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Molecular Analysis of Rising Fluoroquinolone Resistance in Belgian Non-Invasive *Streptococcus pneumoniae* Isolates (1995-2014)

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Abstract

We present the results of a longitudinal surveillance study (1995–2014) on fluoroquinolone resistance (FQ-R) among Belgian non-invasive *Streptococcus pneumoniae* isolates (n = 5,602). For many years, the switch to respiratory fluoroquinolones for the treatment of (a) typical pneumonia had no impact on FQ-R levels. However, since 2011 we observed a significant decrease in susceptibility towards ciprofloxacin, ofloxacin and levofloxacin with peaks of 9.0%, 6.6% and 3.1% resistant isolates, respectively. Resistance to moxifloxacin arised sporadically, and remained <1% throughout the entire study period. We observed classical topoisomerase mutations in *gyrA* (n = 25), *parC* (n = 46) and *parE* (n = 3) in varying combinations, arguing against clonal expansion of FQ-R. The impact of recombination with co-habiting commensal streptococci on FQ-R remains marginal (10.4%). Notably, we observed that a rare combination of DNA Gyrase mutations (GyrA_S81L/GyrB_P454S) suffices for high-level moxifloxacin resistance, contrasting current model. Interestingly, 85/422 pneumococcal strains display MIC_{CIP} values which were lowered by at least four dilutions by reserpine, pointing at involvement of efflux pumps in FQ-R. In contrast to susceptible strains, isolates resistant to ciprofloxacin significantly overexpressed the ABC pump PatAB in comparison to reference strain *S. pneumoniae* ATCC 49619, but this could only be linked to disruptive terminator mutations in a fraction of these. Conversely, no difference in expression of the Major Facilitator PmrA, unaffected by reserpine, was noted between susceptible and resistant *S. pneumoniae* strains. Finally, we observed that four isolates displayed intermediate to high-level ciprofloxacin resistance without any known molecular resistance mechanism. Focusing future molecular studies on these isolates, which are

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also commonly found in other studies, might greatly assist in the battle against rising pneumococcal drug resistance.

Introduction

Streptococcus pneumoniae is a major cause of community-acquired respiratory infections including otitis media and pneumonia, as well of serious invasive infections like septicaemia and meningitis [1]. Penicillins and macrolides were mainstay in the treatment of respiratory diseases for decades [2], but the worldwide spread of drug-resistant clones translated into increased usage of fluoroquinolones [3,4]. Fluoroquinolones are synthetic, broad-spectrum antibiotics targeting the DNA gyrase (GyrA/B) and topoisomerase IV (ParC/E) enzymes, which are critically involved in DNA supercoiling and chromosome segregation, respectively [5]. The early fluoroquinolones ciprofloxacin (CIP) and ofloxacin (OFL) target ParC and display poor potency against pneumococci, rapidly leading to emergence of resistance [6]. In the late 1990s, they were replaced by the so-called "respiratory fluoroquinolones levofloxacin (LVX; the active isomer of ofloxacin) and moxifloxacin (MXF) that acts on both enzymes [2]. In Belgium, this has been reflected by steadily declining sales of OFL and norfloxacin while, in contrast, the use of MXF has markedly increased since 2009 (Fig 1) and will probably further expand as its patent has recently expired. Since the global switch to LVX and MXF was established, the worldwide prevalence of fluoroquinolone resistance (FQ-R) in *S. pneumoniae* remained below 2% [7] Moreover, it seems unrelated to the serotype switches that were observed upon the introduction of 7- and 13-valent pneumococcal conjugate vaccination [8].

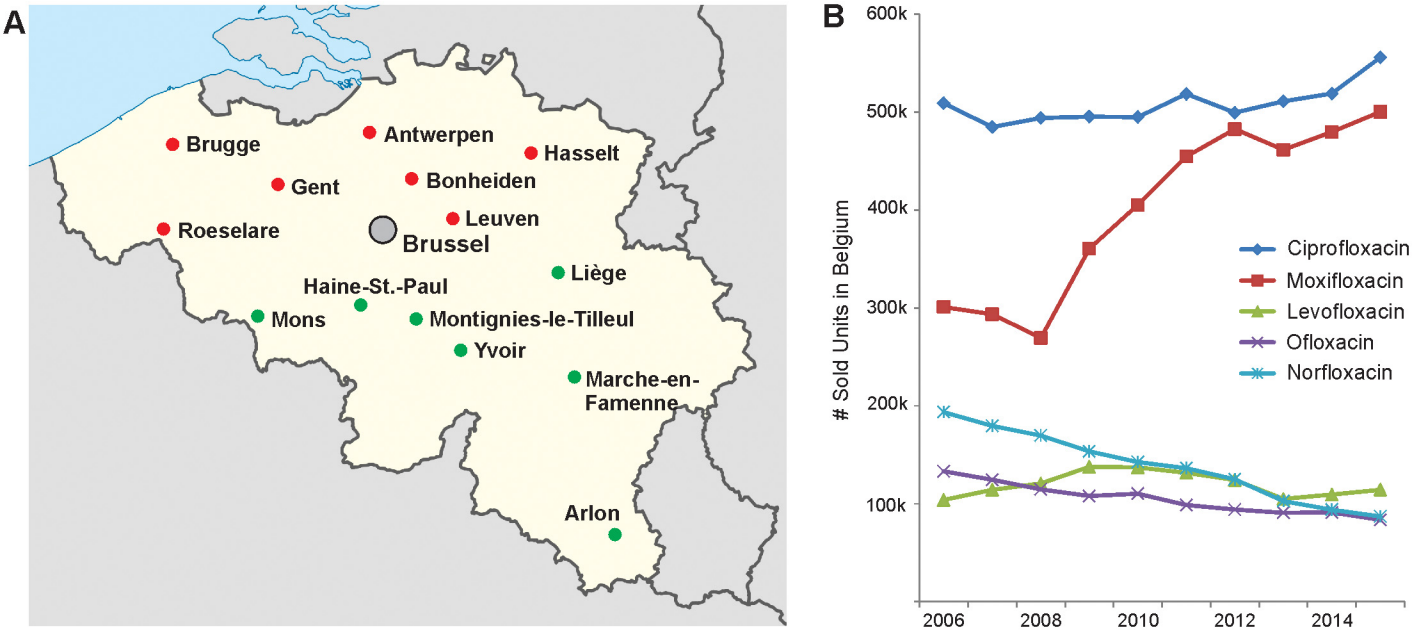

Fig 1. Surveillance of Fluoroquinolone resistance in Belgian non-invasive *S. pneumoniae* isolates. A. Clinical laboratories participating in the survey. Participating Flemish and Walloon laboratories are indicated in red and green, respectively. B. Evolution of the total Belgian fluoroquinolone use over the last decade, expressed as yearly sold units of the five main fluoroquinolones (source: IMS dataview, data December 2015).

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Genetic analyses showed that a first mechanism of FQ-R is through stepwise accumulation of spontaneous mutations in the quinolone resistance determining regions (QRDR) of *gyrA* and *parC*, and rarely *gyrB* and *parE* [9]. The effect of a given mutation depends on the genetic context and the type of fluoroquinolone used [10]. ParC mutations at positions 79 and 83 are most frequently found among pneumococci and are associated with CIP and LVX usage [11]. These first-step mutations lead to a dramatic increase in mutant prevention concentration of all fluoroquinolones [12], enabling prompt selection of secondary and tertiary QRDR mutations in GyrA (mainly positions 81 and 85) required for the FQ-R phenotype [3]. Unlike β-lactam and macrolide resistance mechanisms, QRDR mutations do not appear to be clonally spread and only a minor fraction (0.5–10%) stems from recombination with co-habiting commensal streptococci of the viridans group [13].

In recent years, the role of efflux in low-level *S. pneumoniae* FQ-R has become more and more appreciated. Beyond causing a moderate increase in MIC, increased efflux is indeed associated with rising mutational frequencies in the QRDRs [14]. Gene disruption experiments, expression analyses and susceptibility testing in the presence of the efflux pump inhibitors led to current consensus that two distinct transporters, PmrA and PatA/B, are capable of fluoroquinolone efflux [15–19]. The Major Facilitator Superfamily pump PmrA, however, is reported as intrinsically inactive and non-inducible under CIP pressure [20]. More clinical relevance is therefore attributed to the reserpine-sensitive heterogenic ABC efflux pump PatAB. Deletion of this pump in a laboratory strain led to hypersusceptibility to CIP [21], and its expression is induced in the presence of CIP [20]. Moreover, constitutive overexpression of *patA/B* was observed in roughly one-third of clinical isolates with low-level FQ-R [17], and is linked to gene duplication and disruptive mutations in the transcriptional attenuator upstream *patA* [22–24]. Recently, point mutations in PatA were associated with increased CIP resistance by putative enhanced substrate binding [25].

Although most *S. pneumoniae* surveillance studies focus on bacteraemia, recent work estimated that for every adult bacteraemic case there are three non-invasive infections [26]. In this paper, we present data on FQ-R in non-invasive pneumococci from a longitudinal surveillance program across Belgian clinical laboratories (1995–2014), spanning the world-wide transit era between the use of early (CIP, OFL) and newer (LVX, MXF) fluoroquinolones. We noted that resistance against the early drugs are markedly on the rise since 2011. By studying the molecular background to dissect the relative contribution of target site mutations versus drug efflux, we identified interesting pneumococcal isolates which confer FQ-R through yet uncharacterized mechanisms.

Materials and Methods

Bacterial strains

Non-invasive respiratory clinical isolates of *S. pneumoniae* were collected during winter seasons between 1995 and 2014 in 15 clinical laboratories throughout Belgium by members of The Belgian *Streptococcus pneumoniae* Study Group. The access to patient information was encrypted. All isolates were kept at −70°C in Brain Heart Infusion Broth (Difco) containing 10% (v/v) glycerol until transfer to the Scientific Institute of Public Health for susceptibility testing and downstream molecular analyses. The identification of each isolate made by the participating laboratories was confirmed using PCR targeting the autolysin encoding gene *lytA* [26], slide agglutination (Slidex pneumo Kit™, BioMérieux, Marcy-l'Étoile, France) and Optochin (Opto-F, bioMérieux) tests, all performed according to the manufacturer's instructions. For selected strains, capsular sequence typing (CST) was performed by sequence analysis of the *wzh* gene using a dedicated web application (http://www.rivm.nl/mpf/spn/cst/)[27].

Antibiotic susceptibility testing

For each isolate, the minimal inhibitory concentration (MIC) was determined by broth microdilution as recommended by the US Clinical and Laboratory Standards Institute (CLSI; called National Committee for Clinical Laboratory Standards (NCCLS) at the onset of the study in 1997. The following fluoroquinolones were provided as laboratory standards with known potency by the manufacturers of the original products: levofloxacin and ofloxacin from Aventis Pharma (Mumbai, India), CIP and MXF from Bayer (Leverkusen, Germany). All antibiotics were tested for 16 serial twofold dilutions (0.001–32 μg/mL), with *S. pneumoniae* ATCC 49619 [28,29], *S. pneumoniae* TPN 881, *Staphylococcus aureus* NCTC 11561 and *S. aureus* ATCC 29123 being included as quality control organisms in each series (S1 Table). Interpretation of the results was based on the breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org/). To assess possible synergy between fluoroquinolones and efflux pump inhibitors, commercial E-tests of CIP and MXF (bioMérieux) were applied on MH Blood agar plates containing 0 and 20 μg/mL reserpine. This method was devised after observing that reserpine causes turbidity of broth, preventing a correct reading of the results of the microdilution assay.

Determination of FQ-R related sequences

The DNA sequences of the QRDRs in *gyrA*, *gyrB*, *parC* and *parE* genes, and of the regulatory regions and coding sequences of *patA* and *patB* were determined by PCR sequencing using the primers listed in S2 Table. All sequences were screened for SNPs in comparison to corresponding regions of FQ-sensitive clinical strains using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/); the stability of the *patA* transcriptional attenuator was assessed using the MFold web server (http://unafold.rna.albany.edu/?q=mfold).

Quantitative real-time PCR

All tested *S. pneumoniae* strains were grown overnight in duplicate at 35°C and 5% CO_2 on Mueller-Hinton agar plates supplemented with 5% defibrinated sheep blood (Bio-Rad Laboratories, Hercules, CA, USA). Bacteria were collected using a sterile loop and suspended in 15 mL Todd-Hewitt Broth medium to an $OD_{620\ nm}$ of 0.1–0.2. These samples were incubated at 35°C with occasional stirring to late mid-late exponential phase (OD_{620} ~0.5–0.6), at which point 4 mL of the culture was sampled and cells were harvested by centrifugation (8,000 *x g* for 10 min at 4°C). Cell pellets were rapidly frozen at −80°C until further processing.

Total RNA extraction was performed using the InviTrap® Spin Cell RNA Mini Kit (Stratec Biomedical, Birkenfeld, Germany) according to the manufacturer's instructions and stored at -80°C. Next, the samples were treated two consecutive times with 2 units TURBO™ DNase (Thermo Fisher, Waltham, USA) for 30 min at 37°C, followed by inactivation of the enzyme. To confirm removal of genomic DNA, the *pmrA* gene of *S. pneumoniae* was amplified as described elsewhere [19], and RNA concentrations were determined using Qubit fluorescence (Thermo Fisher).

cDNA was synthesized from 150 ng total RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer's instructions and using random hexamer primers. Residual RNA was removed using RNase III for 30 minutes at 37°C. Finally, real-time PCR was performed in an iQ cycler (Bio-Rad) in 25 μL reaction mixtures containing 12.5 μL of iQ SYBR Green Supermix (2×), 400 nM of forward and reverse primers and 5 μL of cDNA in RNase/DNase-free water. Primers used for amplification of *pmrA*, *patA* and *patB* are listed (S2 Table), and conditions were used as previously described [19]. Differential gene expression was calculated from the two replicates, as described in Pfaffl

et al. [30] and using *rpo*D and *pro*C genes as references to normalize transcript levels, as specified by PrimerDesign (Southampton, UK).

Results

Strain collection

A total of 5,602 unduplicated clinical isolates of *S. pneumoniae* were included in this study. Isolates were obtained from both ambulatory and hospitalized patients presenting a non-invasive respiratory clinical picture. These strains were collected during the winter seasons in 16 surveys spanning the period 1995 and 2014 by 15 participating clinical laboratories, determinedly selected to obtain a representative sampling of the country (Fig 1). Overall, 47.6% (varying between 40.9% and 53.9%) of the isolates were collected in the Southern part of the country, 44.7% (varying between 39.3% and 49.1%) in the Northern part and 7.6% (varying between 4.7% and 10.1%) in the Brussels area.

Annual fluoroquinolone resistance rates (1995–2014)

Annual MIC frequency distributions are presented in Table 1. From the onset of our study in 1995, nearly all isolates were classified as non-susceptible to CIP (96.2–100%) and OFL (97.3–100%). Nonetheless, high-level CIP resistance significantly increased from 0% resistant strains in 1995 and 1.4% in 2009, to 9.0% in 2013 ($P = 0.00025$, χ^2 trend analysis including Bonferroni's correction) (Table 1). In the same time period, the MIC_{50} of OFL significantly increased from 1 to 2 μg/mL ($P < 10^{-6}$; χ^2 linear trend analysis, Extended Mantel-Haenszel method), leading to a peak in resistance (6.6%) in 2013. Regarding the respiratory fluoroquinolones, LVX resistance peaked to 3.3% in 2003 and 3.1% in 2012, but remained in general below 2%. Notably, the levofloxacin MIC_{50} also increased significantly from 0.5 to 1 μg/mL since 2012 ($P < 10^{-6}$). MXF was the fluoroquinolone with the highest intrinsic activity on weight basis, with a stable MIC_{50} at 0.06 μg/mL (P = 0.64). Resistance to MXF arose only sporadically, and remained <1% throughout the entire study period (Table 1).

Next, we investigated the influence of the role of efflux in fluoroquinolone resistance using the efflux pump inhibitor reserpine. Hereto, we selected 422 pneumococcal isolates displaying varying MIC_{CIP} and repeated the MIC testing of CIP and MXF in the presence of reserpine ($MIC_{CIP/MXF+R}$). We observed that for 85 (20.1%) isolates, at least a fourfold decrease in MIC_{CIP} was achieved upon addition of the efflux pump inhibitor (Fig 2), which is a common threshold for the definition of an efflux phenotype [31,32]. For 57 (13.5%) isolates there was no effect, while in 16 cases (3.7%) this reduction was very drastic and caused a decrease to up to nine MIC_{CIP} dilutions, accounting for the entire resistance phenotype. In contrast, MXF MICs were much less decreased by the addition of reserpine as the maximal effect was a two-fold reduction in 45 (10.7%) strains (Fig 2). Of note, we used E-tests for all analyses which included reserpine, and recorded MICs which were generally higher than with the corresponding microdilution method: 52.8% one or less, and 90.2% two or less dilutions difference in CIP MICs, and 56.7% one or less, and 89.2% two or less dilutions difference for MXF (S4 Table). Comparable deviations have been reported elsewhere for other Gram-positive bacteria [33–35], and could be attributed to a conservative interpretation due to insufficient growth of the bacterial lawn.

Analysis of QRDR regions and serotypes

For the same set of 422 isolates, the QRDR of all *gyrA*, *gyrB*, *parC* and *parE* genes were sequenced (Table 2 and S4 Table). To dissect the influence of QRDR from efflux-mediated

Table 1. Yearly percentage of isolates displaying indicated MIC (μg/mL) against four fluoroquinolones. The MIC_{50} values are indicated with an asterisk. The breakpoints (separating the isolates according to their susceptibility to each drug) are those set by EUCAST.

CIPROFLOXACIN		Susceptible					Intermediate					Resistant		
Year	**# strains**	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**% res.**
1995	143	-	-	0.7	2.8	18.9	34.3*	35.7	7.7	-	-	-	-	**0.0**
1997	162	-	-	-	-	6.8	17.9	61.7*	11.7	2.5	0.6	-	-	**3.1**
1999	227	-	-	0.4	0.4	6.2	30.8	47.1*	13.2	1.8	-	-	-	**1.8**
2001	334	-	-	-	0.9	12.9	38*	38	6.6	3	0.6	-	-	**3.6**
2003	391	-	-	0.5	3.1	11.3	25.1	46.3*	9.5	2.6	1.8	-	-	**4.4**
2004	424	-	0.2	1.2	1.9	14.2	37.3*	36.3	6.6	2.1	-	0.2	-	**2.3**
2005	447	-	0.2	1.1	2.5	12.8	35.6*	40.5	6	0.9	0.2	0.2	-	**1.3**
2006	430	-	-	0.2	1.4	7.4	28.6	53.7*	8.1	0.5	-	-	-	**0.5**
2007	413	-	-	0.2	1.5	7.7	30	56.7*	1.7	1.5	0.2	0.5	-	**2.2**
2008	448	-	-	0.2	0.4	4.7	16.1	73.4*	4.7	-	-	0.4	-	**0.4**
2009	413	-	-	-	1.9	6.5	44.1*	44.1	1.9	1	0.2	0.2	-	**1.4**
2010	370	-	-	0.8	2.7	10.8	26.2	55.1*	1.9	2.2	-	-	0.3	**2.5**
2011	368	-	-	0.3	0.5	4.6	14.9	46.2*	29.6	2.2	1.1	0.5	-	**3.8**
2012	351	-	-	-	0.3	1.1	14.2	46.4*	29.9	7.1	0.6	0.3	-	**8.0**
2013	369	-	-	-	-	3	12.5	38.8*	36.9	7.3	1.1	0.3	0.3	**9.0**
2014	312	-	-	-	-	0.6	9.9	49.7*	33	6.4	-	0.3	-	**6.7**
OFLOXACIN		**Susceptible**					**Intermediate**					**Resistant**		
Year	**# strains**	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	**% res.**
1995	143	-	-	-	5.6	22.4	48.9*	15.4	7.7	-	-	-	-	**0.0**
1997	162	-	-	-	-	7.4	43.8*	32.1	15.4	1.2	-	-	-	**1.2**
1999	227	-	-	0.4	0.8	10.6	45.8*	27.3	13.2	1.8	-	-	-	**1.8**
2001	334	-	-	-	2.1	11.4	46.1*	30.2	6.6	3.3	0.3	-	-	**3.6**
2003	391	-	-	0.5	2.8	13.6	40.4*	29.2	9.2	3.1	0.8	0.5	-	**4.4**
2004	424	-	-	1.1	6.8	13	58*	12	6.6	2.1	0.2	-	-	**2.3**
2005	447	-	-	2.7	3.6	25.7	44.5*	16.1	6	1.1	0.2	-	-	**1.3**
2006	430	-	-	0.5	1.8	11.6	51.6*	26	7.9	0.5	-	-	-	**0.5**
2007	413	-	0.5	-	1.5	9.7	51.1*	33.7	1.7	1.3	0.7	-	-	**2.0**
2008	448	-	-	0.2	0.9	4	56*	33.7	4.7	-	0.4	-	-	**0.4**
2009	413	-	-	-	1.9	9	46.7*	39	2.4	0.5	0.5	-	-	**1.0**
2010	370	-	-	1.1	3.5	9.2	48.9*	33	2.7	1.4	0.3	-	-	**1.7**
2011	368	-	0.3	-	1.4	4.3	32.1	50.5*	9.2	1.6	0.5	-	-	**2.1**
2012	351	-	-	-	0.9	2.3	32.8	51.6*	11.1	1.4	-	-	-	**1.4**
2013	369	-	-	-	0.3	4.6	31.7	48.5*	8.4	6	0.3	0.3	-	**6.6**
2014	312	-	-	-	-	1	27.6	64.1*	4.5	2.6	-	0.3	-	**2.9**
LEVOFLOXACIN					**Susceptible**							**Resistant**		
Year	**# strains**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**% res.**
1995	143	-	1.4	2.8	19.6	40.6*	30.1	5.6	-	-	-	-	-	**0.0**
1997	162	-	-	0.6	8	58.6*	26.5	4.9	1.2	-	-	-	-	**1.2**
1999	227	-	0.4	-	2.6	37.9	44.1*	13.2	1.8	-	-	-	-	**1.8**
2001	334	-	-	1.2	9	47.6*	33.2	6.3	2.4	0.3	-	-	-	**2.7**
2003	391	-	0.5	3.6	13.6	31.7*	41.4	5.9	1.8	1.5	-	-	-	**3.3**
2004	424	0.5	0.7	3.8	14.2	42.7*	30.2	5.2	2.6	-	0.2	-	-	**2.7**
2005	447	0.9	2	4.5	22.6	48.1*	15.9	5.4	0.4	-	0.2	-	-	**0.6**
2006	430	0.2	1.2	2.1	9.3	28.6*	53.7	8.1	0.5	-	-	-	-	**0.5**
2007	413	0.2	0.5	2.2	13.8	58.1*	23.5	0.7	0.2	0.7	-	-	-	**0.9**

(*Continued*)

Table 1. (*Continued*)

2008	448	0.2	-	1.1	6.9	60.7*	26.1	4.2	0.2	-	0.4	-	-	**0.6**
2009	413	-	1.2	5.3	30.8	46.2*	15	0.7	0.2	0.5	-	-	-	**0.7**
2010	370	0.3	3.5	4.3	17	55.9*	15.7	2.4	0.5	0.3	-	-	-	**0.8**
2011	368	0.3	0.5	3	10.1	37*	41.3	6.8	0.5	0.5	-	-	-	**1.0**
2012	351	-	-	0.9	3.7	41.3	39*	12	2.8	0.3	-	-	-	**3.1**
2013	369	-	-	1.4	2.7	35	49.3*	10.3	0.8	0.3	0.3	-	-	**1.4**
2014	312	-	-	0.6	2.2	30.8	59.6*	6.1	0.3	-	0.3	-	-	**0.6**
MOXIFLOXACIN					**Susceptible**						**Resistant**			
Year	**# strains**	**0.008**	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**% res.**
1995	143	-	9.1	33.6	38.5*	13.3	0.7	-	-	-	-	-	-	**0.0**
1997	162	-	0.6	12.3	38.9*	44.4	3.1	0.6	-	-	-	-	-	**0.0**
1999	227	0.4	1.8	11	40.1*	30.4	12.8	2.6	0.9	-	-	-	-	**0.9**
2001	334	0.6	6.3	9.3	43.7*	32.3	5.4	1.5	0.6	0.3	-	-	-	**0.9**
2003	391	1	6.6	13.6	30.2*	36.8	10.5	0.8	-	0.3	0.3	-	-	**0.6**
2004	424	0.5	4.5	17	39.4*	30.2	8	0.2	-	0.2	-	-	-	**0.2**
2005	447	1.1	4	18.6	39.6*	28.2	6.9	1.3	-	0.2	-	-	-	**0.2**
2006	430	1.8	4.7	17	41.4*	30.9	-	0.2	-	-	-	-	-	**0.0**
2007	413	0.7	2.9	11.1	43.1*	30	11.4	-	0.5	0.2	-	-	-	**0.7**
2008	448	0.2	0.9	7.4	38.6*	46.4	6.9	-	-	0.2	-	0.2	-	**0.4**
2009	413	0.2	5.3	11.1	51.3*	25.2	6.3	0.2	0.2	-	-	-	-	**0.2**
2010	370	-	5.4	11.6	49.5*	26.8	5.7	0.8	-	-	0.3	-	-	**0.3**
2011	368	0.3	3.3	12.8	48.9*	27.2	6.5	0.5	0.5	-	-	-	-	**0.5**
2012	351	-	2.3	5.4	48.1*	36.5	6.6	0.9	0.3	-	-	-	-	**0.3**
2013	369	-	1.4	9.5	53.9*	30.1	4.3	-	-	0.8	-	-	-	**0.8**
2014	312	-	0.3	8.3	52.6*	34.3	4.2	-	-	0.3	-	-	-	**0.3**

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resistance, various genotypes were grouped according to the MIC of CIP when tested in the presence of reserpine (MIC_{CIP+R}) for each strain. Firstly, this allowed identifying a magnitude of topoisomerase mutations unrelated to FQ-R, most prevalent being ParC K137N, K57T and ParE I460V [8–10] occurring in 14.7, 2.2 and 81.0% pneumococcal isolates with MIC_{CIP+R} <1 μg/mL, respectively. Secondly, we identified signatures of recombination with members of the *S. mitis* group, judged by the presence of ParC S52G, N91D and/or GyrA S114G substitutions [13], in 10.4% of the strains. These recombinant genes were already identified at the onset of our study, but no significant increase in topoisomerase recombination was noted by 2014.

Classical QRDR mutations were retrieved in GyrA at positions 81 ($n = 24$) and 85 ($n = 1$), ParC positions 78 ($n = 1$), 79 ($n = 38$) and 83 ($n = 7$), and ParE position 435 ($n = 3$). These topoisomerase mutations were found in varying combinations, arguing against clonal expansion of FQ-R (Table 2). To investigate this hypothesis, we performed CST typing on 54 FQ-R isolates which showed a wide variety of associated serotypes (S3 Table). In concordance to previous studies, isolates carrying mutations in both topoisomerases unequivocally displayed high-level resistance to CIP ($MIC_{CIP+R} > 12$ μg/mL). In contrast, strains with sole mutations in ParC (50.9% of isolates with mutated QRDR) or GyrA (13.7%) display more variable MIC_{CIP+R} values. For example, four strains carrying a GyrA S81F/G mutation displayed a MIC_{CIP+R} of 32 μg/mL (e.g., 13C28), whereas other strains with the same mutation (*e.g.*, 05A05 and 04L17) only reached 0.5–1 μg/mL.

Finally, some interesting genotypes were observed. For example, we identified a very rare GyrB mutation (P454S) in isolate 05A20 which, in combination with GyrA S81L and wild-type

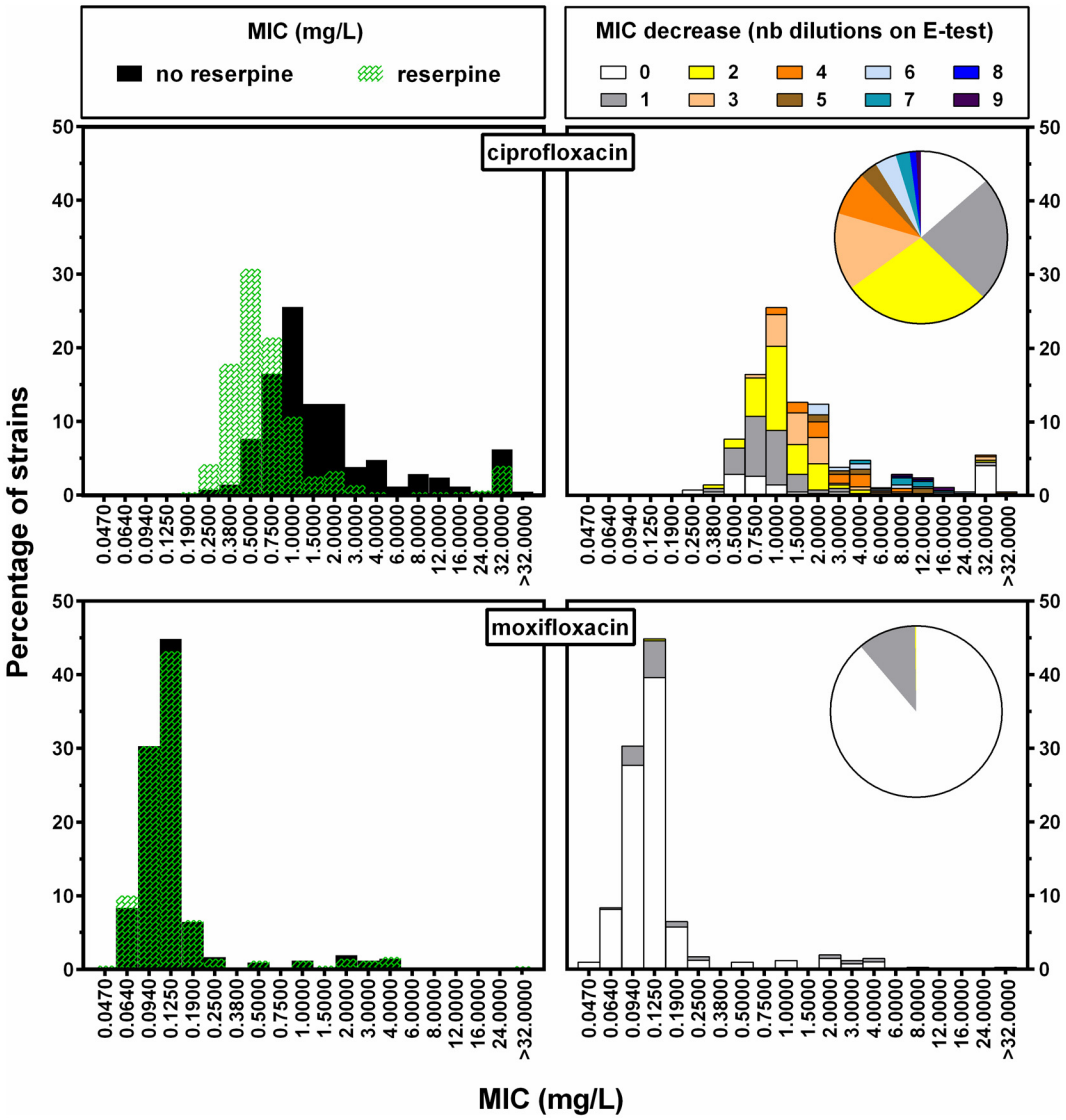

Fig 2. MIC distributions of ciprofloxacin and moxifloxacin (E-test method) for 422 non-invasive *S. pneumoniae* isolates collected in Belgium between 1995 and 2014. Left-hand panels: MIC distributions determined in the absence (control; black) or presence (green) of 20 mg/L reserpine. Right-hand panels: reduction of MIC (in blocks of 0.5 $\log_2$ dilutions from 0 to 3 $\log_2$ dilutions) after addition of 20 mg/L reserpine and plotted as a function of the MIC distribution of the isolates in the absence of reserpine.

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Topoisomerase IV, was correlated with full resistance to MXF (MIC_{MXF+R} = 32 μg/mL) (Table 2). An even more noticeable result was that some isolates with complete wild-type QRDRs (e.g., 13C24, 11I08) were nevertheless fully resistant to CIP, even in the presence of reserpine (Table 2).

Efflux pump expression analysis

Next, we assessed the contribution of PmrA and PatAB efflux transporters to FQ-R by measuring early-log expression levels of *patA*, *patB* and *pmrA* in 94 *S. pneumoniae* isolates grown in the absence of antibiotics, in comparison to the control strain *S. pneumoniae* ATCC 49619 [27,28]. Strains were selected based on various susceptibilities to reserpine, i.e. displaying either

Table 2. Overview of the various FQ-R genotypes encountered in 422 clinical *S. pneumococci* strains. Signature residues of the viridans group of streptococci [13] are indicated in bold.

MIC_{CIP+R} (μg/ml)	MIC_{MXF+R} (μg/ml)	No. isolates	GyrA			GyrB	ParC					ParE
			S81	**E85**	***S114***	**P454**	***S52***	***N91***	**D78**	**S79**	**D83**	**D435**
< 1	0.064–0.19	289	-	-	-	-	-	-	-	-	-	-
(*n* = 311)		10	-	-	*G*	-	-	-	-	-	-	-
		6	-	-	*G*	-	*G*	*D*	-	-	-	-
		2	-	-	-	-	-	*D*	-	-	-	-
		2	-	-	-	-	*G*	*D*	-	-	-	-
		1	-	-	*G*	-	*G*	-	-	-	-	-
		1	F	-	-	-	-	-	-	-	-	-
≤ 2	0.125–0.25*	47	-	-	-	-	-	-	-	-	-	-
(*n* = 80)		12	-	-	-	-	-	-	-	F	-	-
		1	-	-	-	-	-	-	N	-	-	-
		1	F	-	-	-	-	-	-	-	-	-
		1	-	-	-	-	-	*D*	-	F	-	-
		2	-	-	-	-	-	-	-	-	N/Y	-
		1	-	-	-	-	-	-	-	F	-	K
		1	-	-	*G*	-	*G*	*D*	-	F	-	-
		5	-	-	*G*	-	-	-	-	-	-	-
		5	-	-	*G*	-	*G*	*D*	-	-	-	-
		4	-	-	*G*	-	-	*D*	-	-	-	-
2–4	0.19–1	3	-	-	-	-	-	-	-	-	-	-
(*n* = 12)		4	-	-	-	-	-	-	-	Y/F	-	-
		1	F	-	-	-	-	-	-	-	-	N
		1	-	-	-	-	-	*D*	-	F	-	-
		1	-	-	-	-	-	-	-	-	G	-
		1	-	-	*G*	-	-	-	-	-	-	-
		1	-	-	*G*	-	*G*	*D*	-	-	-	-
		1	-	-	-	-	-	-	-	-	-	-
		3	F	-	-	-	-	-	-	-	-	-
≥4	1–32	1	F	-	-	-	-	-	-	-	-	N
(*n* = 29)		12	F	-	-	-	-	-	-	F/Y	-	-
		2	F	-	-	-	-	-	-	-	G/Y	-
		2	-	-	*G*	-	-	*D*	-	F	-	-
		1	Y	-	*G*	-	-	*D*	-	Y	-	-
		1	G	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	N	-
		2	-	-	-	-	-	-	-	F/Y	-	-
		1	-	K	-	-	-	-	-	Y	-	-
		1	L	-	*G*	S	-	-	-	-	-	-

Only shown are amino acid substitutions involved in resistance and recombination with the viridans group. -, wild type. Identified mutations not involved in resistance were **GyrA**: M99I (*n* = 3), G112D (*n* = 2), G103S, L152R, L155F, L155V, L154F, L157F(*n* = 1); **GyrB:** G434R (*n* = 4), D435N, S466G, F480L (*n* = 1); **ParC**: K137N (*n* = 64), K57T (*n* = 11), R95C/G (*n* = 3), E134D, E135D (*n* = 1); **ParE**: I460V (*n* = 232), H534L/Q (*n* = 6), I493L (*n* = 3), A532T/V (*n* = 2), D435N, I431S, I493L, A468T, Y481H, K448P, Q420P (*n* = 1).

*0.38 for the isolate with S81F

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no (n = 2), 1- (n = 8), 2- (n = 20), 3- (n = 24), 4-(n = 25) or ≥5 (n = 14) fold MIC_{CIP} reductions in the presence of this efflux pump inhibitor. The results are shown in Table 3.

Since putative highly different genetic backgrounds preclude reliable strain-to-strain comparison, we performed Kruskal-Wallis testing (with Dunn's multiple comparison test) on three sample groups of strains either susceptible, intermediate or resistant to CIP (S1 Fig). This non-parametric method showed no statistically significant differences in *pmrA* expression among the three groups. In contrast, both *patA* and *patB* expression was significantly upregulated in CIP-resistant, but not in CIP-intermediate *S. pneumoniae* strains. Although this correlated with the observed susceptibility to reserpine, the levels of reserpine-mediated MIC reductions varied strongly among strains with similar transcript levels. For example, strains 99J16 and 13L23 both strongly overexpressed *patA* (6.0±1.3 and 14.8±4.4, resp.) and *patB* (56.5±13.6 and 48.4±10.5, resp.), but showed a sixteen- vs. threefold reduction in MIC_{CIP} in the presence of the efflux pump inhibitor.

Conversely, strain 10N11 also overexpressed *patA* and *patB* but showed no reserpine-dependent reduction of MIC_{CIP} (Table 3). Notably, in 12 and 19 isolates only *patA* or *patB* were overexpressed, respectively, arguing against uniform operon coregulation for these genes. Finally, in 17.0% of the reserpine-susceptible strains both *patA* and *patB* were downregulated. Since the heterogeneous PatAB pump requires both functional subunits to be functional [36], these two last observations strongly indicate the presence of other reserpine-sensitive systems involved in FQ-R.

Constitutive induction of *patA* has very recently been correlated to disruption of the transcriptional terminator of the upstream *hexA* gene [22,24]. We therefore sequenced this upstream region in 103 isolates. Although none of previous described mutations were retrieved, we identified six novel mutations: C(-41)T, G(-40)A, G(-46)T, G(-48)A, G(-49)A and C(-28)T. Each of these mutations could be related to decreased hairpin stability (ΔG increases > 3.2 kCal/mol), leading to increased transcription. The A(-52)G mutation was found not to play a role in *patA* regulation. It is important to note that these mutations were found in only a fraction (15.5%) of the isolates which overexpress *patA*, implying that terminator disruption is only a minor regulatory mechanism in the isolates under study.

Discussion

At the introduction of the respiratory fluoroquinolones LVX and MXF in the treatment of (a) typical pneumonia, there was concern that while treatment success in the short term could be enhanced, highly FQ-R *S. pneumoniae* strains would emerge by accumulation of additional QRDR mutations [37]. The continued high use of CIP for specific respiratory indications, such as the treatment of bronchial infections in cystic fibrosis patients [13], poses an additional risk factor to select for first-step ParC mutations which precede the ones in GyrA under CIP selective pressure. Moreover, the continuous exposure to sub-MIC levels of CIP and levofloxacin has been shown to select for efflux overexpression [38].

In our surveillance data on FQ-R among Belgian non-invasive *S. pneumoniae* isolates (1995–2014), some evidence points in this direction. From 2011 onwards, we observe a trend towards increased resistance to CIP and ofloxacin, and (although only visible at the MIC_{50} level) also for LVX. Our data from CST typing clearly indicates no clonal spread of CIP-R isolates, and thereby suggests there is no direct influence of vaccination campaigns on FQ-R in non-invasive pneumococci. The preference of first-step mutations in ParC is reflected by the 4:1 ratio of single QRDR mutations in the Topoisomerase IV subunits compared to the DNA Gyrase. Although a similar increase in CIP resistance was reported in Canada [10], this was not confirmed in other surveillance studies covering Europe, North America or Asia [8,9]. In

Table 3. Expression analyses of a selection of *S. pneumoniae* strains, with inclusion of the QRDR sequences and phenotypic FQ-R analyses.

Strain Id.	Topoisomerase mutations				$MIC_{CIP + reserpine}$ (μg/ml)[a]		$MIC_{MXF + reserpine}$ (μg/ml)[a]		Gene expression		
	GyrA	GyrB	ParC	ParE	0	20	0	20	*pmrA*	*patA*	*patB*
07A40	S81F	wt	S79F	wt	>32	>32	3	2	0.6 ± 0.2	11.2 ± 4.3	0.7 ± 0.2
10N11	S81F	wt	S79Y	wt	>32	>32	8	4	0.3 ± 0.1	10.4 ± 2.9	281.5 ± 37.7
12K23	wt	wt	wt	I460V	1	0.75	0.125	0.094	1.7 ± 1.1	2.8 ± 1.8	19.9 ± 12.4
ATCC	wt	wt	wt	wt	0.5	0.35	0.064	0.064	1	1	1
99H18	wt	wt	wt	I460V	1.5	1	0.19	0.125	13.1 ± 8.5	0.2 ± 0.1	10.2 ± 7.7
12M03	wt	wt	wt	I460V	1.5	1	0.125	0.125	1.3 ± 0.4	1.3 ± 0.6	10.0 ± 3.5
08 E03	wt	wt	R95C	wt	0.75	0.5	0.094	0.064	1.0 ± 0.2	1.9 ± 0.6	0.7 ± 0.3
05A07	wt	S466G	K57T	I460V	0.75	0.38	0.125	0.094	0.2 ± 0.1	1.0 ± 0.2	10.3 ± 2.8
11I08	wt	wt	wt	I460V	32	16	1	1	1.0 ± 0.3	0.5 ± 0.3	0.7 ± 0.1
11A23	wt	wt	D83N	I460V	>32	16	1.5	1.5	0.3 ± 0.1	3.3 ± 0.8	39.2 ± 10.7
08A02	wt	wt	wt	wt	0.5	0.25	0.125	0.064	0.4 ± 0.06	1.1 ± 0.1	0.4 ± 0.1
09K10	wt	wt	wt	I460V	1	0.5	0.094	0.094	3.8 ± 1.7	0.3 ± 0.1	0.3 ± 0.1
13L15	wt	wt	wt	I460V	1.5	0.75	0.125	0.125	1.7 ± 0.8	1.8 ± 0.9	13.7 ± 7.0
11I10	wt	wt	wt	I460V	1	0.5	0.125	0.125	0.8 ± 0.2	1.9 ± 0.6	16.9 ± 5.5
09K03	wt	wt	wt	I460V	1	0.5	0.094	0.094	1.6 ± 0.4	0.2 ± 0.04	0.7 ± 0.2
97G03	wt	wt	S79F	A532T	2	1	0.19	0.125	4.1 ± 1.3	0.1 ± 0.05	9.1 ± 3.3
11I29	wt	wt	wt	I460V	1.5	0.75	0.125	0.125	0.9 ± 0.4	1.5 ± 0.8	24.4 ± 10.3
10A15	wt	wt	wt	wt	1	0.5	0.125	0.125	1.1 ± 0.4	2.3 ± 0.7	68.0 ± 22.0
07M27	wt	wt	R95C	wt	1	0.5	0.125	0.125	1.6 ± 0.9	4.1 ± 2.5	0.2 ± 0.1
09L20	wt	wt	wt	I460V	1	0.5	0.125	0.094	6.7 ± 5.4	3.6 ± 3.1	2.1 ± 1.2
09N30	wt	wt	wt	I460V	1.5	0.75	0.064	0.064	2.6 ± 0.9	9.1 ± 3.4	8.2 ± 2.8
04F27	wt	wt	wt	I460V	1	0.38	0.094	0.064	1.9 ± 0.9	1.8 ± 0.3	11.8 ± 2.3
05C40	wt	wt	K137N	I460V	1	0.38	0.125	0.094	5.3 ± 3.5	4.5 ± 2.3	25.4 ± 13.0
01H28	S81F	wt	K137N	I460V	32	12	3	2	10.7 ± 4.1	1.6 ± 0.4	15.1 ± 3.3
13A22	wt	wt	wt	I460V	4	1.5	0.125	0.125	0.5 ± 0.3	2.6 ± 1.5	14.8 ± 8.5
09K16	wt	wt	wt	I460V	2	0.75	0.125	0.125	1.8 ± 1.1	0.3 ± 0.1	0.4 ± 0.5
11O31	S114G	wt	wt	wt	4	1.5	0.125	0.125	5.7 ± 5.7	14.3 ± 12.6	24.3 ± 3.9
07O07	S114G	wt	S79F/N91D	I460V	16	6	0.25	0.25	0.6 ± 0.03	1.8 ± 0.0	0.5 ± 0.1
97B14	L154F	wt	wt	I460V	1.5	0.5	0.094	0.094	2.4 ± 0.8	0.08 ± 0.03	9.8 ± 4.1
04A10	wt	wt	wt	I460V	1.5	0.5	0.125	0.125	9.3 ± 3.3	0.5 ± 0.2	0.3 ± 0.2
97I27	wt	wt	wt	I460V	1.5	0.5	0.125	0.094	8.4 ± 3.7	0.1 ± 0.04	9.7 ± 4.2
04A25	wt	wt	wt	I460V	3	1	0.19	0.19	9.4 ± 5.4	0.4 ± 0.2	0.4 ± 0.2
13L23	wt	wt	wt	I460V	3	1	0.19	0.19	1.7 ± 0.5	14.8 ± 4.4	48.4 ± 10.5
06N06	wt	wt	wt	I460V	1.5	0.5	0.125	0.125	1.1 ± 0.2	2.4 ± 0.6	0.2 ± 0.1
13B09	wt	wt	wt	I460V	1.5	0.5	0.094	0.094	0.8 ± 0.5	1.0 ± 0.5	9.6 ± 6.1
95B18	wt	wt	wt	wt	1.5	0.5	0.125	0.125	1.4 ± 0.5	42.4 ± 15.0	198.6 ± 71.1
4E+13	wt	wt	wt	I460V	1.5	0.5	0.125	0.125	3.7 ± 1.5	0.5 ± 0.3	0.1 ± 0.1
95C17	wt	wt	wt	I460V	0.75	0.25	0.094	0.094	2.0 ± 0.2	0.3 ± 0.1	2.8 ± 1.1
13E324	S114G	wt	wt	I460V	1.5	0.5	0.125	0.125	0.8 ± 0.4	1.9 ± 0.9	9.2 ± 4.6
13L14	wt	wt	wt	I460V	3	1	0.19	0.19	1.2 ± 0.5	2.5 ± 1.1	13.3 ± 5.7
04L07	wt	wt	wt	I460V	1.5	0.5	0.094	0.094	3.9 ± 1.9	1.3 ± 0.6	0.5 ± 0.2
06A24	wt	wt	wt	I460V	1.5	0.5	0.125	0.125	0.6 ± 0.2	2.4 ± 1.0	0.5 ± 0.2
10I25	wt	wt	wt	I460V	3	1	0.125	0.125	1.2 ± 0.6	6.6 ± 2.2	72.2 ± 45.7
04C04	wt	wt	wt	I460V	1.5	0.5	0.094	0.094	4.3 ± 1.8	2.8 ± 1.4	1.3 ± 0.5
95C08	wt	wt	S52G/N91D	I460V	1.5	0.5	0.19	0.19	1.6 ± 1.4	6.2 ± 4.6	32.9 ± 24.2

(*Continued*)

Table 3. (*Continued*)

Strain Id.	Topoisomerase mutations				$MIC_{CIP + reserpine}$ (μg/ml)[a]		$MIC_{MXF + reserpine}$ (μg/ml)[a]		Gene expression		
	GyrA	GyrB	ParC	ParE	0	20	0	20	*pmrA*	*patA*	*patB*
04L24	wt	wt	K137N	I460V	1.5	0.5	0.094	0.094	4.5 ± 0.8	1.8 ± 0.3	0.5 ± 0.1
10L18	wt	wt	wt	I460V	1.5	0.5	0.25	0.25	1.1 ± 0.3	1.5 ± 0.5	16.1 ± 5.3
13A12	wt	wt	wt	I460V	1.5	0.5	0.125	0.125	1.4 ± 0.4	3.5 ± 0.3	25.0 ± 3.0
09O15	wt	wt	wt	I460V	0.75	0.25	0.064	0.064	1.2 ± 0.1	0.5 ± 0.07	0.4 ± 0.07
06O04	wt	wt	wt	wt	1.5	0.38	0.094	0.094	0.7 ± 0.4	2.0 ± 1.1	0.3 ± 0.1
08J09	wt	wt	wt	I460V	2	0.5	0.125	0.094	0.7 ± 0.08	0.15 ± 0.0	0.2 ± 0.1
13B01	wt	wt	wt	I460V	3	0.75	0.19	0.19	0.9 ± 0.2	1.5 ± 0.3	8.6 ± 2.6
06A08	S114G	wt	wt	A496T	2	0.5	0.094	0.125	3.6 ± 2.4	2.3 ± 1.4	12.2 ± 7.9
99H17	wt	wt	wt	wt	0.5	0.125	0.064	0.032	7.7 ± 3.0	0.09 ± 0.04	8.9 ± 3.7
06J35	wt	wt	wt	wt	2	0.5	0.125	0.125	1.4 ± 0.8	1.4 ± 0.7	11.0 ± 5.8
12M02	wt	wt	S79F/ N91D	I460V	4	1	0.125	0.125	4.1± 0.6	0.03 ± 0.01	10.8 ± 1.8
95B16	S114G	wt	S52G/N91D	wt	4	1	0.25	0.19	2.7 ± 0.8	0.04 ± 0.0	4.8 ± 1.1
99A09	wt	wt	wt	I460V	2	0.5	0.19	0.125	11.0 ± 9.6	0.2 ± 0.1	9.3 ± 8.4
08G28	wt	wt	wt	I460V	3	0.75	0.125	0.094	0.3 ± 0.03	0.4 ± 0.03	0.2 ± 0.0
06J37	wt	wt	wt	I460V	2	0.5	0.125	0.125	1.4 ± 0.4	1.6 ± 0.9	5.8 ± 0.7
01H21	S81F	wt	K137N	D435N	16	4	1.5	1	9.9 ± 3.3	3.0 ± 0.6	13.3 ± 4.5
03L14	wt	wt	wt	I460V	2	0.5	0.094	0.094	4.4 ± 2.4	0.7 ± 0.4	0.3 ± 0.2
05A28	S114G	wt	S52G/N91D	wt	4	1	0.19	0.19	0.02 ± 0.0	0.8 ± 0.3	0.7 ± 0.2
08G25	wt	G434R	wt	I460V	2	0.5	0.125	0.094	0.2 ± 0.02	2.4 ± 0.4	0.6 ± 0.1
95F08	wt	wt	wt	I460V	2	0.5	0.094	0.094	4.5 ± 2.7	2.0 ± 1.1	53.7 ± 28.7
07H01	wt	wt	S79F	I460V	4	1	0.125	0.125	1.5 ± 0.9	21.4 ± 11.9	5.6 ± 3.0
08G26	wt	wt	wt	I460V	2	0.5	0.094	0.094	1.1 ± 0.5	3.0 ± 1.5	0.4 ± 0.2
08O21	wt	wt	K137N	I460V	2	0.5	0.125	0.125	1.9 ± 0.6	6.5 ± 1.3	1.6 ± 0.3
05D34	wt	wt	wt	I460V	4	1	0.125	0.125	7.0 ± 0.4	0.3 ± 0.04	2.8 ± 0.1
10K19	wt	wt	wt	I460V	6	1.5	0.125	0.125	0.5 ± 0.05	11.1 ± 1.1	208.7 ± 34.9
97A11	wt	wt	wt	I460V	2	0.5	0.125	0.125	1.8 ± 0.6	1.0 ± 0.3	121.4 ± 49.5
97B21	wt	wt	wt	wt	2	0.38	0.125	0.094	1.7 ± 0.8	0.2 ± 0.1	18.7 ± 10.3
05A34	S114G	wt	S52G/N91D	wt	4	0.75	0.19	0.19	0.3 ± 0.1	0.3 ± 0.1	0.04 ± 0.01
99D02	wt	wt	K137N	I460V	4	0.75	0.094	0.064	1.1 ± 0.4	0.4 ± 0.2	34.9 ± 12.1
99J08	wt	wt	wt	I460V	4	0.75	0.125	0.125	3.2 ± 2.2	1.4 ± 1.1	134.0 ± 96.9
97B25	wt	wt	ND	wt	24	4	0.38	0.38	2.2 ± 1.3	0.1 ± 0.08	10.0 ± 5.9
08 E15	wt	wt	wt	I460V	6	1	0.125	0.094	3.5 ± 2.1	0.2 ± 0.01	1.0 ± 0.6
11A17	wt	wt	wt	I460V	3	0.5	0.125	0.125	0.4 ± 0.1	9.9 ± 2.4	79.7 ± 21.4
08L06	wt	wt	wt	I460V	3	0.5	0.094	0.094	1.1 ± 0.2	0.3 ± 0.07	0.3 ± 0.1
01C06	wt	wt	K137N	I460V	3	0.38	0.125	0.125	11.4 ± 4.7	1.3 ± 0.4	12.2 ± 4.4
99H10	wt	wt	wt	wt	1.5	0.19	0.094	0.094	8.2 ± 2.1	0.5 ± 0.08	18.9± 3.1
04A24	wt	wt	wt	I460V	8	1	0.19	0.125	5.6 ± 3.4	0.4 ± 0.1	15.5 ± 4.9
03N11	wt	wt	S79Y	I460V	16	2	0.19	0.19	8.0 ± 3.3	1.4 ± 0.2	41.1 ± 8.7
10A19	wt	wt	wt	I493L	4	0.5	0.125	0.094	0.6 ± 0.3	0.0 ± 0.00	73.2 ± 50.1
13F15	S114G	wt	wt	wt	8	1	0.19	0.19	1.3 ± 0.3	1.1 ± 0.2	10.8 ± 2.6
99G11	wt	wt	wt	I460V	6	0.75	0.125	0.125	9.7 ± 2.4	2.9 ± 0.6	19.5 ± 0.7
11A27	S114G	wt	N91D	I493L	8	1	0.19	0.125	2.8 ± 1.7	3.1 ± 1.9	32.0 ± 17.3
09K15	wt	wt	wt	I460V	4	0.5	0.094	0.094	1.2 ± 0.3	0.6 ± 0.2	0.7 ± 0.2
01C35	S114G	wt	S79Y/N91D	I460V	16	2	0.125	0.125	16.3 ± 5.9	3.4 ± 1.1	0.15 ± 0.05
01G18	wt	wt	wt	I460V	4	0.38	0.125	0.094	14.3 ± 3.8	3.5 ± 0.3	30.8 ± 4.9

(*Continued*)

Table 3. (*Continued*)

Strain Id.	Topoisomerase mutations				$MIC_{CIP + reserpine}$ (μg/ml)[a]		$MIC_{MXF + reserpine}$ (μg/ml)[a]		Gene expression		
	GyrA	GyrB	ParC	ParE	0	20	0	20	*pmrA*	*patA*	*patB*
03 K18	wt	wt	wt	I460V	8	0.75	0.125	0.125	6.2 ± 5.4	13.5 ± 16.6	9.8 ± 11.4
03 L23	wt	wt	S79F	D435K	32	2	0.19	0.19	7.9 ± 4.1	11.5 ± 5.8	153.9 ± 73.3
06H02	S114G	wt	wt	wt	>32	2	0.19	0.125	0.8 ± 0.3	2.0 ± 1.5	0.3 ± 0.1
13G08	S114G	wt	N91D	wt	16	1	0.19	0.19	1.5 ± 0.6	5.4 ± 2.4	34.0 ± 15.8
03L28	wt	wt	K137N	I460V	>32	1.5	0.125	0.125	11.6 ± 3.3	1.7 ± 0.4	18.6 ± 4.6
99J16	wt	wt	wt	I460V	8	0.38	0.094	0.094	6.5 ± 0.4	6.0 ± 1.3	56.5 ± 13.6

[a] Based on E-test.

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contrast, resistance to MXF was only sporadic and globally minimal. As these trends are still very recent, it is critical that resistance rates and their changes are continuously monitored in the near future.

The exceptional case of high-level resistance to MXF (MIC_{MXF+R} of 32 μg/ml) was associated with a GyrB P454S mutation, combined with a mutated GyrA (S81L) subunit but a wild-type ParC/E. Notably, a Chinese group recently reported both ParE_P454S as GyrB_P454S to be associated with MXF resistance in combination with dually mutated GyrA and ParC residues [39,40]. However, our observation of a wild-type Topoisomerase IV QRDR region is important, as it contrasts with the current model which states that mutations in both topoisomerases are a prerequisite for high-level MXF resistance.

Analysis of the contribution of efflux pumps to pneumococcal FQ-R revealed no significant upregulation of the Major Facilitator PmrA in CIP resistant strains. We did observe varying constitutive expression of *pmrA* among clinical isolates, which has been shown before [16], but its contribution to drug resistance in non-invasive pneumococcal strains remains unclear. In contrast, non-parametric analyses showed marked higher expression of the ABC efflux pump PatAB associated with decreasing CIP susceptibility. Unfortunately, the underlying regulatory mechanism behind this upregulation remains unexplored. Although we found novel disruptive mutations in upstream transcriptional terminator sequences [22–24], this mechanism seems rather rare among clinical isolates as the large majority has wild-type upstream regions. A *patA/B* repressor has not been found or seems deleted in comparison to similar operons in related bacteria [41]. Various other levels of regulation can be envisioned at the post-transcriptional, translational or post-translational level.

Another important finding of this study is related to strains which seem deprived of known molecular FQ-R mechanisms, but yet display a resistant phenotype. For example, isolate 11I08 displays a wild-type QRDR yet a MIC_{CIP+R} of 24 μg/mL. A possibility is the involvement of chromosomally encoded qnr-like proteins, which shield topoisomerases from invading fluoroquinolones [42]. In contrast, many reserpine-susceptible strains did not express PatAB pumps, implying the involvement of other reserpine-sensitive efflux mechanisms in pneumococcus. This can be either novel efflux pumps, like the recently identified DinF transporter [43], or any of the five transporter genes found to be consistently induced by fluoroquinolones [44]. In any case, isolates with elevated MICs but without defined resistance mechanism are also commonly reported in other studies [3, 17, 25], and deserve more attention in the future.

We acknowledge limitations of the presented *patAB* expression studies. First of all, putative gene duplication of *patA* [23] could not be detected with the applied methods. Moreover,

although we assessed constitutive gene expression, it has been shown that expression is quickly upregulated upon exposure to CIP, with *patA* being more strongly upregulated than *patB* [19,40]. This might level out the difference we observed in basal expression levels between both genes.

In conclusion, 15 years after the introduction of respiratory fluoroquinolones, we observe a concerning rise in resistance among non-invasive pneumococci. MXF remains a very potent drug with minimal level of resistance, but a combination of rare mutations in the DNA Gyrase was associated with full resistance to this compound. While target topoisomerase mutations and efflux pump (over)expression clearly contribute to FQ-R, we add novel isolates to the existing collection of strains deprived of known molecular mechanisms of fluoroquinolone resistance. It would be of great value to bring these clinical isolates together, and unravel their resistance mechanisms through a profound, comparative molecular characterization at the genomic, transcriptomic and proteomic level.

Supporting Information

S1 Fig. Gene expression analyses.
(DOCX)

S1 Table. MIC distribution of the reference strains used in the broth microdilution experiments.
(DOCX)

S2 Table. Oligonucleotides used in this study.
(DOCX)

S3 Table. Results of CST typing.
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S4 Table. QRDR sequencing and MIC determination of a selection of 422 pneumococcal strains.
(DOCX)

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Author Contributions

Conceived and designed the experiments: PC FVB SB JV PT RV. Performed the experiments: PC WM FF EVB SD HN SDC. Analyzed the data: PC FVB SB RV. Contributed reagents/materials/analysis tools: FVB JV TBSPSG PT. Wrote the paper: PC RV.

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Molecular Analysis of Rising Fluoroquinolone Resistance in Belgian Non-Invasive Streptococcus pneumoniae Isolates (1995-2014)

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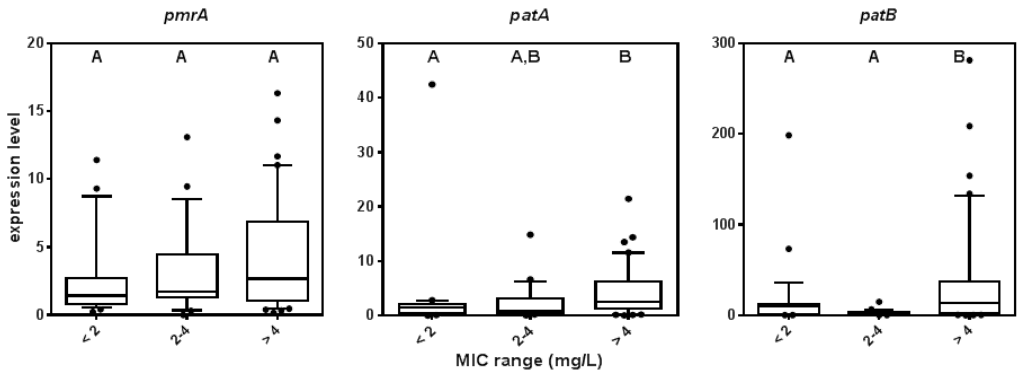
Supporting Information

- S1 Fig. Gene expression analyses.
- S1 Table. MIC distribution of the reference strains used in the broth microdilution experiments.
- S2 Table. Oligonucleotides used in this study.
- S3 Table. Results of CST typing.
- S4 Table. QRDR sequencing and MIC determination of a selection of 422 pneumococcal strains.



S1 Figure. Gene expression analyses. Boxplots showing the expression of *pmrA*, *patA* and *patB* in 94 tested strains, in comparison to the control strain *S. pneumoniae* ATCC 49619 and plotted in function of the MIC_{CIP}. Plots with different letters indicate differences between groups that are statistically significant ($p < 0.05$) by the Kruskal-Wallis non-parametric test with Dunn's multiple test.

S4 Table. MIC distribution of the reference strains used in the broth microdilution experiments.

Strain	**MIC distributions (µg/ml)**			
	Ofloxacin	Ciprofloxacin	Levofloxacin	Moxifloxacin
Streptococcus pneumoniae **TPN 881**	1.70±0.4	1.0±0.3	0.90±0.33	0.14±0.05
Staphylococcus aureus **NCTC 11561**	0.27±0.07	0.27±0.08	0.20±0.06	0.05±0.01
Staphylococcus aureus **ATCC 29123**	0.34±0.12	0.35±0.15	0.22±0.05	0.05±0.01

S1 Table. Oligonucleotides used in this study.

Name	Sequence (5'-3')	Source
patA_up_F	GGCAGAAGAGCATCCTATCCTAG	This study
patA_up_R	CCAATCAACCAAGCCCCGATAC	This study
GyrA_F	CCTGTTCACCGTCGCATTCT	This study
GyrA_R	AGTTGCTCCATTAACCA	This study
GyrB_F	GTGCGCGTGAAGTCACACGTA	This study
GyrB_R	GCATCGGTCATCAAAACGAG	This study
ParC_F	CCGGGCTTTGCCAGATAT	This study
ParC_R	GGCTGCTGGCAAGACCGTT	This study
ParE_F	CAGCCCAATCTAAGAAT	This study
ParE_R	GCAATATAGACATGACCT	This study
rpoD-F	CAGGTAGCAGAATTTATCCGTAATC	PrimerDesign Ltd
rpoD-R	CCCATCAGCGTCCAAGGTA	PrimerDesign Ltd
proC-F	TTATCCCAAGTCAACACCGAAT	PrimerDesign Ltd
proC-R	GCAATTAGGAGACAAGGCATAAC	PrimerDesign Ltd
patB_R	AGGATATCGCCATCTTGTCG	[18]
patB_F	ATGGCAAAGCCTATCAGGAA	[18]
patA_F	TCCTGATGACAGGCTTGATG	[18]
patA_R	TGCGAGGACAACATTGAGTC	[18]
pmrA_F	TCCAGTATGGGCTTTTCCAG	[18]
pmrA_R	CCAATCCAAAGAGGAAACGA	[18]

S2 Table. Results of CST typing. This is based on *wzh* sequences, which was chosen because it varies sufficiently between serotypes, but is conserved enough to amplify the same gene segment of the various serotypes using a single mix of primers. The scores are calculated as described by Elberse KE et al. (2011 PLoS One. 6:e20390).

ID (YEAR/ID)	*Corresponding serotypes*	*Score*
ATCC	19F	99.7921
97a04	35F,47F	99.799194
97b25	23F,15B,15C	100.0
97B27	06A,06B,06C	99.799194
99A16	23A	99.393936
99G11	3	99.79253
99J16	11A,11D,18F	99.8
01A38	23F,15B,15C	99.799194
01C35	09V	97.42063
01D01	06B	100.0
01G34	11A,11D,18F	100.0
01H21	23F,15B,15C	99.59514
01H27	23F,15B,15C	99.798386
01H28	23F,15B,15C	99.799194
01J10	11A,11D,18F	99.7996
03A07	09V	99.5984
03B38f	19A	99.799194
03C18	09V	99.79879
03K18	3	99.2016
03L23	06B,06A	99.79798
03L28	31	99.799194
03N11	21	99.79879
04A24	4	99.5984
04J04	11A,11D,18F	99.8004
05A20	20,13	94.69388
05A36	20,13	96.414345
05K36	22F,15B,22A	99.799194
05M22	15A	99.799194
06H02	09V	97.95082
06H10	09N,09L	99.79339
07A40	06B	99.590164
07B16	3	99.2016
07H04	25F,25A,38	99.799194
07J30	25F,25A,38	99.799194
07O07	19F	98.998
08E15	06A	99.58506
08E16	22F,15B,22A	99.794235
08L33	14	99.7921
09B07	19F	98.998
09K33	19F	99.40239
10D22	18C,18B, 19F	94.02391
10D22	18C,18B, 19F	94.03579
10K19	19A	99.2
10N11	22F,15B,22A	99.5842
11A23	15A	99.198395
11A27	15A	98.75776
13C24	07F,07A	99.393936
13C28	09N,09L	99.79296
13F15	35F,47F	100.0
13G08	35F,47F	99.79879
13J24	19A	100.0
13K19	19F	99.395164
13L04	23B	100.0

S3 Table. QRDR sequencing and MIC determination of a selection of 422 pneumococcal strains.

Strain	MIC (Microdilution)				QRDR Sequencing				MIC (E-tests)			
Id.	CIP	LVX	MXF	OFL	GyrA	GyrB	ParC	ParE	CIP	CIP+R	MOX	MOX+R
95B01	4	2	0.25	4	wt	wt	K137N	I460V	1.5	1	0.19	0.19
95B03	4	2	0.25	4	wt	wt	wt	I460V	1	0.75	0.094	0.094
95B04	4	2	0.25	4	wt	wt	wt	I460V	0.75	0.75	0.125	0.125
95B05	2	2	0.12	2	wt	wt	K137N	I460V	0.38	0.19	0.047	0.047
95B08	4	2	0.25	4	wt	wt	K137N	I460V	1	0.75	0.125	0.125
95B09	4	2	0.25	4	wt	wt	wt	wt	1	0.75	0.19	0.19
95B11	4	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.19	0.19
95B15	4	2	0.25	4	S114G	wt	S52G	wt	1.5	0.75	0.19	0.19
95B16	4	2	0.25	4	S114G	wt	S52G/N91D	wt	4	1	0.25	0.19
95B18	2	2	0.25	2	wt	wt	wt	wt	1.5	0.5	0.125	0.125
95B20	4	2	0.25	4	M99I	wt	wt	I460V	1.5	1	0.19	0.19
95B21	4	2	0.12	4	S114G	wt	wt	K448P	4	1	0.25	0.25
95C02	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
95C08	4	2	0.25	4	wt	wt	S52G/N91D	I460V	1.5	0.5	0.19	0.19
95C12	4	2	0.25	4	wt	wt	wt	I460V	1.5	1.5	0.125	0.125
95C17	2	2	0.12	2	wt	wt	wt	I460V	0.75	0.25	0.094	0.094
95C20	2	2	0.12	2	wt	wt	wt	wt	0.5	0.38	0.125	0.125
95C24	2	2	0.12	2	wt	wt	K137N	I460V	1	0.5	0.125	0.125
95D06	2	2	0.12	2	wt	wt	K137N	I460V	0.5	0.38	0.125	0.094
95D08	2	1	0.12	2	wt	wt	wt	I460V	0.5	0.38	0.094	0.094
95D12	2	1	0.12	2	wt	wt	K137N	wt	0.75	0.38	0.125	0.094
95D19	2	1	0.12	2	M99I	wt	wt	I460V	0.75	0.38	0.094	0.094
95E02	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
95E11	2	1	0.12	2	wt	wt	wt	wt	1	0.5	0.125	0.125
95F03	2	1	0.25	2	G103S	wt	wt	I460V	0.75	0.5	0.125	0.125
95F08	2	1	0.12	2	wt	wt	wt	I460V	2	0.5	0.094	0.094
95G05	2	1	0.12	2	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
95G06	2	1	0.12	2	wt	wt	wt	I460V	1	0.5	0.125	0.094
95H07	2	1	0.06	4	wt	wt	K137N	I460V	0.38	0.19	0.064	0.064
95H10	2	1	0.12	4	wt	wt	wt	I460V	0.75	0.38	0.125	0.125
97A04	8	4	0.25	4	wt	wt	S79F	I460V	8	3	0.25	0.25
97A11	2	1	0.25	4	wt	wt	wt	I460V	2	0.5	0.125	0.125
97B03	2	1	0.06	2	wt	wt	wt	I460V	1	0.38	0.094	0.064
97B14	2	2	0.12	4	L154F	wt	wt	I460V	1.5	0.5	0.094	0.094
97B15	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.094	0.094
97B18	2	2	0.12	4	wt	wt	wt	I460V	0.5	0.38	0.064	0.064
97B21	2	1	0.12	4	wt	wt	wt	wt	2	0.38	0.125	0.094
97B23	4	2	0.12	4	wt	wt	wt	I460V	1	0.75	0.19	0.19
97B25	4	4	0.25	8	wt	wt	ND	wt	24	4	0.38	0.38
97B27	2	2	0.12	4	wt	wt	wt	I460V	8	0.75	0.094	0.094
97C08	2	2	0.12	8	wt		wt	I460V	1	0.75	0.19	0.19
97C12	2	2	0.12	4	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
97C13	2	1	0.06	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
97C16	2	1	0.12	4	wt	wt	wt	wt	2	0.38	0.094	0.094
97C24	2	1	0.12	4	wt	wt	wt	wt	0.5	0.38	0.094	0.094
97D04	2	2	0.25	4	wt	wt	K137N	I460V	1	0.38	0.094	0.094
97D22	2	0.5	0.06	2	wt	wt	wt	I460V	1	0.5	0.094	0.094
97G03	2	0.5	0.06	2	wt	wt	S79F	A532T	2	1	0.19	0.125
97G09	2	1	0.12	2	wt	wt	S79F	wt	4	1.5	0.125	0.125
97H12	2	1	0.12	2	wt	wt	wt	wt	0.75	0.38	0.064	0.064
97H26	2	1	0.12	2	wt	wt	K137N	I460V	0.5	0.38	0.094	0.094
97I27	2	1	0.12	2	wt	wt	wt	I460V	1.5	0.5	0.125	0.094
99A01	0.5	0.06	0.5	0.06	wt	wt	K137N	I460V	0.5	0.25	0.094	0.064
99A05	2	2	0.12	4	wt	wt	wt	wt	1.5	0.5	0.125	0.125
99A09	2	0.5	2	0.5	wt	wt	wt	I460V	2	0.5	0.19	0.125
99A11	2	2	0.12	4	wt	wt	wt	wt	1.5	0.75	0.125	0.125
99A12	2	2	0.06	4	wt	wt	K137N	I460V	0.75	0.38	0.094	0.094
99A16	4	4	4	0.5	S81F	wt	D83G	wt	32	16	2	1.5
99B11	1	0.25	1	0.12	wt	wt	wt	I460V	1	0.38	0.125	0.094
99C21	2	2	0.25	4	wt	wt	wt	I460V	1	0.38	0.094	0.094
99C23	4	1	4	0.25	S114G	wt	S52G/N91D/ E134D	opnieuw	0.75	0.38	0.125	0.094
99C25	1	0.5	2	0.25	wt	wt	wt	I460V	1.5	0.38	0.094	0.094
99D02	2	0.12	2	0.06	wt	wt	K137N	I460V	4	0.75	0.094	0.064

99D21	2	2	0.06	4	wt	wt	N91D	I460V	0.75	0.25	0.064	0.064
99D22	2	2	0.12	4	wt	wt	wt	wt	0.75	0.38	0.064	0.064
99D25	2	0.5	2	0.25	wt	wt	K137N	I460V	1	0.5	0.125	0.094
99E12	2	1	2	0.25	wt	wt	K137N	I460V	1	0.5	0.094	0.094
99 E22	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
99 E24	2	2	0.12	4	wt	wt	wt	I460V	4	1	0.094	0.094
99G09	2	2	0.5	4	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
99G10	2	2	0.25	4	wt	wt	wt	I460V	2	0.5	0.125	0.125
99G11	2	2	0.5	4	wt	wt	wt	I460V	6	0.75	0.125	0.125
99G16	2	2	0.5	4	wt	wt	wt	I460V	0.5	0.38	0.094	0.094
99G17	2	0.5	2	0.25	wt	wt	K137N	I460V	1	0.5	0.125	0.125
99G21	2	0.5	2	0.25	wt	wt	wt	I460V	1	0.5	0.125	0.125
99G24	4	2	0.5	8	wt	wt	wt	I460V	2	0.5	0.125	0.125
99H10	2	0.25	2	0.12	wt	wt	wt	wt	1.5	0.19	0.094	0.094
99H17	2	0.5	2	0.25	wt	wt	wt	wt	0.5	0.125	0.064	0.032
99H18	2	1	2	0.5	wt	wt	wt	I460V	1.5	1	0.19	0.125
99I23	2	0.5	2	0.25	wt	wt	wt	I460V	1	0.38	0.125	0.125
99I24	2	2	0.12	4	L155F	wt	wt	I460V	0.5	0.38	0.094	0.094
99J07	2	2	0.25	4	wt	wt	K137N	I460V	0.75	0.5	0.125	0.125
99J08	2	1	0.12	4	wt	wt	wt	I460V	4	0.75	0.125	0.125
99J13	2	0.5	2	0.25	wt	wt	wt	I460V	0.75	0.38	0.094	0.094
99J16	4	1	4	0.5	wt	wt	wt	I460V	8	0.38	0.094	0.094
99J20	2	1	4	0.5	wt	wt	K137N	I460V	1	0.5	0.125	0.125
01A01	2	1	0.25	4	wt	wt	wt	Q420P/I460V	1	0.38	0.125	0.125
01A05	2	1	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
01A07	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
01A09	4	2	0.12	8	wt	wt	wt	wt	0.75	0.5	0.125	0.094
01A38	4	2	0.12	8	wt	wt	K137N	I460V	12	2	0.25	0.25
01C01	4	4	0.5	8	wt	wt	wt	I460V	1	0.75	0.125	0.125
01C02	4	4	0.5	8	wt	wt	wt	I460V/D435N	0.75	0.75	0.125	0.19
01C03	4	2	0.5	8	S114G	wt	wt	H534L	1	0.25	0.064	0.094
01C06	4	2	0.5	8	wt	wt	K137N	I460V	3	0.38	0.125	0.125
01C24	1				S114G	wt	wt	I460V	0.75	0.25	0.094	0.094
01C30	2	2	0.25	4	wt	wt	K137N	I460V	1	0.38	0.094	0.094
01C33	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
01C35	4	4	0.25	8	S114G	wt	S79Y/N91D/E213D	I460V	16	2	0.125	0.125
01D01	2	2	0.25	4	wt	wt	K137N	wt	6	0.75	0.125	0.125
01D02	2	2	0.12	4	wt	wt	wt	I460V	0.75	0.5	0.125	0.094
01D04	2	2	0.12	4	wt	wt	wt	I460V	0.5	0.5	0.125	0.125
01D33	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
01F05	2	2	0.12	4	wt	wt	wt	I460V	0.5	0.38	0.094	0.064
01G07	2	2	0.25	4	wt	wt	wt	wt	0.75	0.38	0.094	0.094
01G18	2	2	0.12	4	wt	wt	wt	I460V	4	0.38	0.125	0.094
01G34	8	4	0.25	8	wt	wt	D83Y	I460V	12	1	0.125	0.125
01H12	2	2	0.25	4	wt	wt	N91D / E135D	I460V	1	0.5	0.125	0.125
01H21	4	4	1	8	S81F	wt	K137N	I460V/D435N	16	4	1.5	1
01H27	4	4	0.5	8	S81F	wt	K137N	I460V/D435N	32	12	3	2
01H28	4	4	1	8	S81F	wt	K137N	I460V	>32	12	3	2
01I04	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
01J06	2	2	0.25	4	wt	wt	K137N	I460V	1	0.5	0.125	0.125
01J10	8	8	2	16	S81F	wt	S79F	I460V	32	32	2	3
01J38	2	1	0.06	4	wt	wt	wt	I460V	4	1	0.125	0.125
03 A05	2	0.5	2	4	wt	wt	wt	I460V	1.5	1	0.125	0.125
03 A07	8	8	8	8	S81Y/S114G	wt	S79Y/N91D/E134D	ND	32	32	4	4
03 A22	2	0.12	1	4	S114G	wt	wt	I460V	1.5	0.5	0.094	0.094
03 B29	2	0.25	1	4	wt	wt	wt	I460V	0.75	0.75	0.094	0.094
03B38	2	1	0.25	4	wt	wt	S79Y	I460V	8	2	0.125	0.125
03 C09	2	0.25	2	4	M99I	wt	wt	I460V	0.75	0.75	0.094	0.125
03 C10	2	0.12	1		wt	wt	wt	I460V	2	0.75	0.064	0.125
03 C14	2	0.12	2	4	wt	wt	wt	wt	3	0.75	0.094	0.125
03 C18	8	2	8	16	E85K	wt	S79Y/K137N	I460V	32	32	3	4
03 D03	2	0.12	2	4	wt	wt	wt	I460V	0.75	0.5	0.064	0.064

03 D06	4	0.25	4	8	S114G/ F161L	wt	N91D/S52G	wt	1	0.75	0.094	0.125
03 D11	2	0.12	2	4	wt	wt	wt	I460V	0.75	0.75	0.047	0.094
03 D13	2	0.25	1	4	wt	wt	wt	I460V	1.5	0.75	0.094	0.094
03 D17	2	0.12	1	4	wt	wt	wt	I460V	1	0.5	0.064	0.064
03 D27	2	0.06	1	4	wt	wt	wt	I460V	0.75	0.5	0.094	0.094
03 E04	2	0.12	1	4	wt	wt	wt	wt	0.75	0.5	0.094	0.125
03 E10	4	0.12	2	8	wt	wt	wt	I460V	1	0.75	0.094	0.094
03 E11	8	0.25	8	32	wt	wt	wt	I460V	0.5	0.38	0.047	0.094
03 E12	4	0.25	4	8	wt	wt	wt	I460V	1	0.75	0.094	0.125
03 E13	4	0.12	2	8	wt	wt	wt	ND	0.75	0.75	0.064	0.064
03 E14	2	0.06	2	4	wt	wt	wt	I460V	1.5	0.38	0.094	0.094
03 E15	4	0.12	4	8	wt	wt	wt	I460V	0.38	0.38	0.047	0.094
03 E17	8	0.12	8	16	wt	wt	wt	wt	2	0.75	0.094	0.125
03 E18	8	0.25	8	32	wt	wt	S79F/K137N	I460V	3	1.5	0.125	0.19
03 E19	8	0.12	8	16	S114G/ L152R	wt	S52G/K57Q/ N91D	wt	2	0.5	0.094	0.125
03 E21	4	0.12	4	8	wt	wt	wt	wt	0.75	0.75	0.094	0.125
03 G16	2	0.12	2	4	wt	wt	wt	wt	2	0.75	0.125	0.125
03 H10	2	0.12	2		wt	wt	wt	I460V	2	0.5	0.125	0.094
03H12	4	0.12	4	8	wt	wt	wt	I460V	0.5	0.5	0.064	0.064
03 H32	4	0.12	4	8	S114G/L1 55V	wt	S52G/N91D	Y481H/ I493L	1.5	0.75	0.094	0.125
03 I06	2	0.12	1	4	wt	wt	wt	I460V	2	1	0.125	0.125
03 I08	2	0.5	1	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
03 I12	2	0.25	1	4	wt	wt	wt	I460V	2	0.75	0.125	0.125
03 I21	2	0.12	2	4	wt	wt	wt	I460V	1.5	0.75	0.125	0.125
03 I23	2	0.25	2	4	wt	wt	wt	I460V	2	0.75	0.125	0.125
03 J25	2	0.12	1	4	wt	wt	wt	I460V	1	0.38	0.094	0.064
03 K18	4	0.12	2	8	wt	wt	wt	I460V	8	0.75	0.125	0.125
03K32	2	2	0.25	4	wt	wt	S79F	wt	2	1.5	0.125	0.125
03K33	2	2	0.25	4	wt	wt	K137N	I460V	0.75	0.75	0.125	0.125
03K36	2	2	0.5	4	wt	wt	wt	I460V	1	0.5	0.064	0.125
03K38	2	1	0.25	4	wt	wt	wt	wt	2	0.5	0.125	0.125
03L03	2	2	0.25	4	wt	wt	wt	I460V	0.5	0.5	0.094	0.064
03L14	2	1	0.25	4	wt	wt	wt	I460V	2	0.5	0.094	0.094
03L15	2	2	0.25	4	wt	wt	wt	I460V	0.5	0.5	0.125	0.125
03 L23	8	0.12	4	8	wt	wt	S79F	D435K/H 534L	32	2	0.19	0.19
03L28	2	1	0.12	4	wt	wt	K137N	I460V	>32	1.5	0.125	0.125
03L31	2	1	0.12	4	wt	F480L	wt	wt	0.5	0.25	0.064	0.064
03N04	2	1	0.12	2	wt	wt	K137N	I460V	1.5	0.75	0.125	0.125
03N06	2	1	0.12	4	wt	wt	K137N	I460V	1.5	0.75	0.125	0.064
03N11	2	1	0.12	4	wt	wt	S79Y	I460V	16	2	0.19	0.19
03 O03	2	0.12	2	4	wt	wt	S52G/N91D/ R95G	I460V	1	0.75	0.94	0.94
03 O06	4	0.12	2	8	wt	wt	wt	I460V	0.5	0.5	0.094	0.125
03O10	2	1	0.12	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
03O22	2	1	0.25	4	wt	wt	wt	I460V	3	1	0.19	0.19
04A06	2	2	0.25	4	wt	wt	K137N	I460V	0.75	0.5	0.125	0.125
04A07	2	2	0.25	4	wt	wt	wt	I460V	0.5	0.5	0.125	0.125
04A10	2	2	0.25	4	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
04A24	2	2	0.25	4	wt	wt	wt	I460V	8	1	0.19	0.125
04A25	2	2	0.12	4	wt	wt	wt	I460V	3	1	0.19	0.19
04A30	2	2	0.12	4	S114G	wt	K137N	I460V	1	0.38	0.125	0.125
04B04	4	4	0.25	8	S114G	F491I/S 494T	wt	I460V	0.75	0.38	0.064	0.064
04B11	4	4	0.25	8	wt	wt	K137N	I460V	0.5	0.25	0.094	0.064
04B15	2	2	0.06	4								
04B16	4	4	0.25	8	wt	wt	wt	I460V	1.5	0.75	0.125	0.125
04C03	4	4	0.06	8	wt	wt	wt	I460V	0.38	0.38	0.064	0.064
04C04	4	4	0.12	8	wt	wt	wt	I460V	1.5	0.5	0.094	0.094
04D19	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.125	0.094
04E02	4	4	0.25	8	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
04E05	2	4	0.25	4	wt	wt	wt	wt	0.5	0.38	0.094	0.094
04E10	2	4	0.25	4	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
04F27	4	4	0.12	8	wt	wt	wt	I460V	1	0.38	0.094	0.064
04H30	2	2	0.12	4	wt	wt	wt	I460V	1	0.38	0.094	0.094
04H32	2	4	0.12	4	wt	wt	wt	A532V	1	0.38	0.094	0.094

04I06	2	2	0.12	4								
04I29	2	1	0.06	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
04I40	2	1	0.12	4	wt	wt	wt	I460V	0.5	0.38	0.125	0.125
04J04	16	16	2	16	S81Y	wt	S79F	I460V	>32	>32	1	1
04J07	2	1	0.06	4	wt	wt	K137N	I460V	0.38	0.25	0.064	0.064
04J15	2	2	0.06	4	wt	wt	wt	I460V	0.75	0.38	0.094	0.094
04J18	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
04K11	4	2	0.12	8	wt	wt	wt	I460V	1	0.38	0.094	0.094
04L02	2	1	0.12	4	wt	wt	wt	I460V	0.75	0.5	0.094	0.094
04L03	2	1	0.12	4	wt	wt	wt	I460V	0.75	0.38	0.094	0.094
04L06	2	2	0.25	4	wt	wt	wt	wt	0.5	0.5	0.094	0.094
04L07	2	2	0.12	4	wt	wt	wt	I460V	1.5	0.5	0.094	0.094
04L17	4	4	0.5	8	S81F	wt	K137N	I460V	2	1	0.5	0.38
04L24	2	2	0.12	4	wt	wt	K137N	I460V	1.5	0.5	0.094	0.094
04L31	2	2	0.12	4	wt	wt	wt	I460V	2	0.75	0.125	0.125
04M10	2	2	0.12	4	wt	wt	wt	I460V	0.38	0.25	0.064	0.064
04N12	2	2	0.06	4	wt	wt	wt	I460V	0.5	0.38	0.094	0.094
04N39	2	2	0.12	4	wt	wt	K137N	I460V	0.5	0.5	0.094	0.094
04O22	2	2	0.12	4	wt	wt	wt	I460V	0.5	0.38	0.094	0.094
05A02	2	2	0.25	4	wt	wt	K137N	I460V	0.75	0.38	0.094	0.094
05A05	2	2	0.25	4	S81F	wt	K57T	I460V	1	0.5	0.125	0.094
05A07	2	2	0.25	4	wt	S466G	K57T	I460V	0.75	0.5	0.125	0.094
05A13	4	2	0.25	8	S114I	wt	K57T	I460V	1.5	0.5	0.125	0.125
05A15	2	2	0.25	4	wt	wt	K57T	I460V	1.5	0.75	0.125	0.125
05A20	64	64	64	64	S81L\ S114G	P454S	wt	wt	>32	>32	>32	>32
05A28	2	2	0.25	4	S114G	wt	S52G/N91D/ T54N/K57T	wt	4	1	0.19	0.19
05A34	2	2	0.25	4	S114G	wt	S52G/N91D/ K57L/	wt	4	0.75	0.19	0.19
05A36	8	2	0.25	8	wt	wt	S79Y/K137N/ K57T	wt	16	3	0.25	0.19
05B29	2	2	0.25	4	wt	wt	K57T	I460V	1	0.38	0.125	0.125
05C03	2	1	0.25	4	wt	wt	K57T	I460V	1.5	0.75	0.125	0.125
05C07	2	1	0.12	4	wt	wt	K137N/K57M	I460V	0.75	0.5	0.125	0.125
05C32	2	1	0.12	4	wt	wt	wt	I460V	1.5	0.5	0.064	0.047
05C40	2	1	0.06	4	wt	wt	K137N	I460V	1	0.38	0.125	0.094
05D25	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
05D26	2	2	0.12	4	wt	wt	wt	I460V/ I431S	1	0.75	0.125	0.125
05D28	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
05D30	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
05D31	2	2	0.25	4	wt	wt	wt	I460V	1.5	0.5	0.094	0.094
05D32	2	2	0.5	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
05D34	4	2	0.25	8	wt	wt	wt	I460V	4	1	0.125	0.125
05D36	4	4	0.5	8	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
05D39	2	2	0.25	4	wt	wt	wt	I460V	0.5	0.5	0.094	0.094
05D40	4	4	0.5	8	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
05E02	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
05E04	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
05E10	2	1	0.12	4	wt	wt	K137N	wt	1.5	0.5	0.125	0.125
05E30	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
05E36	2	2	0.25	4	wt	wt	D78N	I460V	2	1	0.19	0.19
05F15	2	1	0.12	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
05I37	2	2	0.12	4	wt	wt	wt	I460V	0.75	0.5	0.19	0.19
05J31	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.38	0.094	0.094
05K34	2	2	0.25	4	wt	wt	S79F	wt	4	1.5	0.19	0.19
05K36	16	16	2	16	S81F	wt	S79F	wt	>32	32	4	3
05M22	2	1	0.12	4	wt	wt	S79F	I460V	6	2	0.19	0.19
06A02	2	1	0.12	4	wt	wt	wt	I460V	0.5	0.5	0.064	0.094
06A03	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
06A05	2	1	0.12	4	wt	wt	wt	wt	1	0.5	0.125	0.125
06A07	2	2	0.12	2	wt	wt	K137N	I460V	0.75	0.38	0.094	0.094
06A08	2	2	0.12	4	S114G	wt	wt	M467I/ A496T	2	0.5	0.094	0.125
06A10	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
06A11	2	1	0.25	4	wt	wt	wt	wt	0.5	0.5	0.125	0.094
06A12	2	1	0.12	4	wt	wt	K137N	I460V	0.75	0.5	0.064	0.094
06A13	2	2	0.25	4	wt	wt	K137N	I460V	0.5	0.5	0.125	0.094

06A15	2	2	0.12	4	wt	wt	wt	I460V	2	1	0.125	0.125
06A19	2	1	0.12	4	wt	wt	wt	I460V	0.75	0.5	0.094	0.125
06A20	2	2	0.12	4	wt	wt	wt	I460V	1.5	1	0.5	0.5
06A22	2	1	0.12	4	wt	wt	wt	I460V	0.75	0.38	0.125	0.125
06A24	2	2	0.12	4	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
06A25	2	2	0.12	4	wt	wt	wt	I460V	1	0.5	0.094	0.125
06B18	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.38	0.094	0.094
06H02	4	4	0.25	8	S114G	wt	wt	wt	>32	2	0.19	0.125
06H06	2	1	0.25	4	wt	wt	wt	wt	0.75	0.5	0.094	0.094
06H10	4	4	1	8	S81G	wt	K137N	I460V	>32	>32	3	2
06J34	2	1	0.06	4	wt	wt	wt	I460V	2	0.5	0.094	0.125
06J35	2	2	0.25	4	wt	wt	wt	wt	2	0.5	0.125	0.125
06J37	2	1	0.25	4	wt	wt	wt	I460V	2	0.5	0.125	0.125
06K02	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.094
06K08	2	2	0.12	4	wt	wt	K137N	I460V	0.75	0.5	0.094	0.094
06K13	2	1	0.25	4	wt	wt	wt	I460V	2	0.75	0.125	0.125
06K15	2	2	0.25	4	wt	wt	K137N	I460V	0.75	0.5	0.094	0.094
06K19	2	2	0.25	4	wt	D435N	K137N	I460V	1	0.5	0.094	0.094
06K23	2	2	0.12	4	wt	wt	K137N	I460V	0.75	0.38	0.094	0.094
06L08	2	1	0.25	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
06L09	2	2	0.25	4	wt	wt	wt	I460V	1.5	0.75	0.125	0.125
06N06	2	1	0.12	4	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
06O04	2	1	0.12	4	wt	wt	wt	wt	1.5	0.38	0.094	0.094
06O06	2	1	0.12	4	wt	wt	wt	wt	1.5	0.75	0.125	0.125
06O09	2	1	0.12	4	wt	wt	K137N	I460V	0.75	0.38	0.125	0.25
06O15	2	2	0.12	4	wt	wt	wt	I460V	2	0.75	0.125	0.125
06O17	2	2	0.12	4	wt	wt	K137N	I460V	0.5	0.25	0.125	0.125
06O19	2	1	0.06	4	wt	wt	wt	I460V	2	0.75	0.094	0.094
07A01	2	1	0.25	2	wt	wt	wt	wt	0.25	0.25	0.064	0.125
07A40	16	8	1	16	S81F	wt	N49L/K50N/ S79F	wt	>32	>32	3	2
07B16	4	2	0.12	4	wt	wt	wt	I460V	12	0.75	0.125	0.125
07C12	2	1	0.25	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
07C24	2	1	0.12	4	wt	G434R	K137N	I460V	0.75	0.5	0.064	0.125
07F04	2	1	0.12	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
07F07	4	2	0.12	8	wt	wt	wt	I460V	2	0.75	0.125	0.125
07F08	4	1	0.12	8	wt	wt	wt	I460V	1	0.75	0.125	0.125
07G18	4	1	0.12	8	wt	wt	K137N	I460V	1	0.75	0.125	0.125
07H01	4	2	0.12	8	wt	wt	S79F	I460V	4	1	0.125	0.125
07H04	8	8	2	16	S81F	wt	S79F	I460V	>32	>32	4	3
07I02	2	1	0.12	4	wt	wt	K137N	I460V	0.75	0.75	0.125	0.064
07J30	16	8	1	16	S81F	wt	S79F	wt	>32	>32	2	2
07L34	4	4	0.25	8	wt	wt	wt	I460V	1	0.5	0.125	0.125
07M27	2	1	0.25	4	wt	wt	R95C	wt	1	0.5	0.125	0.125
07O07	2	1	0.12	4	S114G	wt	S79F/N91D	I460V	16	6	0.25	0.25
08A02	2	1	0.12	4	wt	wt	wt	wt	0.5	0.25	0.125	0.064
08A03	2	2	0.25	4	wt	wt	K137N	I460V	0.5	0.25	0.125	0.125
08A12	2	2	0.12	4	wt	wt	wt	I460V	2	0.5	0.094	0.094
08A15	2	2	0.25	4	wt	G434R	wt	I460V	1.5	0.75	0.125	0.5
08C15	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.064	0.064
08 E03	2	2	0.25	4	wt	wt	R95C	wt	0.75	0.5	0.094	0.064
08 E13	2	2	0.12	4	wt	G434R	wt	I460V	1.5	0.5	0.094	0.094
08 E15	2	2	0.25	4	wt	wt	wt	I460V	6	1	0.125	0.094
08 E16	32	16	8	16	S81F	wt	S79F	wt	>32	12	3	3
08G22	2	1	0.12	4	wt	wt	wt	wt	0.25	0.25	0.125	0.125
08G25	2	4	0.12	4	wt	G434R	wt	I460V	2	0.5	0.125	0.094
08G26	2	2	0.12	4	wt	wt	wt	I460V	2	0.5	0.094	0.094
08G28	2	2	0.12	4	wt	wt	wt	I460V	3	0.75	0.125	0.094
08I01	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.094	0.094
08J01	2	2	0.25	4	wt	wt	K137N	I460V	2	1	0.125	0.125
08J07	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.38	0.094	0.094
08J09	2	2	0.25	4	wt	wt	wt	I460V	2	0.5	0.125	0.094
08L06	2	2	0.12	4	wt	wt	wt	I460V	3	0.5	0.094	0.094
08L13	2	2	0.25	4	wt	wt	wt	wt	0.75	0.5	0.125	0.125
08L33	16	16	2	16	S81F	wt	S79F / K137N	I460V	>32	>32	2	4
08L34	2	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.5
08O21	2	2	0.12	4	wt	wt	K137N	I460V	2	0.5	0.125	0.125
09B07	4	2	0.25	8	S114G	wt	S79F / N91D	wt	>32	4	0.125	0.125
09K03	2	1	0.25	4	wt	wt	wt	I460V	1	0.5	0.094	0.094

09K10	4	1	0.12	4	wt	wt	wt	I460V	1	0.5	0.094	0.094
09K15	4	2	0.12	4	wt	wt	wt	I460V	4	0.5	0.094	0.094
09K16	2	1	0.12	4	wt	wt	wt	I460V	2	0.75	0.125	0.125
09K22	2	1	0.06	4	wt	wt	S79F	I460V	3	2	0.125	0.125
09K32	16	8	1	16	wt	wt	S79F	I460V	>32	>32	2	2
09K33	8	8	0.5	16	wt	wt	S79Y	I460V	>32	>32	2	2
09L20	2	1	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.094
09L23	2	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.094	0.094
09L24	2	1	0.25	4	wt	wt	S79F	I460V	2	1.5	0.125	0.125
09N30	4	4	0.12	8	wt	wt	wt	I460V	1.5	0.75	0.064	0.064
09O15	2	1	0.06	4	wt	wt	wt	I460V	0.75	0.25	0.064	0.064
10A09	4	2	0.25	4	wt	wt	wt	I460V	1.5	1	0.125	0.125
10A11	4	2	0.5	4	wt	wt	wt	I460V	1.5	1	0.19	0.19
10A13	2	1	0.25	4	wt	wt	wt	wt	0.75	0.75	0.125	0.125
10A15	2	1	0.25	4	wt	wt	wt	wt	1	0.5	0.125	0.125
10A19	2	1	0.12	4	wt	wt	wt	A468T / I493L	4	0.5	0.125	0.094
10A23	4	2	0.25	8	wt	wt	wt	wt	3	3	0.5	0.5
10A34	4	2	0.25	8	wt	wt	S79F	I460V	3	3	0.19	0.19
10D22	2	2	0.25	4	wt	wt	S79I / N91D / E135D	wt	12	6	0.25	0.25
10I25	2	1	0.25	4	wt	wt	wt	I460V	3	1	0.125	0.125
10K19	4	4	0.12	8	wt	wt	wt	I460V	6	1.5	0.125	0.125
10L01	2	1	0.5	4	wt	wt	wt	wt	0.75	0.5	0.125	0.125
10L03	4	4	0.25	8	wt	wt	wt	wt	0.75	0.38	0.125	0.125
10L16	4	2	0.25	8	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
10L18	4	2	0.5	4	wt	wt	wt	I460V	1.5	0.5	0.25	0.25
10L36	2	2	0.25	4	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
10N11	32	8	4	16	S81F	wt	S79Y	wt	>32	>32	8	4
11A01	8	4	0.25	8	wt	wt	wt	I460V	4	0.5	0.125	0.125
11A17	8	4	0.25	8	wt	wt	wt	I460V	3	0.5	0.125	0.125
11A21	8	2	0.5	4	wt	wt	wt	I460V	1.5	0.75	0.19	0.19
11A23	16	8	1	16	wt	wt	D83N	I460V	>32	16	1.5	1.5
11A27	4	2	0.25	4	S114G	wt	N91D	I493L	8	1	0.19	0.125
11A28	8	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
11A36	16	8	1	16	wt	wt	D83N	I460V	>32	24	1	1
11G08	4	2	0.25	4	wt	wt	wt	I460V	0.75	0.5	0.125	0.125
11I08	4	2	0.25	8	wt	wt	wt	I460V	>32	32	1	1
11I10	4	2	0.12	8	wt	wt	wt	I460V	1	0.5	0.125	0.125
11I29	4	2	0.12	4	wt	wt	wt	I460V	1.5	0.75	0.125	0.125
11I30	4	2	0.12	4	wt	wt	wt	wt	0.75	0.38	0.094	0.094
11L12	4	2	0.5	8	wt	wt	wt	I460V	1	0.75	0.125	0.125
11O31	4	2	0.25	8	S114G	wt	wt	wt	4	1.5	0.125	0.125
12C10	16	8	2	8	S81F	wt	S79F	wt	>32	>32	3	3
12C16	4	1	0.12	8	S114G	wt	wt	I460V	4	3	0.5	0.5
12D07	4	2	0.25	8	S114G	wt	N91D	I460V	3	2	0.125	0.125
12J05	4	1	0.25	4	wt	wt	wt	I460V	1.5	1	0.125	0.125
12J12	4	2	0.25	4	wt	wt	wt	I460V	1	1	0.094	0.094
12K21	4	2	0.5	4	wt	wt	wt	I460V	1	1	0.125	0.125
12K22	4	2	0.25	4	wt	wt	wt	I460V	1	0.75	0.064	0.064
12K23	4	1	0.12	4	wt	wt	wt	I460V	1	0.75	0.125	0.094
12K25	4	2	0.25	4	wt	wt	wt	I460V	1	1	0.125	0.125
12K32	4	4	0.12	4	wt	wt	wt	I460V	0.5	0.5	0.125	0.125
12L01	4	4	0.25	4	wt	wt	wt	I460V	1	1	0.125	0.125
12L03	4	2	0.12	4	wt	wt	wt	I460V	1	0.75	0.094	0.094
12L10	4	4	0.12	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
12L13	4	4	0.5	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
12L14	4	4	0.12	4	wt	wt	wt	wt	0.25	0.25	0.094	0.094
12L18	4	4	0.5	4	wt	wt	wt	I460V	1	0.75	0.094	0.094
12L20	4	2	0.12	2	wt	wt	wt	wt	0.75	0.5	0.094	0.094
12L36	4	2	0.12	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
12L37	4	2	0.12	4	wt	wt	wt	I460V	1	1	0.094	0.094
12L38	4	2	0.06	2	wt	wt	wt	I460V	1.5	1	0.094	0.094
12M02	4	4	0.25	4	wt	wt	S79F / N91D	I460V	4	1	0.125	0.125
12M03	4	2	0.25	4	wt	wt	wt	I460V	1.5	1	0.125	0.125
12M06	4	4	0.12	4	wt	wt	wt	I460V	0.75	0.75	0.064	0.064
12M09	8	4	0.12	8	wt	wt	wt	I460V	1	0.75	0.125	0.094
12N02	4	2	0.25	4	S114G	wt	wt	I460V	3	1.5	0.25	0.19
12O33	4	2	0.12	4	wt	wt	wt	I460V	1	0.75	0.125	0.125

12O34	4	2	0.12	4	wt	wt	wt	I460V	1	1	0.125	0.125
12O40	8	4	0.25	8	wt	wt	wt	wt	2	1	0.125	0.125
13A12	4	2	0.12	8	wt	wt	wt	I460V	1.5	0.5	0.125	0.125
13A21	4	2	0.25	8	wt	wt	wt	I460V	3	1	0.19	0.19
13A22	8	2	0.25	8	wt	wt	wt	I460V	4	1.5	0.125	0.125
13A26	4	2	0.25	4	wt	wt	wt	I460V	2	1	0.125	0.125
13A30	4	2	0.25	4	wt	wt	wt	I460V	2	1.5	0.125	0.125
13A35	4	2	0.06	8	wt	wt	D83N	I460V	4	2	0.125	0.125
13B01	8	2	0.5	8	wt	wt	wt	I460V	3	0.75	0.19	0.19
13B02	4	2	0.25	4	wt	wt	wt	I460V	1	0.75	0.125	0.125
13B06	4	2	0.25	4	wt	wt	wt	I460V	1	0.5	0.125	0.125
13B08	4	2	0.25	8	wt	wt	wt	wt	2	1	0.125	0.125
13B09	4	2	0.12	4	wt	wt	wt	I460V	1.5	0.5	0.094	0.094
13C24	4	2	0.12	8	wt	wt	wt	I460V	8	4	0.19	0.19
13C28	8	4	2	8	S81F	wt	wt	I460V	>32	>32	4	4
13D16	4	2	0.12	4	wt	wt	wt	I460V	2	1	0.19	0.125
13E07	4	2	0.25	4	wt	wt	wt	I460V	2	1	0.125	0.125
13E24	4	4	0.12	4	S114G	wt	wt	I460V	1.5	0.5	0.125	0.125
13F15	4	2	0.12	8	S114G	wt	wt	wt	8	1	0.19	0.19
13G08	4	1	0.06	8	S114G	wt	N91D / E135D	wt	16	1	0.19	0.19
13J24	32	16	2	32	S81F	wt	wt	I460V	>32	>32	4	4
13J29	4	2	0.12	8	wt	wt	wt	I460V	1.5	0.75	0.125	0.125
13K02	4	1	0.25	8	wt	wt	wt	I460V	2	1	0.19	0.19
13K03	4	2	0.12	8	wt	wt	wt	I460V	1	0.75	0.125	0.125
13K06	4	1	0.12	8	wt	wt	wt	I460V	1	0.5	0.125	0.125
13K07	4	0.5	0.12	8	wt	wt	K137N	I460V	1.5	0.75	0.125	0.125
13K13	4	2	0.12	8	wt	wt	K137N	I460V	2	1	0.125	0.125
13K14	4	2	0.12	4	wt	wt	wt	I460V	1.5	1	0.125	0.125
13K18	4	2	0.25	8	wt	wt	wt	wt	1.5	1	0.19	0.19
13K19	8	4	0.25	8	wt	wt	S79F	I460V	6	3	0.25	0.25
13L03	4	2	0.12	8	wt	wt	wt	I460V	1.5	0.75	0.125	0.125
13L04	16	8	2	16	S81Y	wt	S79F	I460V	24	24	4	4
13L14	4	1	0.12	8	wt	wt	wt	I460V	3	1	0.19	0.19
13L15	4	2	0.12	8	wt	wt	wt	I460V	1.5	0.75	0.125	0.125
13L23	4	2	0.12	8	wt	wt	wt	I460V	3	1	0.19	0.19