Europe-wide expansion and eradication of multidrug-resistant *Neisseria gonorrhoeae* lineages: a genomic surveillance study

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Summary

Background Genomic surveillance using quality-assured whole-genome sequencing (WGS) together with epidemiological and antimicrobial resistance (AMR) data is essential to characterise the circulating *Neisseria gonorrhoeae* lineages and their association to patient groups (defined by demographic and epidemiological factors). In 2013, the European gonococcal population was characterised genomically for the first time. We describe the European gonococcal population in 2018 and identify emerging or vanishing lineages associated with AMR and epidemiological characteristics of patients, to elucidate recent changes in AMR and gonorrhoea epidemiology in Europe.

Methods We did WGS on 2375 gonococcal isolates from 2018 (mainly Sept 1–Nov 30) in 26 EU and EEA countries. Molecular typing and AMR determinants were extracted from quality-checked genomic data. Association analyses identified links between genomic lineages, AMR, and epidemiological data.

Findings Azithromycin-resistant *N gonorrhoeae* (8·0% [191/2375] in 2018) is rising in Europe due to the introduction or emergence and subsequent expansion of a novel *N gonorrhoeae* multi-antigen sequence typing (NG-MAST) genogroup, G12302 (132 [5·6%] of 2375; *N gonorrhoeae* sequence typing for antimicrobial resistance [NG-STAR] clonal complex [CC]168/63), carrying a mosaic *mtrR* promoter and *mtrD* sequence and found in 24 countries in 2018. CC63 was associated with pharyngeal infections in men who have sex with men. Susceptibility to ceftriaxone and cefixime is increasing, as the resistance-associated lineage, NG-MAST G1407 (51 [2·1%] of 2375), is progressively vanishing since 2009–10.

Interpretation Enhanced gonococcal AMR surveillance is imperative worldwide. WGS, linked to epidemiological and AMR data, is essential to elucidate the dynamics in gonorrhoea epidemiology and gonococcal populations as well as to predict AMR. When feasible, WGS should supplement the national and international AMR surveillance programmes to elucidate AMR changes over time. In the EU and EEA, increasing low-level azithromycin resistance could threaten the recommended ceftriaxone–azithromycin dual therapy, and an evidence-based clinical azithromycin resistance breakpoint is needed. Nevertheless, increasing ceftriaxone susceptibility, declining cefixime resistance, and absence of known resistance mutations for new treatments (zoliflodacin, gepotidacin) are promising.

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Introduction

The 2020 global estimates for gonorrhoea indicated 82 million annual cases among adults.[1] Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* is threatening gonorrhoea treatment. *N gonorrhoeae* has developed or acquired resistance to every antimicrobial used for empiric therapy, including the first-line extended-spectrum cephalosporin ceftriaxone and azithromycin.[2] Fortunately, inability to cure gonorrhoea with ceftriaxone, in recommended monotherapy or together with azithromycin,[2] remains rare,[3,4] despite the international spread of a ceftriaxone-resistant clone (FC428)[3] and the prevalent decreased susceptibility or resistance to ceftriaxone and azithromycin internationally.[2,4]

The European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) monitors AMR in the EU and EEA by analysing annual, quality-assured AMR data in conjunction with epidemiological and clinical data of patients with gonorrhoea.[5–8] Molecular epidemiological characterisation of gonococcal isolates was done in two previous Euro-GASP surveys. First, 1066 Euro-GASP isolates from 2009–10[7] were genotyped using *N gonorrhoeae* multi-antigen sequence typing (NG-MAST) and, second, 1054 Euro-GASP isolates from 2013[8] were subjected to whole-genome sequencing (WGS), which revealed the distribution of particular sequence types and AMR clones across different patient groups. In 2009–10,[7] the major EU and EEA lineage was *N gonorrhoeae*

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Research in context

Evidence before this study

Gonorrhoea is a major public health problem internationally, and antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* compromises the effective management and accordingly, the control of gonorrhoea. Phenotypic AMR surveillance is imperative, nationally and internationally. Whole-genome sequencing (WGS) can further support phenotypic AMR surveillance by, for example, elucidation of gonococcal population dynamics and identification of AMR (or antimicrobial susceptible) lineages or clones, and by providing understanding of AMR determinants conferring AMR and molecular prediction of phenotypic AMR. We searched PubMed using the terms "*Neisseria gonorrhoeae*" OR "gonorrhoea" with "genome sequencing" for papers published in English between Jan 1, 2000, and June 25, 2021. WGS of *N gonorrhoeae* has mainly been used to investigate molecular epidemiology at national or local level in, for example, the USA, Canada, Argentina, Brazil, the UK, China, Japan, Vietnam, and Australia. Most of these studies have focused on isolates selected because of their AMR (with relatively few isolates sequenced in many studies) or for examining the spread of relatively few AMR strains or local outbreaks (including determining transmission chains). Gonococcal genomes with available metadata from most of these studies are available and can be compared in Pathogenwatch. To our knowledge, except for our smaller study that examined 75 azithromycin-resistant isolates from 17 EU and EEA countries and our 2013 WGS study (1054 consecutive antimicrobial-resistant and antimicrobial-susceptible isolates from 20 EU and EEA countries), there have been no other regional studies that used WGS of selected or consecutive *N gonorrhoeae* isolates.

Added value of this study

We report WGS data for 2375 gonococcal isolates cultured in 2018 in 26 EU and EEA countries, in conjunction with AMR data and epidemiological data for the patients with gonorrhoea, and compare with *N gonorrhoeae* multi-antigen sequence typing (NG-MAST) data from 2009–10 and WGS data from 2013 for gonococcal isolates from 20 EU and EEA countries. We describe increasing azithromycin resistance and increasing susceptibility to extended-spectrum cephalosporins in the EU and EEA, and elucidate the reasons for these changes. Increasing low-level azithromycin resistance among gonococci in the EU and EEA is largely due to the introduction and subsequent expansion of a novel NG-MAST genogroup, G12302 (NG-STAR clonal complex [CC]168 or CC63), carrying a mosaic *mtrR* promoter and *mtrD* sequence resulting in low-level azithromycin resistance. CC63 isolates were associated with pharyngeal infections in men who have sex with men. Susceptibility to extended-spectrum cephalosporins (ceftriaxone and cefixime) is increasing, mainly because the NG-MAST G1407 lineage, associated with decreased susceptibility and resistance to extended-spectrum cephalosporins, has been progressively vanishing in the EU and EEA since our studies in 2009–10 and 2013. We additionally provide an updated genomic baseline of the EU and EEA gonococcal population. Finally, we describe the prevalence of AMR determinants and their associations with phenotypic AMR, and we show that AMR can be predicted with high precision in most cases using WGS data. In general, our results substantially improve the understanding of the distribution of AMR and antimicrobial-resistant gonococcal clones in different risk groups for gonorrhoea nationally and regionally in the EU and EEA.

Implications of all the available evidence

To our knowledge, we report the second project using WGS in conjunction with relevant epidemiological and AMR data in an international programme for regional surveillance of gonorrhoea. AMR gonococcal strains are spreading internationally, hence, enhanced international AMR surveillance is essential. Where feasible, WGS, in conjunction with epidemiological and AMR data, is imperative in the international AMR surveillance programmes to additionally elucidate the changes in AMR prevalence and transmission of gonococcal lineages (AMR and antimicrobial susceptible ones), their association to patient groups, and in general the dynamics in gonorrhoea epidemiology. Increasing azithromycin resistance, which in the EU and EEA was largely due to the introduction or emergence and subsequent expansion of a single new gonococcal lineage, might threaten the use of ceftriaxone and azithromycin dual therapy for gonorrhoea. Accordingly, detailed understanding of the increasing azithromycin resistance internationally and an evidence-based clinical azithromycin resistance breakpoint are essential. Nevertheless, increasing ceftriaxone susceptibility, declining cefixime resistance, and absence of known target resistance mutations for new potential treatment options, such as zoliflodacin or gepotidacin, are promising. Finally, WGS with appropriate bioinformatic analysis provides a substantially higher and more accurate resolution of gonococcal isolates than traditional molecular epidemiological typing methods, such as NG-MAST and multilocus sequence typing; discrepant isolates, mixed infections, and contaminants can be identified for exclusion to not bias the results in surveillance programmes and AMR can be accurately predicted in most cases.

multi-antigen sequence typing (NG-MAST) genogroup 1407 (G1407), associated with decreased susceptibility and resistance to extended-spectrum cephalosporins and men who have sex with men (MSM). This lineage threatened the recommended empirical cefixime monotherapy in the EU and EEA until 2012, when cefixime monotherapy was replaced by dual therapy (ceftriaxone plus azithromycin).[9] In 2013, the NG-MAST G1407 incidence had substantially decreased and its association switched from MSM to heterosexual groups.[8] Since the change in therapy recommendation, the incidence of resistance to cefixime and ceftriaxone has decreased, but the level of azithromycin resistance has increased,[6,10] illustrating that recommended treatments impact on the gonococcal

population dynamics, together with other factors—eg, antimicrobial overuse and misuse, changes in sexual behaviour in or between sexual networks, and expansion or eradication of specific gonococcal lineages.[8,11]

WGS proved in the 2013 survey[8] valuable for detailed surveillance of gonorrhoea by providing a genomic baseline of the EU and EEA gonococcal population, in conjunction with associated AMR and epidemiological data, and by informing public health and associated interventions. WGS provided a more ideal resolution and accuracy compared with other typing schemes.[8] WGS allows the identification of high-risk or AMR clones, transmission chains or outbreaks, known and novel AMR determinants, and new targets for therapy, vaccines, and diagnostics, including molecular tests for AMR prediction.

In this study, we analysed WGS results on 2375 *N gonorrhoeae* isolates from 26 EU and EEA countries in 2018 together with quality-assured AMR data and epidemiological and clinical information from the patients with gonorrhoea. We identified novel genomic lineages and their association with AMR and patient metadata, predicted AMR, and monitored existing high-risk lineages through a longitudinal comparison with the Euro-GASP 2013 WGS[8] and 2009–10 NG-MAST[7] surveys.

Methods

Euro-GASP sampling and antimicrobial susceptibility testing

In Euro-GASP 2018, 3299 gonococcal isolates linked to metadata of patients were collected in 27 EU and EEA countries, mostly from Sept 1 to Nov 30, 2018.[6] 2653 (80·4%) of 3299 isolates from 26 countries were available for this study and for 2375 (72·0%) of 3299, quality-controlled genomic data linked to AMR and metadata were obtained (table 1). One isolate per infection episode was included in accordance with sampling priority rules (appendix 1 pp 1–6).

All countries were part of the annual Euro-GASP external quality assessment (EQA).[12] 19 (73%) of 26 countries did decentralised AMR testing in their country while isolates from 7 (27%) of 26 countries were tested centrally at UK Health Security Agency (UKHSA, London, UK) or Örebro University Hospital (ÖUH, Örebro, Sweden). Minimum inhibitory concentration (MIC) gradient strip tests or agar dilution were done for ceftriaxone, cefixime, azithromycin and ciprofloxacin as previously described.[6] Current (v11.0) breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied. AMR testing was repeated for some isolates with discrepancies between phenotypic and genotypic resistance (appendix 1 pp 8–9).

Genomic sequencing and analyses

Total DNA was extracted from pure cultures using a QIAsymphony instrument (Qiagen, Hilden, Germany) at UKHSA and ÖUH, frozen and sent to the Wellcome Sanger Institute (UK) for WGS. Raw genomic data was quality-checked, assembled and processed to obtain molecular typing information (NG-MAST, multilocus sequence typing [MLST], and *N gonorrhoeae* sequence typing for antimicrobial resistance [NG-STAR]) and to detect genetic AMR determinants. Association analyses among the genotypes in each country and the phenotypic AMR or patient epidemiological data was performed. For details regarding WGS, quality checks, molecular typing, bioinformatics and statistical analyses, see appendix 1.

See **Online** for appendix 1

For **EUCAST clinical breakpoints** see https://www.eucast.org/clinical_breakpoints/

Role of the funding source

The funders had no role in study design, data collection, analysis, interpretation, or report writing.

	Number of isolates in Euro-GASP 2018[6]†	Number of isolates in genomic survey (%)	Azithromycin resistance (%)‡	Ciprofloxacin resistance (%)‡	Cefixime resistance (%)‡	Ceftriaxone resistance (%)‡
Austria	267	183 (68·5%)	19 (10·4%)	103 (56·3%)	5 (2·7%)	0
Belgium	180	76 (42·2%)	7 (9·2%)	32 (42·1%)	2 (2·6%)	0
Croatia	10	9 (90·0%)	5 (55·6%)	7 (77·8%)	0	0
Cyprus	5	4 (80·0%)	1 (25·0%)	3 (75·0%)	1 (25·0%)	0
Czech Republic	95	88 (92·6%)	12 (13·6%)	44 (50·0%)	1 (1·1%)	0
Denmark	114	99 (86·8%)	1 (1·0%)	40 (40·4%)	0	0
Estonia	7	7 (100·0%)	1 (14·3%)	2 (28·6%)	0	0
Finland	168	50 (29·8%)	4 (8·0%)	26 (52·0%)	0	0
France	109	37 (33·9%)	2 (5·4%)	22 (59·5%)	0	0
Germany	201	114 (56·7%)	4 (3·5%)	80 (70·2%)	0	0
Greece	83	79 (95·2%)	1 (1·3%)	46 (58·2%)	5 (6·3%)	0
Hungary	89	89 (100·0%)	3 (3·4%)	35 (39·3%)	2 (2·2%)	0
Iceland	45	41 (91·1%)	7 (17·1%)	21 (51·2%)	0	0
Ireland	200	169 (84·5%)	9 (5·3%)	105 (62·1%)	0	0
Italy	100	98 (98·0%)	14 (14·3%)	55 (56·1%)	5 (5·1%)	0
Latvia	5	3 (60·0%)	0	1 (33·3%)	0	0
Luxembourg	1	0	..	..	..	..
Malta	25	6 (24·0%)	2 (33·3%)	4 (66·7%)	0	0
Netherlands	402	190 (47·3%)	12 (6·3%)	88 (46·3%)	0	0
Norway	126	113 (89·7%)	16 (14·2%)	62 (54·9%)	1 (0·9%)	0
Poland	73	64 (87·7%)	1 (1·6%)	31 (48·4%)	1 (1·6%)	0
Portugal	122	97 (79·5%)	11 (11·3%)	33 (34%)	0	0
Slovakia	77	76 (98·7%)	3 (3·9%)	27 (35·5%)	0	0
Slovenia	155	104 (67·1%)	4 (3·8%)	63 (60·6%)	0	0
Spain	189	173 (91·5%)	22 (12·7%)	83 (48·0%)	7 (4·0%)	1 (0·6%)
Sweden	200	199 (99·5%)	14 (7·0%)	120 (60·3%)	3 (1·5%)	0
UK	251	207 (82·5%)	16 (6·4%)	86 (41·5%)	3 (1·4%)	0
Total	3299	2375 (72·0%)	191 (8·0%)	1219 (51·3%)	36 (1·5%)	1 (0·04)

Euro-GASP=European Gonococcal Antimicrobial Surveillance Programme. *After retesting of isolates with discrepant results (appendix 1 pp 8–9). †Euro-GASP aims to collect 100 or more isolates per country and year. Countries with low incidence of gonorrhoea or gonococcal culture include isolates from outside the official window (Sept–Nov). The Euro-GASP has been shown to reflect the gonococcal antimicrobial resistance situation in the region;[6] however, the low number of isolates in some countries is a major concern. ‡Phenotypic resistance in the isolates included in the Euro-GASP 2018 genomic survey.

Table 1: **Phenotypic antimicrobial resistance* of *Neisseria gonorrhoeae* isolates and isolates included in the Euro-GASP 2018 genomic survey,[6]† by country**

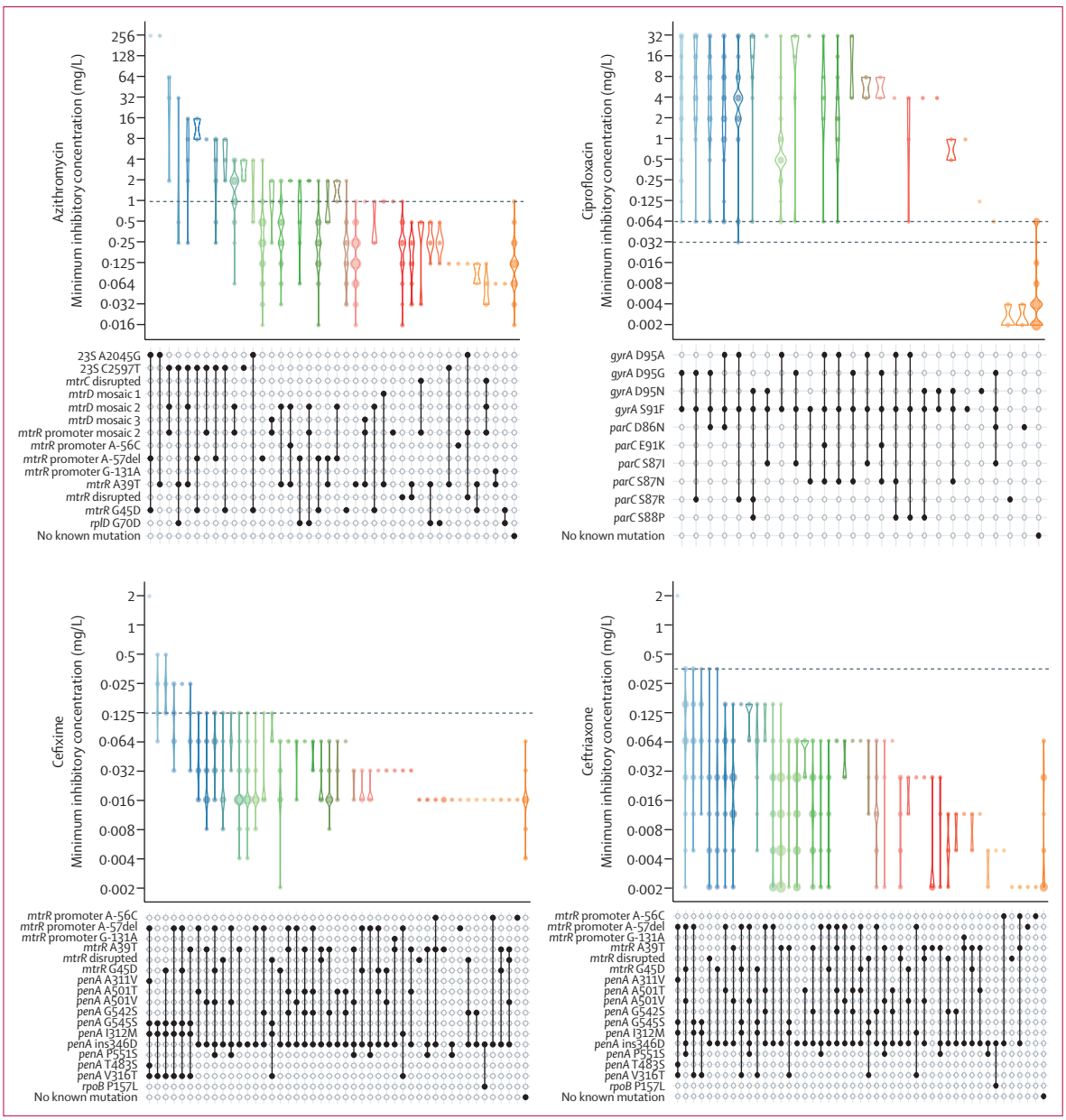

Figure 1: **Concordance between antimicrobial minimum inhibitory concentrations (phenotypes) and genetic determinants of AMR (genotypes, single or in combination) in the Euro-GASP 2018 genomic dataset**

Minimum inhibitory concentrations (mg/L) of four key antibiotics showed by the combinations of known genetic AMR determinants observed in 2375 *Neisseria gonorrhoeae* isolates. X-axis shows the AMR determinants (black dots linked with a solid black line to indicate their presence in combination) and y-axis the minimum inhibitory concentrations (coloured dots, where size of dot indicates number of isolates). Violins are coloured to indicate different combinations of AMR determinants. Dashed horizontal lines represent the epidemiological cut-off for azithromycin and clinical resistance breakpoints for ciprofloxacin, cefixime, and ceftriaxone established by the European Committee on Antimicrobial Susceptibility Testing. AMR=antimicrobial resistance. Euro-GASP=European Gonococcal Antimicrobial Surveillance Programme.

Results

The gonococcal isolates from 26 EU and EEA countries with WGS data and patient metadata linked to AMR passing quality check represented 72·0% (2375 of 3299) of the Euro-GASP 2018 isolates (table 1).[6] Of these gonorrhoea patients, 2343 (98·7%) of 2375 reported sex, of which 1992 (85·0%) of 2343 were men and 351 (15·0%) of 2343 were women. Age was reported for 2319 (97·6%) of 2375 patients, and the largest age group was 25–34 years (872 [37·6%] of 2319) followed by 24 years or younger (660 [28·5%] of 2319). The median age for women was 24 years (IQR 21–32), with 24 years or younger (181 [51·7%] of 350 women with information about sex and age) being the largest group. For men, the median age was 30 years

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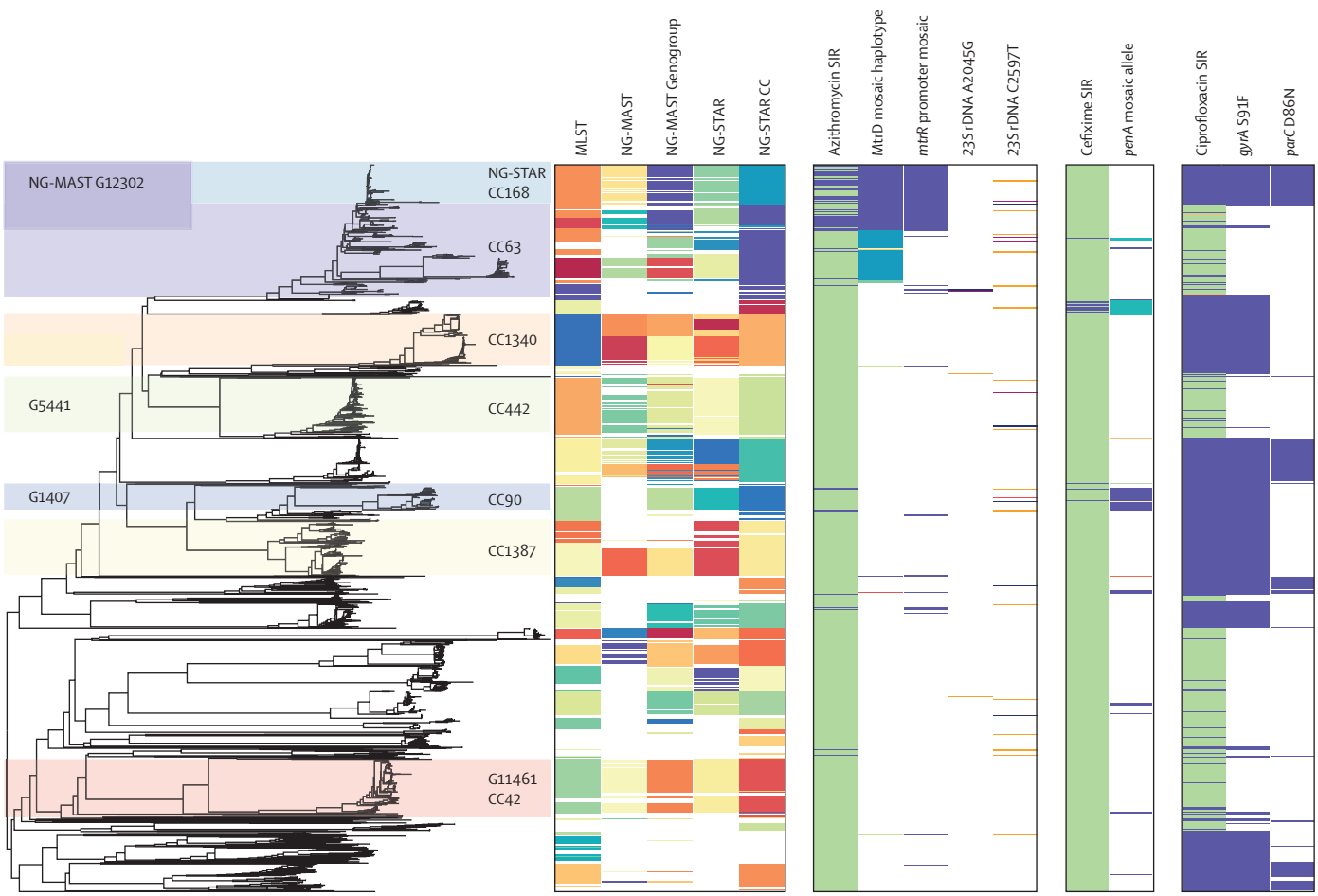
(IQR 25–39) with 25–34 years old (782 [39·7%] of 1969 men with information about sex and age) being the largest group. Sexual orientation was reported in 1494 (62·9%) of 2375 patients, of whom 785 (52·5%) were MSM, 483 (32·3%) were heterosexual men, and 225 (15·1%) were heterosexual women. 230 (9·7%) of 2375 patients reported having a concurrent sexually transmitted infection, with chlamydia the most frequent in 188 (81·7%) of 230 patients, followed by syphilis (23 [10·0%] of 230). Of the 973 (41·0%) of 2375 patients reporting HIV status, 151 (15·5%) patients were HIV positive.

The final phenotypic AMR information for the 2375 isolates is summarised by country in table 1. Ciprofloxacin resistance was the most common (1219 [51·3%] of 2375), as in the 2013 survey (562 [53·3%] of 1054).[8] Azithromycin resistance was observed in 191 (8·0%) of 2375 isolates, which is an increase compared with 2013 (71 [6·7%] of 1054, using the abandoned EUCAST clinical resistance breakpoint of MIC>0·5 mg/L).[8] Contrarily, cefixime resistance was detected in 36 (1·5%) of 2375 isolates, a decrease compared with 2013 (51 [4·8%] of 1054).[8] In 2375 isolates, ceftriaxone resistance was observed in only one (0·04%) isolate (from Spain). In the 2013 survey, five (0·5%) ceftriaxone-resistant isolates were observed in 1054 isolates.[8] Furthermore, from 2013 to 2018, the proportion of isolates with MICs of ceftriaxone of 0·016 mg/L or less increased and the proportion of isolates with MICs of ceftriaxone of 0·064 mg/L or greater decreased.[6] Ciprofloxacin resistance was detected in all 26 countries; ranging from 28·6% (two of seven) in Estonia to 77·8% (seven of nine) in Croatia. Resistance to azithromycin ranged from 0% in Latvia to 55·6% (five of nine) in Croatia. Cefixime resistance was detected in 12 (46·2%) of 26 countries; ranging from 0·9% (one of 113) in Norway to 25·0% (one of four) in Cyprus. Three (8·3%) of the 36 cefixime-resistant isolates were also azithromycin resistant.

Of the ciprofloxacin-resistant isolates, 1209 (99·2%) of 1219 carried the *gyrA* S91F mutation (MICs>0·064–32 mg/L), which was additionally present in one ciprofloxacin-susceptible isolate (MIC=0·032 mg/L) and six isolates with intermediate ciprofloxacin susceptibility (MIC=0·064 mg/L; figure 1, 2). All azithromycin-resistant isolates had at least one known azithromycin resistance determinant (figure 1, 2), with those isolates with the highest MICs having four 23S rDNA copies with the A2045G (MIC≥256 mg/L, n=2) or C2597T mutations (MIC=2–64 mg/L, n=39). Four isolates with one (n=3) or two (n=1) 23S rDNA copies with A2045G and nine isolates with one (n=7) or two (n=2) 23S rDNA copies with C2597T remained azithromycin susceptible (MIC=0·125–0·5 mg/L), which has also been previously observed.[13] 133 isolates without 23S rDNA mutations but containing the previously described[14,15] *Neisseria lactamica*-like mosaic 2 *mtrR* promoter and *mtrD* sequences (*mtr* mosaic, figure 2) showed low-level azithromycin resistance (MIC=2–4 mg/L; figure 1), whereas 80 isolates with this *mtr* mosaic remained azithromycin susceptible (MIC=0·032–1 mg/L). These 133 azithromycin-resistant isolates formed a single lineage constituted by mainly NG-MAST G12302 isolates (figure 2), which was found in 24 EU and EEA countries (figure 3; appendix 1 pp 11, 20). Some azithromycin-resistant isolates carried mutations in the *mtrR* promoter (n=26), *mtrR* (n=25), or the *rplD* G70D (n=4) mutation, or any combination of these (figure 1). The GC deletion in *mtrC* that increases antimicrobial susceptibility[16] was found in 17 isolates, of which 13 had the *mtr* mosaic[14,15] mentioned here (MICs=0·032–0·125 mg/L), and four had only a mosaic *mtrR* (MICs=0·032–0·5 mg/L). None of them had any 23S rDNA azithromycin resistance mutations. 24·1% (46 of 191) of azithromycin-resistant cases were explained by considering only 23S rDNA resistance mutations and 69·6% (133 of 191) by considering only the *mtr* mosaic (appendix 1 pp 11, 18–19).[14,15] The only ceftriaxone-resistant isolate (MIC=0·5 mg/L; azithromycin MIC=0·25 mg/L) carried the mosaic *penA* 60.001, described in the internationally spreading ceftriaxone-resistant clone FC428.[3] This strain was genomically similar to FC428 and only 131 single nucleotide polymorphisms (SNPs)-distant (49 loci) in the cgMLST scheme (appendix 1 pp 22–24), compared with over 1800 SNPs to other isolates in the same clade. All 36 cefixime-resistant isolates (MICs=0·25–2 mg/L) carried a mosaic *penA* (figure 1): 25 isolates carried mosaic *penA* 10.001 (MICs=0·25–0·50 mg/L), nine mosaic *penA* 34.001 (MIC=0·25 mg/L), 1 mosaic *penA* 60.001 (MIC=2 mg/L) and 1 mosaic *penA* 171.001 (MIC=0·25 mg/L). Notably, 114 cefixime-susceptible isolates also carried a mosaic *penA* allele. Of those, 85 carried different variants of *penA* 34 (MICs=0·032–0·125 mg/L), 25 *penA* 10.001 (MICs=0·032–0·125 mg/L), 2 *penA* 188.001 (MIC=0·064 mg/L), 1 *penA*-63·001 (MIC=0·032 mg/L), and 1 *penA* 171.001 (MIC=0·125 mg/L; figure 2).

No known target resistance mutations for the new potential treatment options zoliflodacin or gepotidacin were found.[17–19]

MLST, NG-MAST, and NG-STAR sequence types across countries are shown in appendix 1 (pp 14–15, 24–25). 189 MLST (34 with >10 isolates), 419 NG-STAR (33 with >10 isolates) and 273 NG-MAST (30 with >10 isolates) sequence types were detected (figure 2, table 2). The most abundant sequence types in the 2375 isolates were MLST sequence type(ST)9363, found in 221 (9·3%) isolates, NG-STAR ST442, in 162 (6·8%) isolates, and NG-MAST ST11461, in 112 (4·7%) isolates (table 2). Of the 2375 isolates, 1592 (67·0%) isolates were assigned to an NG-MAST genogroup and 2329 (98·1%) to an NG-STAR CC (appendix 1 pp 10, 14–15). Using an Analysis of Molecular Variance, most of the genotypic variation was found within countries (96·9%) rather than between them (3·0%). No significant population structure was detected by randomising population structure (p=0·55). The three most abundant NG-MAST genogroups were G12302 (132 [5·6%] of 2375; 89 of



Typing

MLST
1580, 1583, 1587, 1588, 1594, 1596, 1599, 1901, 7359, 7363, 7822, 7827, 8135, 8143, 8156, 9363, 10314, 11177, 11422, 13292

NG-MAST
387, 1993, 3935, 5441, 9208, 10386, 11461, 12302, 14700, 14769, 14994, 15589

NG-MAST Genogroup
G12302, G384, G10386, G2400, G4186, G2992, G1407, G5441, G10800, G15589, G14994, G387, G14769, G11461, G14700, G9208, G1993

NG-STAR
20, 38, 63, 90, 158, 168, 193, 231, 439, 442, 520, 539, 568, 729, 1225, 1340, 1387, 3543

NG-STAR CC
CC63, CC90, CC168, CC38, CC158, CC231, CC442, CC307, CC20, CC1387, CC352, CC1340, CC426, CC3087, CC42, CC348

AMR

AMR (SIR)
S, I, R

Presence genetic AMR determinant

23S mutations copy number
1, 2, 3, 4

MtrD mosaic allele
1, 2, 3, 4, 5, 6, 7, 8

***penA* mosaic allele**
penA-34, *penA*-10, *penA*-171, *penA*-188, *penA*-63, *penA*-60

Figure 2: **Main *Neisseria gonorrhoeae* molecular sequence types and antimicrobial-resistant lineages in the EU and EEA in 2018**
Phylogenetic reconstruction of 2375 *N gonorrhoeae* isolates from 26 European countries from 2018. Main NG-MAST genogroups (G) and NG-STAR CCs are highlighted in different colours. First block of columns shows the assignment of each isolate to the three typing schemes: MLST, NG-MAST, and NG-STAR, including NG-MAST genogroups and NG-STAR CCs. Only types with more than 30 isolates are shown for visualisation purposes. The blocks represent AMR data in the form of SIR for azithromycin, cefixime, and ciprofloxacin followed by themain genetic resistance determinants for each. MtrD mosaic alleles represent eight different haplotypes found for the amino acid sequence of the *mtrD* gene in this dataset. AMR=antimicrobial resistance. CC=clonal complex. MLST=multi locus sequence typing. NG-MAST=*N gonorrhoeae* multi-antigen sequence typing. NG-STAR=*N gonorrhoeae* sequence typing for antimicrobial resistance. SIR=susceptible, intermediate (susceptible, increasedexposure), resistant.

them NG-STAR CC168 and 35 CC63), G5441 (132 [5·6%] of 2375; 120 CC442) and G11461 (128 [5·4%] of 2375; 123 CC42), which all increased by 5% or more compared with previous Euro-GASP surveys[7,8] (figure 2; appendix 1 pp 24–25, appendix 2 pp 1–2). NG-MAST ST12302 (mostly MLST ST9363 and NG-STAR ST168) is an emerging clone that was not found in the previous surveys (table 2),[7,8] whereas the second major sequence

See Online for appendix 2

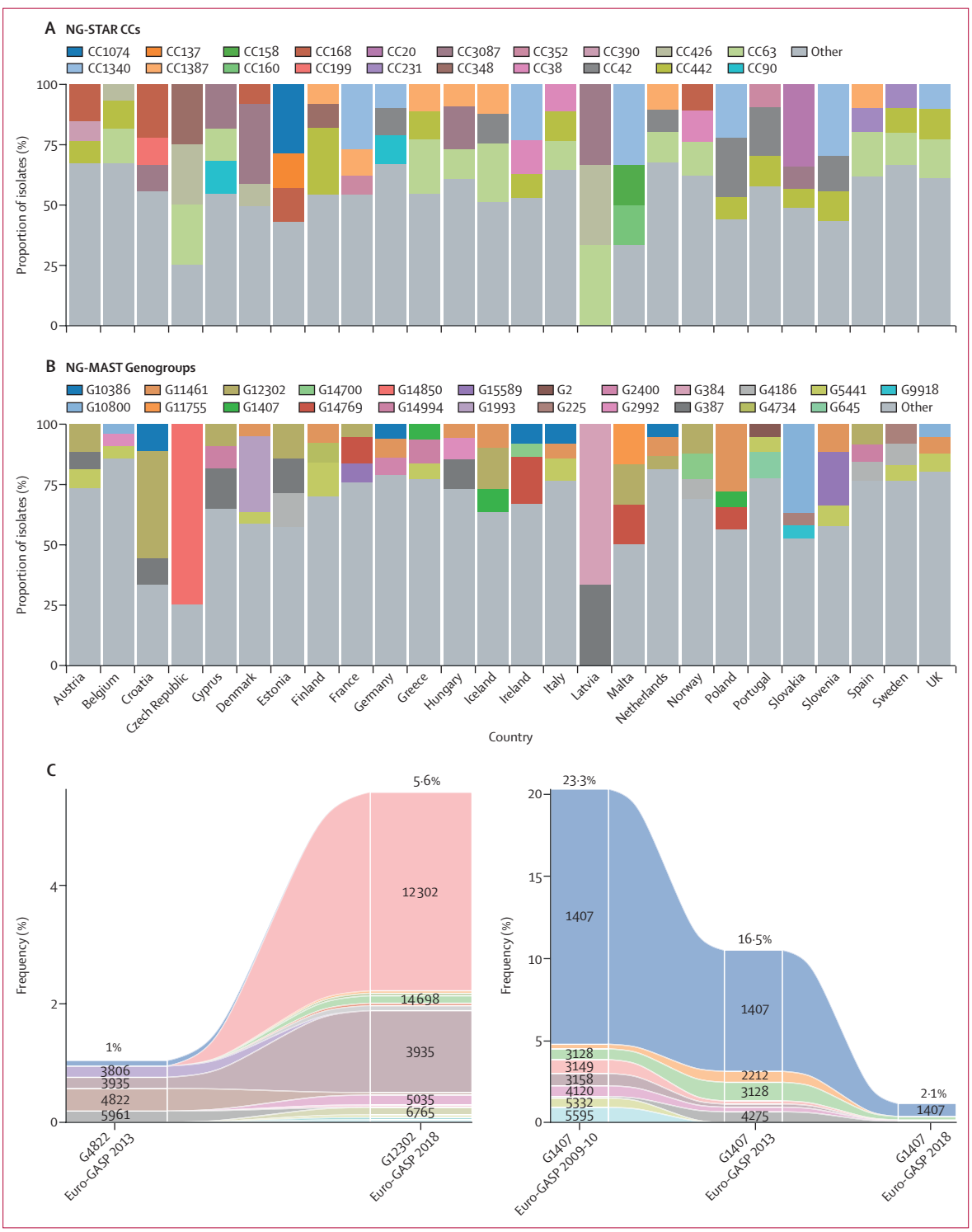


Figure 3: **Distribution of the most prevalent NG-STAR CCs and NG-MAST genogroups in Europe in 2018 compared to 2009–10[7] and 2013;[8] expansion of NG-MAST G12302 and remission of G1407**

(A) Proportion of isolates assigned to the top three NG-STAR CCs (in each country, and 'Other' includes all other CCs in the specific country) found in the Euro-GASP 2018 genomic study in each participating country. (B) Proportion of isolates assigned to the top three NG-MAST genogroups (in each country, and 'Other' includes all other genogroups in the specific country), found in the Euro-GASP 2018 genomic study in each participating country. (C) Left panel shows the increase of 5% in frequency of NG-MAST G12302 from 2013 to 2018 (reaching 5·6% (132/2375) of all isolates in the present Euro-GASP 2018 WGS survey) mostly due to the substantial expansion of NG-MAST ST12302. Other NG-MAST STs within this genogroup, including the second major ST3935 were part of G4822 in the Euro-GASP 2013 survey[8] and absent in 2009–10.[7] Right panel shows the sharp decrease in frequency of NG-MAST G1407 with resistance or decreased susceptibility to cefixime in the three Euro-GASP surveys, which decreased by >14% from 2013[8] to 2018 (reaching 2·1% [51/2375] of all isolates in the present Euro-GASP 2018 WGS survey). Notably, the frequencies between the molecular study time points (2009–10,[7] 2013[8] and 2018) are unknown, and only drawn to illustrate possible changes over time. CC=clonal complex. Euro-GASP=European Gonococcal Antimicrobial Surveillance Programme. NG-MAST=*Neisseria gonorrhoeae* multi-antigen sequence typing. NG-STAR=*N gonorrhoeae* sequence typing for antimicrobial resistance.

NG-MAST ST (n)	Mean patient age (range)	Number of patients with known gender	% male patients (n)	Number of patients with known sexual orientation (n men)	% heterosexual men and women (n)	% men who have sex with men (n)	Most common MLST STs (n)	Most common NG-STAR types (n)
11461 (112)	31·4 (18–55)	110	95·5% (105)	61 (60)	18·0% (11)	82·0% (50)	1599 (109)	520 (107)
5441 (88)	33·3 (19–63)	87	90·8% (79)	60 (54)	36·7% (22)	63·3% (38)	8156 (76)	442 (72)
12302 (80)	32·2 (16–69)	79	87·3% (69)	45 (41)	40·0% (18)	60·0% (27)	9363 (80)	168 (79)
14994 (77)	31·7 (19–67)	75	96·0% (72)	56 (53)	26·8% (15)	73·2% (41)	7822 (72)	1387 (74)
14769 (71)	32·7 (18–74)	71	97·2% (69)	52 (51)	9·6% (5)	90·4% (47)	1583 (71)	539 (35), 3543 (34)
15589 (67)	35·0 (18–68)	66	100% (66)	50 (50)	12·0% (6)	88·0% (44)	1583 (66)	1340 (62)
10386 (47)	32·7 (21–58)	46	95·7% (44)	29 (28)	17·2% (5)	82·8% (24)	7827 (47)	38 (46)
9208 (42)	32·3 (19–67)	42	97·6% (41)	35 (35)	17·1% (6)	82·9% (29)	13292 (39)	439 (42)
387 (40)	30·5 (19–53)	39	71·8% (28)	15 (9)	80·0% (12)	20·0% (3)	8135 (32)	729 (30)
14700 (34)	32·7 (20–50)	34	97·1% (33)	16 (16)	..	100% (16)	7827 (19), 13489 (15)	1225 (33)
1993 (33)	25·5 (18–44)	33	45·5% (15)	29 (14)	100% (29)	..	11177 (31)	568 (32)
3935 (32)	34·2 (21–55)	32	96·9% (31)	18 (17)	27·8% (5)	72·2% (13)	11422 (23), 9363 (9)	193 (26)
4186 (30)	29·6 (18–58)	29	69·0% (20)	15 (11)	100% (15)	..	7359 (29)	231 (23), 2171 (6)
10800 (28)	30·2 (0–66)	28	75·0% (21)	18 (14)	100% (18)	..	1594 (25)	20 (24)
2400 (23)	32·9 (17–58)	21	90·5% (19)	13 (11)	76·9% (10)	23·1% (3)	7363 (21)	158 (16)
2992 (22)	36·9 (21–72)	22	90·9% (20)	13 (12)	53·8% (7)	46·2% (6)	11428 (12), 11864 (6)	63 (15), 439 (6)
12547 (20)	29·8 (16–52)	19	94·7% (18)	13 (12)	15·4% (2)	84·6% (11)	10314 (12)	1387 (20)
1407 (19)	28·4 (19–61)	19	47·4% (9)	3 (1)	100% (3)	..	1901 (19)	90 (19)
9909 (19)	28·5 (19–45)	19	63·2% (12)	17 (12)	58·8% (10)	41·2% (7)	7359 (19)	231 (19)
13489 (19)	36·7 (21–67)	19	100% (19)	9 (9)	22·2% (2)	77·8% (7)	8156 (18)	442 (13)
225 (17)	34·1 (19–55)	17	88·2% (15)	15 (13)	26·7% (4)	73·3% (11)	1580 (15)	884 (15)
51 (16)	25·8 (16–41)	16	50·0% (8)	14 (7)	100% (14)	..	11990 (11)	20 (6)
2 (15)	25·2 (16–44)	14	71·4% (10)	7 (4)	71·4% (5)	28·6% (2)	11975 (9)	84 (14)
5743 (15)	23·9 (15–37)	15	73·3% (11)	12 (8)	100% (12)	..	8135 (15)	729 (15)
5624 (14)	37·3 (24–62)	13	100% (13)	7 (7)	..	100% (7)	8143 (12)	426 (11)
13252 (14)	32·2 (22–48)	12	100% (12)	10 (10)	20·0% (2)	80·0% (8)	1596 (14)	307 (14)
14764 (14)	34·8 (23–59)	12	100% (12)	7 (7)	..	100% (7)	1599 (14)	520 (14)
645 (12)	31·3 (17–47)	12	83·3% (10)	3 (2)	100% (3)	..	15680 (11)	520 (12)
9918 (12)	27·2 (19–41)	12	83·3% (10)	6 (5)	66·7% (4)	33·3% (2)	8143 (9)	436 (10)
5793 (11)	31·6 (21–45)	11	100% (11)	7 (7)	..	100% (7)	11516 (9)	55 (11)

Euro-GASP=European Gonococcal Antimicrobial Surveillance Programme. NG-MAST=*Neisseria gonorrhoeae* multi-antigen sequence typing. NG-STAR=*N gonorrhoeae* sequence typing for antimicrobial resistance. MLST=multi-locus sequence typing. ST=sequence type.

Table 2: **Characteristics of patients infected with the most frequently observed NG-MAST, MLST, and NG-STAR sequence types, Euro-GASP 2018 genomic survey**

type in G12302, ST3935 (mostly MLST ST9363 or ST11422 and NG-STAR ST193), was found within G4822 in 2013[8] (figure 3C). Genogroup G1407 (CC90) was the most abundant in 2009–10[7] (248 [23·3%] of 1066) and 2013[8] (174 [16·5%] of 1054), but only included 51 (2·1%) of 2375 isolates in 2018, showing a significant decrease (figure 3B, C). NG-MAST ST1407 (MLST ST1901 and NG-STAR ST90) also significantly decreased; 15·6% (166 of 1066) in 2009–10,[7] 7·4% (78 of 1054) in 2013,[8] and 0·8% (19 of 2375) in 2018 (appendix 2 pp 1–2). The four major NG-STAR CCs found in 2375 isolates in 2018 were NG-STAR CC63 (270 [11·4%]), CC442 (219 [9·2%]), CC1340 (166 [7·0%]) and CC1387 (165 [6·9%]). These results contrast with a recent study that mostly included data on isolates from Euro-GASP 2013 and where CC90 was the major complex (194 [18·0%] of 1075)[20] followed by CC63 (166 [15·4%] of 1075). In 2018, 90 (3·8%) of 2375 isolates clustered in CC90, also supporting the 14% decrease in this AMR lineage as calculated for the associated NG-MAST G1407.

Of the three largest NG-MAST genogroups, G12302 did not show any significant association with an age group, gender, sexual orientation, or site of infection. However, NG-STAR CC63, one of the major CCs within

this genogroup, was associated with MSM (odds ratio [OR] 1·8 [95% CI 1·3–2·5], p<0·0001), men (OR 2·4 [1·5–3·9], p<0·0001), and pharyngeal (OR 2·2 [1·5–3·2], p<0·0001) infections (appendix 2 p 7, 10). Both NG-MAST G5441 and G11461 were associated with men, and G11461 also with MSM (OR 4·9 [2·6–9·6], p<0·0001; appendix 2 p 6). NG-MAST ST11461 was the major sequence type found in 2018 (table 2) and was associated with men and MSM (OR 4·3 [2·2–8·4], p<0·0001; appendix 2 p 3). The major NG-STAR CCs associated with NG-MAST G5441 (CC442) and G11461 (CC42) also supported an association with men and MSM (appendix 2 p 7, 10). NG-MAST G1407 was associated with MSM in 2009–10[7] and heterosexuals in 2013.[8] In 2018, G1407 (CC90), was exclusively found in heterosexual patients and was associated with women (OR 2·9 [1·6–5·3], p<0·0001; appendix 2 p 6, 9).

The gonococcal lineages with the strongest association with azithromycin resistance were within NG-MAST G12302 (OR 102·6 [61·6–170·1], p<0·0001), including the two major sublineages characterised by NG-MAST ST12302 (MLST ST9363, NG-STAR CC168) and NG-MAST ST3935 (MLST ST9363 or ST11422, NG-STAR CC63; appendix 2 p 11). Cefixime-resistant isolates were strongly associated with NG-MAST G13876 (OR 452·1 [109·8–2342·7], p<0·0001), NG-STAR CC348, and several others, including NG-MAST G1407 (OR 14·5 [5·3–39·6], p<0·0001) (MLST ST1901, NG-STAR CC90). Resistance to ciprofloxacin was associated with several lineages, including STs of the three schemes exclusively formed by ciprofloxacin-resistant isolates. An increased MIC of ceftriaxone was associated with several sequence types, with the top lineage being NG-STAR ST38 (OR 18·2 [13·3–24·9], p<0·0001; MLST ST7827, NG-STAR CC38, and NG-MAST G10386). NG-MAST G13876 (CC348) and G1407 (CC90) were also associated with an increased ceftriaxone MIC (appendix 2 p 11).

Discussion

We report the genomic analysis of the Euro-GASP 2018 survey in the EU and EEA in comparison with the 2013 genomic and 2009–10 NG-MAST surveys.[7,8] Since 2013,[8] cefixime resistance has declined from 4·8% (51 of 1054) to 1·5% (36 of 2375) and azithromycin resistance increased from 6·7% (71 of 1054, using the abandoned EUCAST clinical azithromycin resistance breakpoint of MIC>0·5 mg/L)[8] to 8·0% (191 of 2375). Promisingly, only one isolate was ceftriaxone resistant in 2018. The changes in AMR levels were associated with fluctuations in the gonococcal population. In particular, the introduction or emergence and subsequent expansion of a single novel NG-MAST genogroup, G12302 (NG-STAR CC168 and CC63), was responsible for the European-wide spread of azithromycin resistance, mostly associated with pharyngeal infections in MSM (especially in CC63). Azithromycin resistance in this genogroup is driven by mosaic sequences in the *mtrR* promoter and *mtrD* gene of the MtrRCDE efflux pump system.[14,15] Likewise, the progressive reduction in NG-MAST G1407 (NG-STAR CC90) largely explains the increasing susceptibility to extended-spectrum cephalosporins. This genogroup was the most abundant in the 2009–10[7] (248 [23·3%] of 1066) and 2013 (174 [16·5%] of 1054) surveys,[8] but sparse in 2018, with only 51 (2·1%) of 2375 NG-MAST G1407 isolates. Of note, G1407 was associated with MSM in 2009–10[7] and with heterosexual patients in 2013,[8] whereas it was exclusively found in heterosexuals and associated with women in 2018. The decline in G1407 and cefixime resistance, exceedingly low level of ceftriaxone resistance (one [0·04%] of 2375), and a ceftriaxone MIC distribution shifting to lower MICs, is reassuring for the continued use of ceftriaxone as first-line gonorrhoea treatment.[21] However, the emergence and spread of the frequently azithromycin-resistant NG-MAST G12302 with an *mtr* mosaic sequence could threaten the future effectiveness of the ceftriaxone–azithromycin dual therapy.[21] Nevertheless, only three (0·1%) of 2375 isolates in 2018 were resistant to both azithromycin and cefixime and concomitant resistance to ceftriaxone and azithromycin has been exceedingly rare internationally, which indicate that the occasional ceftriaxone-resistant gonorrhoea cases identified are possible to cure with azithromycin. Furthermore, it remains unknown whether the predominantly low-level azithromycin resistance (MIC=2–4 mg/L; 166 [86·9%] of 191 in 2018) causes treatment failure with azithromycin 2 g.[21] An evidence-based clinical azithromycin resistance breakpoint is needed.

In *N gonorrhoeae,* azithromycin resistance has frequently been associated with 23S rDNA mutations sporadically acquired by isolates with limited phylogenetic relationship.[8,13] Recombination in the loci encoding the MtrRCDE efflux pump have also been proven to confer resistance to azithromycin.[14] Here, we explain the genetic basis of azithromycin resistance for 179 (93·7%) of 191 isolates by only considering 23S rDNA mutations and presence of *N lactamica*-like mosaic 2 *mtrR* promoter and *mtrD* sequences.[14,15] Many isolates (80 [37·6%] of 213) with identical or similar mosaic *mtrR* promoter and *mtrD* sequences remained azithromycin susceptible; however, most of these isolates had an increased azithromycin MIC close to the epidemiological cutoff value (65 [81·3%] of 80; MIC=0·5–1 mg/L). The mosaic *mtrR* promoter and *mtrD* sequences found in this collection were similar to those found in other studies,[14,15] *N lactamica*-like mosaic 2 being the most frequent *mtr* mosaic in Europe. This emerging lineage was recently described in a study that combined worldwide public *N gonorrhoeae* genomic data and has been reported in several countries.[15,22] The *rplD* G70D mutation in the 50S ribosomal protein L4 is another azithromycin resistance determinant.[23] We found *rplD* G70D in 34 isolates but only four (11·8%) of 34 were azithromycin resistant and three (75·0%) of these four isolates had

four 23S rDNA C2597T mutated alleles (n=1) or mosaic *mtrR* promoter and *mtrD* sequences (n=2). Accordingly, we did not observe an obvious effect of *rplD* G70D on azithromycin resistance.

Only one ceftriaxone-resistant isolate was found. This Spanish isolate indicates international transmission of the ceftriaxone-resistant FC428 strain[24] rather than an isolate that has independently acquired the *penA* 60.001 allele as the genomic comparison with FC428 showed that both isolates are genomically very close. FC428 was initially described in Japan[24] in 2015 and subsequently in Canada, Denmark, France, and Australia in 2017.[3] Soon after, FC428 was also detected in the UK and Ireland, proving the international dissemination of this clone.[2–4] Since then, sustained transmission of this clone has been reported in China and Japan.[25,26] Mutations in subunits of the RNA polymerase have been associated with cephalosporin resistance,[27] including *rpoB* P157L, which was found in one isolate in combination with the PBP2 (*penA*) D345 insertion (ins346D in Pathogenwatch).[15] This isolate, however, was highly susceptible to cefixime (MIC<0·016 mg/L) and ceftriaxone (MIC=0·003 mg/L).

Ciprofloxacin resistance was by far the most extended and sustained in time. The gonococcal population in the EU and EEA includes several lineages that have acquired and maintained the *gyrA* S91F and D95A, D95G, or D95N mutations, causing high-level ciprofloxacin resistance. Of particular worry is also the presence of the *parC* D86N mutation in some of these lineages (397 [16·7%] of 2375). This mutation might not substantially increase the MIC of ciprofloxacin on its own (figure 1; ie, when mutations in the main ciprofloxacin target [GyrA] are absent). However, the *parC* D86N mutation increases the ciprofloxacin MICs when the *gyrA* S91F mutation is present, and can additionally predispose for the development of resistance to the new topoisomerase II inhibitor gepotidacin,[17] currently in phase 3 randomised controlled clinical trial (RCT) for treatment of uncomplicated gonorrhoea. Gepotidacin, as ciprofloxacin, is targeting GyrA and ParC and gonococcal isolates with the *parC* D86N mutation can select a gepotidacin-resistance mutation also in the GyrA target (*gyrA* A92T) during treatment,[18] which causes clinical gepotidacin resistance.[17,18] No *gyrA* A92 mutations were found in the Euro-GASP 2018 dataset. Another promising new treatment for uncomplicated gonorrhoea in phase 3 RCT is zoliflodacin, a topoisomerase II inhibitor whose main target is GyrB.[19] Zoliflodacin-resistance mutations in *gyrB* D429 and K450 have been selected in vitro. However, in the Euro-GASP 2018 material no mutations in *gyrB* D429 and K450 were found, indicating full susceptibility to zoliflodacin.[19] Regarding spectinomycin resistance, no 16S rDNA C1192T or *rpsE* mutations were found,[28,29] indicating that this antimicrobial can also be continuously used for the treatment of gonorrhoea where available.

The main limitations of Euro-GASP include the low number and suboptimal representativeness of isolates from some countries, and low coverage of reporting of several epidemiological variables—eg, sexual orientation.[5,6,8,10,12] However, it has been recently shown that the overall AMR prevalence reported by Euro-GASP appropriately reflects the AMR situation in the EU and EEA.[5] Nevertheless, increased numbers of representative isolates remain crucial in Euro-GASP, which is also a continuous work of Euro-GASP. Furthermore, the selection of one isolate per patient or infection episode through a sampling hierarchy could have slightly biased conclusions regarding associations with infection sites. Finally, in the present Euro-GASP 2018 WGS study some isolates were not available or viable for confirmatory WGS and phenotypic AMR testing (including several of the discrepant ciprofloxacin-resistant isolates).

In summary, the EU and EEA gonococcal population in 2018 shows the emergence and subsequent expansion of an azithromycin-resistant clone (NG-MAST G12302), which largely explains the increase in azithromycin resistance during recent years, associated with pharyngeal infections among MSM (NG-STAR CC63), and carrying mosaic *mtrR* promoter and *mtrD* sequences. Nevertheless, many isolates with similar mosaic *mtrR* promoter and *mtrD* sequences remained azithromycin susceptible, despite an increased azithromycin MIC. Continued research to identify and verify novel determinants associated with resistance to azithromycin and other current and future gonorrhoea treatment options remains imperative. Contrarily, NG-MAST G1407 (NG-STAR CC90), a frequently extended-spectrum cephalosporin-resistant clone, carrying a mosaic *penA*, that was predominant in previous Euro-GASP molecular surveys,[7,8] is progressively disappearing, which largely elucidates the decrease in cefixime resistance and increase in ceftriaxone susceptibility in recent years. The spread of the emerged azithromycin-resistant clone could threaten the effectiveness of the current recommended dual treatment for gonorrhoea (ceftriaxone plus azithromycin)[21] but fortunately, cases of ceftriaxone resistance in the EU and EEA are scarce and combined ceftriaxone and azithromycin resistance is exceedingly rare (so azithromycin might eradicate the occasional ceftriaxone-resistant strains). Furthermore, most azithromycin-resistant isolates have low-level azithromycin resistance (MIC=2–4 mg/L) and it remains unknown whether these MICs translate into clinical resistance when using azithromycin 2 g treatment (an evidence-based clinical resistance breakpoint is required). Ultimately, new gonorrhoea treatments such as zoliflodacin[19] or gepotidacin,[17,18] which are both in phase 3 RCTs and for which the EU and EEA gonococcal population appears susceptible, will be crucial. The Euro-GASP genomic surveys show that WGS is essential to complement epidemiological and AMR information for quality-assured molecular epidemiology and surveillance of gonococci, including effective molecular AMR prediction, locally, nationally, and internationally.

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Contributors
LSB, MJC, GS, DMA, and MU designed, initiated, and coordinated the study. The European STI network members supplied gonococcal isolates and patient data. MD, SJ, DG, and NS did the main laboratory work. LSB analysed and interpreted the data with support by MD, MJC, SJ, DMA, and MU. LSB with support by MU wrote a first draft of the paper. LSB, MJC, GS, MD, SJ, DG, NS, CAY, KA, AU, BB, DMA, and MU read, commented on, and approved the final manuscript. The first author (LSB) and corresponding authors (DMA and MU) had full access to and verified all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests
We declare no competing interests.

Data sharing
Most data collected and analysed in this study are included in the main paper or appendices. However, remaining datasets can be made available from the corresponding author after publication on reasonable request.

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