is possible that HL-AziR leads to a fitness cost, which may explain why this phenotype has previously been seen only sporadically or in non-sustained clusters. We do not know why there has been sustained transmission of ST9768 in England; perhaps compensatory mutations to preserve this HL-AziR phenotype may be present. Further work to investigate this is needed.

Cases from both heterosexual and MSM populations were seen across the phylogenetic tree, providing evidence of transmission between MSM and heterosexual networks on several occasions during the course of this relatively short time period. This suggests that there may be greater fluidity between sexual networks than previously supposed, which, given the relatively large numbers of partners reported in both populations in this outbreak, have implications for infection control. It also suggests that when resistance emerges in one population it can soon spread to the other. It is of particular concern that 80% of contacts were not traceable, and as 77% of those traced were positive, this might mean a substantial burden of unidentified cases.

Fortunately, there were no confirmed treatment failures in any of these cases, probably because the isolates were all susceptible to ceftriaxone. The cluster of seven cases of HL-AziR *N. gonorrhoeae* reported in Hawaii in 2016[16] is concerning because these isolates also showed decreased susceptibility to ceftriaxone (MIC 0·125 mg/L). It is reassuring that all of the patients in Hawaii were successfully treated with 250 mg ceftriaxone plus 1 g azithromycin, and there have been no further cases.

Given that the prevalence of low-level azithromycin resistance (MICs >0·5 mg/L) in England is now 5%,[7] there is concern that HL-AziR *N. gonorrhoeae* may arise more frequently if a proportion of the low-level resistant isolates already harbour an A2059G mutation in a single allele. It is also likely that

even low-level azithromycin resistance renders the azithromycin component of dual therapy ineffective. In a Japanese study, treatment failures in men with gonococcal urethritis treated with an extended-release 2g azithromycin single dose were associated with azithromycin MICs >0·5 mg/L.[27] In addition, the *in vitro* MIC of azithromycin does not necessarily correlate with clinical treatment outcome, and treatment failures occur in patients with isolates identified as azithromycin susceptible in the laboratory.[28]

In conclusion, sustained transmission of a successful HL-AziR *N. gonorrhoeae* clone has been observed across England. WGS provided characterisation of an outbreak significantly beyond that achieved by NG-MAST. It also progressed our understanding of the emergence and mechanisms of resistance, particularly the finding that high-level resistance may actually emerge from low-level resistance. Dual therapy for gonorrhoea using azithromycin with ceftriaxone is clearly under threat and we may not be able to rely on azithromycin to 'protect' ceftriaxone. Surveillance of resistance, regular review of treatment guidelines and detection of treatment failures are critical.

Contributors

HF prepared the manuscript with input from MC, GH, SP, NW and AU. AU and US prepared the figures. All other authors contributed to the review of the final manuscript. MC managed the laboratory work and assisted with the analysis. NM prepared the cultures for WGS. US, RM and AU developed and performed the bioinformatic analysis. HF and AU analysed the data. HF and GH co-ordinated the national outbreak control team. SP, AW and CS performed data collection, analysis and interpretation for epidemiological information for the outbreak cases. KT and JS managed the Scottish Reference service and identified ST9768 isolates in their laboratory.

Conflicts of Interest

PHE's AMRHAI Reference Unit has received financial support for conference attendance, lectures, research projects or contracted evaluations from numerous sources, including: Accelerate, Achaogen, Allecra, Amplex, AstraZeneca, Basilea, Becton Dickinson Diagnostics, bioMérieux, Bio-Rad Laboratories, the BSAC, Cepheid, Check-Points, Cubist Pharmaceuticals, Department of Health, European Centre for Disease Prevention and Control, Enigma Diagnostics, Food Standards Agency, GlaxoSmithKline, Henry Stewart Talks, IHMA, Kalidex, Melinta, Merck Sharpe & Dohme, Meiji, Mobidiag, Momentum Biosciences, Nordic, Norgine, Rempex, Roche, Rokitan, Smith & Nephew, Trius, VenatoRx and Wockhardt. HF is a member of the Scientific Advisory Board for Discuva Ltd. The other authors have declared no conflicts of interest.

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