Characterization of demographics and NS5A genetic diversity for hepatitis C virus genotype 4-infected patients with or without cirrhosis treated with ombitasvir/paritaprevir/ritonavir

G. Schnell | R. Tripathi | J. Beyer | T. Reisch | P. Krishnan | T. Dekhtyar | M. Irvin | C. Hall | Y. Yu | N. Mobashery | R. Redman | T. Pilot-Matias | C. Collins

Research & Development, AbbVie Inc., North Chicago, IL, USA

Correspondence
G. Schnell, AbbVie Inc., North Chicago, IL, USA.
Email: gretja.schnell@abbvie.com

Funding information
AbbVie

Summary
Hepatitis C virus (HCV) genotype 4 (GT4) is genetically diverse with 17 confirmed and 4 provisional subtypes. In this report, HCV GT4-infected patient samples from Phase 2/3 clinical studies were analysed to characterize global demographics and genetic diversity of GT4 infection among patients treated with ombitasvir (OBV, NS5A inhibitor) plus paritaprevir/r (NS3/4A inhibitor codosed with ritonavir). Among 17 subtypes isolated from GT4-infected patients in the PEARL-I and AGATE-I studies, subtype prevalence by country of enrolment and country of origin suggested that subtypes 4a and 4d were likely circulating in Europe, while heterogeneous GT4 subtypes and a portion of GT4a detected in European and North American countries were likely due to immigration of HCV-infected patients from Africa. The distributions of birth cohort and race were also significantly different across GT4 subtypes 4a, 4d, and non-4a/4d. In addition, phylogenetic analyses of NS5A sequences revealed clustering within subtype 4a which segregated by the patient-reported country of origin and the presence of the L30R/S polymorphism. HCV NS5A sequences derived from GT4a-infected patients who originated from Europe and the United States clustered separately from sequences derived from patients who originated from Egypt, suggesting that genetically distinct strains of subtype 4a may be circulating globally. Finally, NS5A baseline polymorphisms were frequently detected at amino acid positions of interest for the inhibitor-class and OBV retained activity against 37 of 39 NS5A GT4 clinical isolates, with no impact on treatment outcome in the PEARL-I and AGATE-I studies.

KEYWORDS
genotype 4, hepatitis C virus, NS5A

1 | INTRODUCTION

Hepatitis C virus (HCV) genotype 4 (GT4) is responsible for 13% of infections worldwide, and is genetically diverse with 17 confirmed

and 4 provisionally assigned subtypes. The global distribution of HCV GT4 infection varies by geographic region. Prevalence of GT4 is low in Europe and North America, comprising around 1%-6% of the HCV-infected patient population. High incidence of GT4 infection has been reported in the regions of Central, Eastern and Western sub-Saharan Africa, where GT4 represents approximately 97%, 31% and 11% of the HCV-infected patient population, respectively.

Abbreviations: DAA, direct-acting antiviral; DCV, daclatasvir; HCV, hepatitis C virus; IFN, interferon; NGS, next-generation sequencing; OBV, ombitasvir; PTV, paritaprevir; RBV, ribavirin; SOF, sofosbuvir.
In the regions of North Africa and the Middle East, GT4 has been reported to represent 65% of HCV infections; this includes the country of Egypt where 90% of HCV infections are caused by GT4 and around 15% of the general population is seropositive for HCV antibodies. Subtype distribution of GT4 also varies by country, with a high incidence of subtype 4a in Egypt, while subtypes 4a and 4d are common in Europe and more heterogeneous GT4 subtypes have been reported throughout Central and Eastern sub-Saharan Africa.

In the era of direct-acting antiviral (DAA) therapies for HCV, treatment options for GT4-infected patients have expanded to interferon (IFN)-free regimens with or without ribavirin (RBV), including elbasvir/grazoprevir, glecaprevir/pibrentasvir, omibitasvir/paritaprevir/ritonavir (OBV/PTV/r), sofosbuvir (SOF), SOF plus daclatasvir (DCV), SOF plus simeprevir, SOF/ledipasvir (LDV) and SOF/velpatasvir. Ombitasvir (OBV) is a non-structural protein 5A (NS5A) inhibitor, and paritaprevir (PTV) is an NS3/4A inhibitor identified by AbbVie and Enanta. In the phase 2b/3 PEARL-I and AGATE-I studies, high sustained virologic response rates 12 weeks after the last dose of study drug (SVR12) were achieved in HCV GT4-infected patients without cirrhosis or with compensated cirrhosis treated with OBV/PTV/r plus RBV for 12 weeks of duration. This report utilizes a large database of NS5A gene sequences that included 17 GT4 subtypes isolated from the baseline samples of GT4-infected patients in the PEARL-I and AGATE-I studies to assess the genetic diversity of GT4 by geographic region, analyse patient demographics, examine NS5A sequence variability across GT4 subtypes and impact on treatment outcome in GT4-infected patients treated with OBV/PTV/r plus RBV, and assess OBV activity against a panel of NS5A GT4 clinical isolates.

2 | MATERIALS AND METHODS

2.1 | Clinical study design

The PEARL-I study (ClinicalTrials.gov identifier NCT01685203) was a randomized, open-label phase 2b study that evaluated the safety and efficacy of omibitasvir plus paritaprevir/r with or without RBV in noncirrhotic, treatment-naïve and pegylated-interferon (pegIFN)-RBV treatment-experienced HCV GT4-infected patients for 12 weeks of duration. The AGATE-I study (ClinicalTrials.gov identifier NCT02265237) was a randomized, open-label phase 3 study that evaluated the safety and efficacy of OBV/PTV/r plus RBV in treatment-naïve, IFN-RBV treatment-experienced, and SOF plus RBV ± pegIFN treatment-experienced HCV GT4-infected patients.

| TABLE 1 | HCV GT4 subtype by country of enrolment in AGATE-I and PEARL-I |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Subtype        | Austria | Belgium | France | Germany | Greece | Italy | Poland | Spain | Canada | United States | Total |
| 4a             | 15      | 2       | 48     | 2       | 18     | 3     | 18     | 14    | 27     | 147             |
| 4b             | 1       | 4       |        |         |        |       |        |       |        | 5               |
| 4c             | 1       | 5       |        |         |        | 1     |        |       |        | 6               |
| 4d             | 1       | 6       | 23     | 1       | 2      | 28    | 16     | 32    | 2      | 2               | 113              |
| 4e             | 1       |        |        |         |        |       |        |       |        | 1               |
| 4f             | 1       | 9       | 1      |        |        | 1     | 1      |       | 11     | 11              |
| 4g             | 1       | 1       |        |         |        |       |        |       |        | 1               |
| 4h             | 1       | 1       |        |         |        |       |        |       |        | 2               |
| 4k             | 1       | 4       | 3      | 1       |        |       |        |       | 8      | 8               |
| 4l             | 1       | 1       | 1      |        |        |        |        |       | 3      | 3               |
| 4n             | 1       | 1       | 1      |        |        |        |        |       | 3      | 3               |
| 4o             | 1       | 1       | 1      |        | 1      | 1     | 1      | 1     | 4      | 4               |
| 4p             | 1       | 1       |        |         |        |       |        |       | 2      | 2               |
| 4q             | 1       |        |        |         |        |       |        |       | 1      | 1               |
| 4r             | 1       | 1       |        |         |        |       |        |       | 2      | 2               |
| 4t             | 1       |        |        |         |        |       |        |       | 1      | 1               |
| 4v             | 1       |        |        |         |        |       |        |       | 1      | 1               |
| 4a             | 1       | 2       |        |         |        |       |        |       | 3      | 3               |

*GT4 subtype could not be determined by phylogenetic analysis due to lack of homology with any known subtype.

bPatient sample was not available for analysis.
with compensated cirrhosis for 12, 16 or 24 weeks of duration. The PEARL-I and AGATE-I studies were conducted in accordance with the World Medical Association Declaration of Helsinki. The study protocols were approved by the relevant institutional review boards and regulatory agencies, and all patients provided written informed consent.

### 2.2 | Sample processing and sequence analysis

Hepatitis C virus genotype was determined by the Versant HCV Genotype Inno-LiPA Assay v2.0, and HCV subtype was identified by neighbour-joining phylogenetic analyses using a 329-nucleotide region of NS5B and full-length NSSA nucleotide sequences isolated from patient baseline samples, as previously described.

The final HCV GT4 subtype listed in Tables 1-3 was determined by consensus between the NS5B and NSSA subtyping results. Overall, 97.5% (306/314) of samples were concordant between the NS5B and NSSA subtyping results, while 4 samples were missing subtyping data for 1 of the targets due to technical issues, and 4 samples had a specific subtype assigned for 1 target but the other target could only be assigned as GT4. Viral RNA isolation, reverse transcriptase (RT)-PCR and nested PCR were conducted as previously described for NS5A on plasma samples with HCV RNA ≥1000 IU/mL. Full-length NS5A genes were PCR amplified from the baseline samples of 132/135 noncirrhotic and 182 of 184 cirrhotic GT4-infected patients, and sequenced by either population nucleotide sequencing for samples from PEARL-I, or next-generation sequencing (NGS) for samples from AGATE-I. The baseline samples from 5 patients were not available for analysis.

Next-generation sequencing analysis of NS5A amplicons was conducted by DDL Diagnostic Laboratory (Rijswijk, Netherlands). Briefly, NS5A amplicons were purified and quantified using Ampure XP beads (Beckman Coulter Genomics, Danvers, MA) and the Quant-iT PicoGreen dsDNA kit (Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. The amplicons were then fragmented and tagged using the Nextera XT sample preparation kit and the Nextera XT Index kit (Illumina, San Diego, CA) per the manufacturer’s instructions. Paired-end sequencing was conducted using the Illumina MiSeq system and the MiSeq v2 sequencing kit with 300 cycles (Illumina, San Diego, CA, USA). FASTQ files were mapped against a subtype-specific reference sequence using CLC Genomics Workbench software (CLCBio, Denmark), and sequences were trimmed to remove nucleotides with a Q-score <30. Amino acid substitutions relative to a subtype-specific HCV reference sequence were then reported by DDL using Athena pipeline proprietary software.

NS5A amino acid positions 24, 28, 29, 30, 31, 32, 58, 92 and 93 were considered signature positions for the NS5A inhibitor class in HCV GT4. Baseline polymorphisms were identified by comparing translated baseline sequences to prototypic reference sequence 4a ED43 (GenBank accession number GU814265) for GT4a and all non-4a/4d sequences, or prototypic reference sequence 4d QC382 (GenBank accession number FJ462437) for GT4d sequences. For samples sequenced by NGS, a 15% detection threshold was implemented to identify baseline polymorphisms relative to the respective reference sequence.

### 2.3 | Phylogenetic analysis

Population nucleotide sequences and consensus NGS nucleotide sequences (generated with an ambiguity setting of 0.25) for NS5A were aligned using the MAFFT sequence alignment method. To increase the number of sequences from Egypt, 7 additional GT4a NS5A sequences from GenBank identified as originating from Egypt

#### TABLE 2  HCV GT4 subtype by country of origin in patients with cirrhosis (AGATE-I)

| Country of origin | Subtypes (no. of patients) | Patient total |
|-------------------|--------------------------|--------------|
| North America     |                          | 9            |
| Canada            | 4d                       | 1            |
| United States     | 4a                       | 8            |
| Europe            | 78                       |              |
| Belgium           | 4a (1), 4b (1), 4d (3)    | 5            |
| Bulgaria          | 4d                       | 1            |
| France            | 4a (9), 4d (7), 4p (1)    | 17           |
| Greece            | 4a (12), 4a/1a (1), 4d (1), 4k (1) | 15 |
| Italy             | 4d                       | 17           |
| Romania           | 4d                       | 1            |
| Spain             | 4a (11), 4d (11)         | 22           |
| Middle East       |                          | 3            |
| Iraq              | 4a                       | 2            |
| Saudi Arabia      | 4a                       | 1            |
| Africa            | 92                       |              |
| Burundi           | 4k (1), 4v (1)           | 2            |
| Cameroon          | 4f (3), 4p (1), 4t (1)    | 5            |
| Central African Republic | 4e (1), 4f (1) | 2 |
| Congo             | 4h (1), 4b (1), 4c (3), 4h (2), 4k (5), 4l (1), 4r (1) | 14 |
| Cote d’Ivoire    | 4c                       | 1            |
| Egypt             | 4a (50), 4d (1), 4l (2), 4n (3), 4o (3) | 59 |
| Eritrea           | 4d                       | 1            |
| Gabon             | 4c                       | 1            |
| Morocco           | 4a (2), 4r (1)           | 3            |
| Mozambique        | 4d                       | 1            |
| Rwanda            | 4k (1), 4q (1)           | 2            |
| Tunisia           | 4a                       | 1            |

aNumber of patients per subtype is listed in parenthesis for countries with more than 1 identified subtype.

bGT4 subtype could not be determined by phylogenetic analysis due to lack of homology with any known subtype.
(GenBank Accession numbers: DQ988073, DQ988074, DQ988075, DQ988076, DQ988077, DQ988078, DQ988079) were included in the phylogenetic analyses; these sequences were excluded from the NS5A baseline polymorphism prevalence calculations. Maximum likelihood phylogenetic trees were constructed using PHYML31,32 with the following parameters: HKY85 nucleotide substitution model,33 four substitution rate categories, estimation of the transition/transversion rate ratio, estimation of the proportion of invariant sites, estimation of the gamma distribution parameter and 100 bootstrapping replicates, using Geneious software (Biomatters Ltd., Auckland, New Zealand).34

2.4 | HCV transient replicons containing NS5A from GT4 clinical samples

The NS5A gene encompassing amino acids 1-214 from HCV GT4 clinical samples were amplified using subtype-specific PCR primers for subtypes 4a, 4f, 4o and 4r. For subtype 4b, 4d, 4g and 4k clinical samples, the NS5A consensus sequence (comprising amino acids 1-214) from each clinical sample was generated as a synthetic gene (IDT, Coralville, IA, USA) and internal NotI and Bpl restriction sites were removed without modification of the amino acid sequence. The PCR amplicons or synthetic genes were digested with restriction enzymes NotI and Bpl, and ligated into an HCV GT 1b-Con1 strain (GenBank accession number AJ238799) subgenomic transient replicon vector containing a luciferase reporter gene, as previously described.29 Ombitasvir activity against GT4 chimeric transient replicons was assessed as previously described.26 Briefly, in vitro transcribed replicon RNA was transfected via electroporation into a Huh-7-derived cell line, and luciferase expression was measured 4 days post-transfection.35 The EC$_{50}$ value for each isolate was calculated using a nonlinear regression curve fitting to the 4-parameter logistic equation in Prism5 software (GraphPad Software, Inc., La Jolla, CA, USA). The average EC$_{50}$ value for each isolate was calculated from 2 independent experiments.

2.5 | Statistical analysis

Fisher’s exact test with a two-sided significance level of 0.05 was used to compare the prevalence of baseline polymorphisms in NS5A between subject groups, impact of baseline polymorphisms in NS5A on treatment outcome, as well as the distribution of patient demographics across GT4 subtypes.

3 | RESULTS

3.1 | HCV GT4 subtype prevalence by country

Among 314 GT4-infected patients with available sequences out of 319 patients enrolled in the combined cohort from the PEARL-I and AGATE-I studies, 17 GT4 subtypes were identified from patient baseline samples. HCV sequences from three patient samples did not map to any known GT4 subtype, and may represent novel or previously undetermined subtypes. Based on country of enrolment (Table 1), the majority of patients from North America were infected with subtype 4a, and patients from Europe were predominantly infected with subtypes 4a or 4d. Patients infected with subtypes other than 4a or 4d were generally from Belgium and France, indicating that heterogeneous, less common GT4 subtypes can be detected in some European countries.

The patient-reported country of origin (Table 2) was collected in addition to the country of enrolment in study AGATE-I. Of the 97 patients infected with subtype 4a, 51.5% originated from Egypt, 34.0% from Europe, 8.2% from North America and 6.2% originated from other African or Middle Eastern countries. In addition, while 92.5% of the patients infected with a heterogeneous non-4a/4d subtype were enrolled in Europe, 90% (36/40) of these patients...
originated from an African country with the remainder (4 of 40) originating from Europe. These data indicate that a portion of GT4a and most of the heterogeneous GT4 subtypes detected in European and North American countries were likely due to immigration of HCV-infected patients from Africa. On the other hand, 91.1% of the 45 GT4d-infected patients both enrolled in and originated from Europe, indicating that subtype 4d is likely circulating within Europe.

3.2 Baseline demographic characteristics by GT4 subtype

The distributions of patient birth cohort and race were significantly different across GT4 subtypes 4a, 4d and non-4a/4d (P-values < .001, Table 3). A large proportion (71%) of the GT4d-infected patients were born after 1959, while 74% of patients with a non-4a/4d subtype were born prior to 1960. Patients of black race comprised 90% and 98% of the 4a- and 4d-infected populations, respectively, and patients of black race comprised 68% of the non-4a/4d-infected patient population. Of the 37 non-4a/4d-infected patients of black race, 27 patients had a known country of origin and were from an African country.

3.3 Phylogenetic analysis of NS5A sequences by geographic region

NS5A nucleotide sequences isolated from the baseline samples of 132/135 noncirrhotic and 181/184 cirrhotic GT4-infected patients were included in phylogenetic analyses to assess genetic relationships within GT4 subtypes. Phylogenetic analysis of NS5A sequences from study AGATE-I revealed sequence clustering within subtype 4a which segregated by the patient-reported country of origin (Figure 1A; bootstrap value = 81 at node of divergence) but not by country of enrolment. Although country of origin was not collected for patients in study PEARL-I, a combined phylogenetic analysis of 143 NS5A sequences from GT4a-infected patients in PEARL-I and AGATE-I confirmed the presence of 2 main sequence clusters within subtype 4a (Figure 1B; bootstrap value = 69 at node of divergence). Among the GT4a-infected patients, HCV NS5A sequences derived from patients who originated from Europe and the United States clustered separately from sequences derived from patients who originated from Egypt. A third subpopulation containing 7 patients from Greece was also strongly supported. Three patients who originated from either Morocco (enrolled in France) or Saudi Arabia (enrolled in the United States) had NS5A sequences that were genetically similar to European and North American isolates. Fourteen patients from Europe or North America had NS5A sequences that sorted with AGATE-I sequences isolated from patients who originated from Egypt, including five patients in AGATE-I from Greece or the United States, two patients who identified as Egyptian in PEARL-I, and 7 additional PEARL-I patients enrolled in France or the United States.

NS5A sequence clustering within subtype 4d was not detected among 113 NS5A sequences from GT4d-infected patients, while clustering within other GT4 subtypes was unable to be assessed due to limited number of sequences per subtype. The phylogenetic separation within subtype 4a between sequences isolated from patients who originated from Europe and the United States, and sequences isolated from patients who originated from Egypt, suggests that genetically distinct strains of subtype 4a may be circulating globally.

3.4 NS5A baseline polymorphism prevalence and lack of impact on SVR12 rates

Prevalence of baseline polymorphisms in NS5A at amino acid positions important for the inhibitor-class (amino acid positions 24, 28, 29, 30, 31, 32, 58, 92, and 93) was analysed using either population sequencing or NGS data with a detection threshold of 15%. Baseline polymorphisms at these amino acid positions were frequently detected in NS5A across GT4 subtypes (Figure 2, 34.2% of the samples, excluding the common T/P58 polymorphism in GT4d). Based on phylogenetic clustering among subtype 4a sequences (Figure 1), NS5A polymorphisms were more prevalent in the group of sequences isolated from patients who originated from Egypt (49.3%, 33/67) compared to either the cluster of sequences isolated from patients who originated from either Europe and North America (17.4%, 12/69; P-value = .0001), or from Greece (0%, n = 7; P-value = .015), and the difference between the groups was statistically significant. The most common polymorphisms in subtype 4a were L28M and L30R/S, detected in 17.8% and 11.0% of the GT4a sequences, respectively. The L28M polymorphism was detected at a higher prevalence (22.4%, 15/67) in the group of sequences isolated from patients who originated from Egypt, compared to 14.5% (10/69) of sequences isolated from patients who originated from Europe and North America, although the association was not statistically significant (P-value = .27). In contrast, the L30R/S polymorphism was only detected in the phylogenetic subpopulation of sequences isolated from patients who originated from Egypt (23.9% of the sequences, 16/67), and the association was statistically significant (P-value < .0001).

In the PEARL-I study, HCV GT4-infected patients without cirrhosis treated with OBV/PTV/r plus RBV for 12 weeks of duration achieved an SVR12 of 100% (91/91). In study AGATE-I,
Antiviral activity of OBV was assessed against a panel of 39 NS5A GT4 clinical isolates that included subtypes 4a, 4b, 4d, 4f, 4g, 4k, 4o, and 4r (Table 4). The median EC₅₀ of OBV against 9 subtype 4a clinical isolates was 0.21 pmol/L (range = 0.10-0.36 pmol/L), including 5 samples with NS5A polymorphisms at positions important for the inhibitor class. OBV also retained activity against subtypes 4d (n = 7), 4f (n = 5), 4k (n = 2), 4o (n = 5), and 4r (n = 5), with median EC₅₀ values of 0.38, 0.38, 0.37, 0.21 and 1.4 pmol/L, respectively. The majority of these clinical isolates contained polymorphisms in NS5A at resistance-associated amino acid positions (Table 4). OBV had a slight reduction in activity against one subtype 4d clinical isolate with M31V + T58P in NS5A (EC₅₀ = 4.1 pmol/L; patient achieved SVR12 on a regimen of OBV/PTV/r plus RBV), and two subtype 4r clinical isolates with either L28M + L30R + P58A (EC₅₀ = 0.53 pmol/L; patient achieved SVR12 on a regimen of OBV/PTV/r plus RBV) or L28V + L30R + M31L and Y93H/Y in NS5A (EC₅₀ = 7.0 pmol/L; patient achieved SVR12 on a regimen of glecaprevir/pibrentasvir). Among 3 subtype 4b clinical isolates, OBV retained activity against NS5A with P58T (EC₅₀ = 0.14 pmol/L) or P58S + Y93H (EC₅₀ = 0.53 pmol/L); one clinical isolate with L30S + Y93H in NS5A was resistant to OBV (EC₅₀ = 71.1 pmol/L) even though this patient achieved SVR12 on a regimen of OBV/PTV/r plus RBV. Among 3 subtype 4g clinical isolates, OBV retained activity against 2 isolates with L30C + M31L in NS5A (EC₅₀ = 0.24 pmol/L for both isolates). The 4g clinical isolate with L28M + L30R + M31L + P58S in NS5A was resistant to OBV (EC₅₀ = 675 pmol/L); this patient was enrolled in a clinical study evaluating the NS3/4A and NS5A inhibitors glecaprevir/pibrentasvir, and achieved SVR12 with this regimen.

3.5 Activity of ombitasvir against a panel of NS5A GT4 clinical isolates

SVR12 on a regimen of OBV/PTV/r plus RBV, and two subtype 4r clinical isolates with either L28M + L30R + P58A (EC₅₀ = 4.5 pmol/L; patient achieved SVR12 on a regimen of OBV/PTV/r plus RBV) or L28V + L30R + M31L and Y93H/Y in NS5A (EC₅₀ = 7.0 pmol/L; patient achieved SVR12 on a regimen of glecaprevir/pibrentasvir). Among 3 subtype 4b clinical isolates, OBV retained activity against NS5A with P58T (EC₅₀ = 0.14 pmol/L) or P58S + Y93H (EC₅₀ = 0.53 pmol/L); one clinical isolate with L30S + Y93H in NS5A was resistant to OBV (EC₅₀ = 71.1 pmol/L) even though this patient achieved SVR12 on a regimen of OBV/PTV/r plus RBV. Among 3 subtype 4g clinical isolates, OBV retained activity against 2 isolates with L30C + M31L in NS5A (EC₅₀ = 0.24 pmol/L for both isolates). The 4g clinical isolate with L28M + L30R + M31L + P58S in NS5A was resistant to OBV (EC₅₀ = 675 pmol/L); this patient was enrolled in a clinical study evaluating the NS3/4A and NS5A inhibitors glecaprevir/pibrentasvir, and achieved SVR12 with this regimen.

4 DISCUSSION

Subtype distribution of HCV GT4 varies substantially by geographic region and country in Europe, the Middle East, North America, Northern Africa and sub-Saharan Africa.1,3 Among 17 HCV GT4 subtypes isolated from 314 GT4-infected patients in the PEARL-I and AGATE-I studies, GT4 subtype prevalence was examined by country of enrolment and patient-reported country of origin to distinguish between current circulating strains of GT4 and probable country of original infection. An analysis by country of enrolment demonstrated that 84% (41/49) of patients from North America were infected with subtype 4a, and 40% (106/265) or 41% (109/265) of patients from Europe were infected with subtypes 4a or 4d, respectively (Table 1). This is consistent with previous reports of high prevalence of subtypes 4a and 4d throughout Europe among GT4-infected patients.36 The majority of GT4d-infected patients in our cohort were born after 1959, which coincides with the spread of GT4d across Europe via intravenous drug use in recent decades.5,7,9-11,37 Our analysis also revealed that 19% (50/265) of GT4 infections in Europe resulted from subtypes other than 4a or 4d, and the majority of these patients were enrolled in Belgium and France. In addition, patients infected with non-4a/4d subtypes were generally born prior to 1960 and were of black race. Previous studies examining the epidemiological profile of GT4 infection in France and Belgium have reported an association between heterogeneous GT4 subtypes and patients of African origin with an unknown route of infection.11,38 In Africa, high prevalence of subtype 4a exists in Egypt,5,7,8 while heterogeneous GT4 subtypes (4b, 4c, 4e, 4f, 4g, 4h, etc.) account for the majority of HCV infections throughout Central and Eastern sub-Saharan Africa7,12-15 where GT4 is thought to have originated.39 Our analysis by patient-reported country of origin (collected for study AGATE-I) revealed that of the 97 patients infected with subtype 4a, 51.5% originated from Egypt, while 42.2% originated from Europe or North America (Table 2). In addition, 90% (36/40) of the
patients infected with a heterogeneous non-4a/4d subtype who enrolled in Europe actually originated from an African country. This differed from the GT4d-infected patients, where 91.1% both enrolled in and originated from Europe. These observations suggested that heterogeneous GT4 subtypes and a portion of GT4a detected in European and North American countries were likely due to immigration of HCV-infected patients from Africa, while subtypes 4a and 4d were likely actively circulating in Europe. The detection of a

| HCV subtype | NS5A amino acid polymorphismsa | Number of isolates | Median EC50 (range), pmol/L |
|-------------|---------------------------------|--------------------|---------------------------|
| 4a<sup>d</sup> | None<sup>b</sup> | 4 | 0.24 (0.21-0.35) |
|             | K24R                           | 1 | 0.19 |
|             | L28M                           | 1 | 0.21 |
|             | L30R                           | 1 | 0.36 |
|             | P58T                           | 1 | 0.10 |
|             | L28M + L30R                     | 1 | 0.24 |
|             | Total<sup>c</sup>              | 9 | 0.21 (0.10-0.36) |
| 4b          | L30S + Y93H                     | 1 | 71.1 |
|             | P58S + Y93H                     | 1 | 0.53 |
|             | P58T                            | 1 | 0.14 |
| 4d          | None<sup>b</sup>               | 2 | 0.27 (0.16-0.38) |
|             | T58L                            | 1 | 0.80 |
|             | T58P                            | 2 | 0.26 (0.25-0.26) |
|             | T58S                            | 1 | 0.50 |
|             | M31V + T58P                     | 1 | 4.1 |
|             | Total                           | 7 | 0.38 (0.16-4.1) |
| 4f          | L30R                            | 4 | 0.38 (0.28-1.6) |
|             | L30R + M31L                     | 1 | 1.4 |
|             | Total                           | 5 | 0.38 (0.28-1.6) |
| 4g          | L28M + L30R + M31L + P58S       | 1 | 675 |
|             | L30C + M31L                     | 2 | 0.24 (0.24-0.24) |
| 4k          | L30R                            | 1 | 0.37 |
|             | L30R + M31L                     | 1 | 0.37 |
| 4o          | L28M + L30T                     | 4 | 0.22 (0.18-0.23) |
|             | L28M, L30A/V                    | 1 | 0.12 |
|             | Total                           | 5 | 0.21 (0.12-0.23) |
| 4r          | L28I + L30R + M31L              | 2 | 1.2 (0.91-1.4) |
|             | L28M + L30R + P58A              | 1 | 4.5 |
|             | L28V + L30H + M31L              | 1 | 0.98 |
|             | L28V + L30R + M31L, Y93H/Y      | 1 | 7.0 |
|             | Total                           | 5 | 1.4 (0.91-7.0) |
| GT4         | Total                           | 39 | 0.35 (0.10-675) |

EC<sub>50</sub>: half-maximal effective concentration.

<sup>a</sup>N55A amino acid polymorphisms were assessed at positions 24, 28, 30, 31, 32, 58, 92 and 93 relative to the designated reference sequence. The patient sequences were compared to HCV reference sequence 4a-ED43 (GenBank accession number GU814265) for subtypes 4a, 4b, 4f, 4g, 4k, 4o and 4r. Reference 4d-QC382 (GenBank accession number FJ462437) was used for subtype 4d sequences. For patient sequences analysed by NGS, only polymorphisms detected at ≥15% prevalence within a patients’ viral population are listed. Polymorphisms detected at ≥90% prevalence are separated with a “+”; polymorphisms detected at <90% are separated with a “,” and listed as a mixture of amino acids.

<sup>b</sup>None indicates that there were no polymorphisms relative to the designated reference sequence.

<sup>c</sup>Mean EC<sub>50</sub> and range for GT4a total were previously published in reference. 29

<sup>d</sup>The GT4a N55A clinical isolates had an unknown country of origin, but were genetically similar to 4a sequences isolated from patients who originated from Egypt.
substantial proportion of diverse GT4 subtypes among GT4-infected patients in Europe (19%) and, to a lesser extent, North America (8%) suggests that global distribution of GT4 subtypes is shifting with immigration patterns; this was particularly apparent in Belgium and France.

We observed that over 51% of the GT4a-infected patients in study AGATE-I originated from Egypt where subtype 4a is predominant. Previous studies examining the molecular evolution of HCV in Egypt reported an exponential expansion of subtype 4a beginning in the 1940s due to antischistosomiasis campaigns. Rapid amplification and spread of GT4a due to unsafe injection practices resulted in low diversity of GT4 in the Egyptian HCV-infected population. We examined genetic diversity in subtype 4a by geographic region and country by conducting phylogenetic analyses of NS5A sequences, which revealed clustering within subtype 4a that segregated by the patient-reported country of origin and presence of the L30R/S polymorphism (Figure 1). HCV NS5A sequences derived from GT4a-infected patients who originated from Europe and the United States clustered separately from sequences derived from patients who originated from Egypt, suggesting that genetically distinct strains of subtype 4a may be circulating globally.

Further analysis of NS5A sequence variability based on phylogenetic clustering in subtype 4a revealed that NS5A baseline polymorphisms at amino acid positions of interest for the inhibitor-class were more prevalent in the phylogenetic group of sequences isolated from patients who originated from Egypt (49.3%, 33/67) compared to patients who originated from Europe and North America (17.4%, 12/69). The most common polymorphisms in subtype 4a were L28M and L30R/S, which were detected at frequencies similar to previously published studies. Our analysis demonstrated that the L30R/S polymorphism was significantly associated (P-value <.0001) with the phylogenetic subpopulation of sequences isolated from patients who originated from Egypt (23.9% of the sequences, 16/67). The combined evidence suggests that the founder effect in Egypt may have resulted in a strain of HCV subtype 4a that is genetically distinct from other strains of subtype 4a that are circulating in Europe and North America.

Egypt historically had the highest prevalence of HCV worldwide, with approximately 90% of infections caused by GT4. Prior to the availability of DAA therapies for HCV, standard of care in Egypt included pegIFN plus RBV. In 2014, SOF became the first DAA treatment option in Egypt, and since then other DAA regimens have become available through the national HCV treatment programmes, including DCV, OBV/PTV/r, simeprevir and SOF/LDV. Given the higher rate of NS5A baseline polymorphisms detected in patients who originated from Egypt in our analysis, it is important to assess the impact of baseline polymorphisms on treatment outcome for regimens containing an NS5A inhibitor. In GT4-infected patients treated with the NS5A inhibitor DCV (20 mg) plus pegIFN/RBV, L28M in combination with L30H/S in NS5A emerged in patients experiencing virologic failure. DCV has a 10-fold reduction in antiviral activity against the L28M or L30R NS5A polymorphisms, while L30S or the combination of L28M plus L30R results in a 150- or 350-fold reduction in DCV activity, respectively. Therefore, treatment of GT4 infection with DCV plus pegIFN/RBV may not be a suitable regimen for regions with a high prevalence of NS5A baseline polymorphisms. A study examining the combination of DCV plus SOF for treatment of GT4 infection reported a per-protocol SVR12 rate of 99% (117/118) in Egyptian patients, suggesting that DCV should be combined with another DAA to overcome the reduction in antiviral activity against NS5A baseline polymorphisms in GT4.

High SVR12 rates of 96.2% or 95.4% were reported in HCV GT4-infected patients treated with either OBV/PTV/r (n = 122) or SOF/LDV (n = 130), respectively, with or without RBV in a real-world setting, but an analysis of the prevalence of NS5A baseline polymorphisms was not included in the study results. Another study reported SVR12 rates of 93% (41/44) in GT4-infected patients treated with SOF/LDV, which included 25 patients with baseline polymorphisms L28M and/or L30R/S in NS5A; all 3 virologic failures had L28M/V with or without L30R in NS5A at baseline, which corresponds with a 37- to 67-fold change in LDV activity against L28M/V and L30R in GT4. In our combined cohort analysis, SVR12 rates of 98.9% (94/95) were observed for HCV GT4-infected patients with an NS5A polymorphism at baseline treated with OBV/PTV/r plus RBV in the PEARL-I and AGATE-I studies, indicating that there was no impact of NS5A baseline polymorphisms on treatment outcome, even though 35.8% (95/265, excluding the common T/P58 polymorphism in GT4d) of the patient sequences had an NS5A polymorphism at a resistance-associated amino acid position; this included 78 patients with L28M and/or L30R/S in NS5A (not counting subtype 4d where R30 is wild-type). In addition, high SVR12 rates (93%-97%) were reported in HCV GT4-infected Egyptian patients treated with OBV/PTV/r in Egypt, where NS5A polymorphisms may occur at a higher prevalence. Finally, OBV retained activity against 37 of 39 NS5A GT4 clinical isolates with or without polymorphisms, including those at amino acid positions 28 and 30 (Table 4).

In conclusion, 17 GT4 subtypes were identified in the PEARL-I and AGATE-I studies among GT4-infected patients treated with OBV/PTV/r with or without RBV. Subtype prevalence by country of enrolment and country of origin suggested that global immigration patterns are changing the distribution of GT4 subtypes, which could be extrapolated to include genetically distinct viral strains within a given subtype. The distributions of birth cohort and race were also significantly different across GT4 subtypes 4a, 4d, and non-4a/4d, which correlated with the known transmission route of GT4d and the historical origin of non-4a/4d subtypes from Africa. In addition, phylogenetic and baseline polymorphism analyses of NS5A sequences suggested that a genetically distinct strain of subtype 4a may be circulating in Egypt vs Europe and North America, and the L30R/S polymorphism in NS5A was significantly associated with the Egyptian strain, although this observation needs to be confirmed with a larger sample size. Finally, NS5A baseline polymorphisms
were frequently detected at resistance-associated amino acid positions for the inhibitor-class, with no impact on treatment outcome in GT4-infected patients treated with OBV/PTV/r plus RBV in the PEARL-I and AGATE-I studies. A regimen of OBV/PTV/r plus RBV for 12 weeks of duration has been approved for the treatment of HCV GT4 infection without cirrhosis or with compensated cirrhosis in the United States, European Union, and other global countries including Egypt.

ACKNOWLEDGEMENTS

The authors acknowledge the clinical providers and patients for their study participation, and the study coordinators for assistance provided in the preparation and operation of the study. All authors are employees of AbbVie and may hold AbbVie stock or stock options. The design, study conduct and financial support for these studies were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

DISCLOSURES

Authors’ declaration of personal interests: Greitja Schnell, Rakesh Tripathi, Jill Beyer, Thomas Reisch, Preethi Krishnan, Tatyana Dekhtyar, Michelle Irvin, Coleen Hall, Yao Yu, Niloufar Mobashery, Rebecca Redman, Tami Pilot-Matias, and Christine Collins are employees of AbbVie and may hold AbbVie stock or stock options. Declaration of funding interests: The design, study conduct, and financial support for these studies were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

ORCID

G. Schnell http://orcid.org/0000-0002-2085-7314

REFERENCES

1. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol. 2014;61:545-557.
2. Smith DB, Bukh J, Kuiken C, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology. 2014;59:318-327.
3. Messina JP, Humphreys I, Flaxman A, et al. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology. 2015;61:77-87.
4. Mahmud S, Al-Kanaani Z, Chemaitez H, Chaabna K, Kouroumijian SP, Abu-Raddad LJ. Hepatitis C virus genotypes in the Middle East and North Africa: distribution, diversity, and patterns. J Med Virol. 2018;90:131-141.
5. Frank C, Mohamed MK, Strickland GT, et al. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet. 2000;355:887-891.
6. Kamal SM. Improving outcome in patients with hepatitis C virus genotype 4. Am J Gastroenterol. 2007;102:2582-2588.
7. Kamal SM, Nasser IA. Hepatitis C genotype 4: what we know and what we don’t yet know. Hepatology. 2008;47:1371-1383.
8. Tanaka Y, Agha S, Saudy N, et al. Exponential spread of hepatitis C virus genotype 4a in Egypt. J Mol Evol. 2004;58:191-195.
9. Cantaloube JF, Gallian P, Laperche S, et al. Molecular characterization of genotype 2 and 4 hepatitis C virus isolates in French blood donors. J Med Virol. 2008;80:1732-1739.
10. Di Lello FA, Neukam K, Parra-Sanchez M, et al. Hepatitis C virus genotype 4 in Southern and Central Spain does not originate from recent foreign migration waves. J Med Virol. 2013;85:1734-1740.
11. Morice Y, Roulot D, Grando V, et al. Phylogenetic analyses confirm the high prevalence of hepatitis C virus (HCV) type 4 in the Seine-Saint-Denis district (France) and indicate seven different HCV-4 subtypes linked to two different epidemiological patterns. J Gen Virol. 2001;82(Pt 5):1001-1012.
12. Cantaloube JF, Gallian P, Bokilo A, et al. Analysis of hepatitis C virus strains circulating in Republic of the Congo. J Med Virol. 2010;82:562-567.
13. Ndjomou J, Pybus OG, Matz B. Phylogenetic analysis of hepatitis C virus isolates indicates a unique pattern of endemic infection in Cameroon. J Gen Virol. 2003;84(Pt 9):2333-2341.
14. Ndong-Atome GR, Mukwu M, Njouom R, et al. Hepatitis C virus prevalence and genetic diversity among pregnant women in Gabon, central Africa. BMC Infect Dis. 2008;8:82.
15. Nerrienet E, Pouillot R, Lachenal G, et al. Hepatitis C virus infection in Cameroon: a cohort-effect. J Med Virol. 2005;76:208-214.
16. Zeuzem S, Ghalib R, Reddy KR, et al. Grazoprevir-Elbasvir combination therapy for treatment-naive cirrhotic and noncirrhotic patients with chronic hepatitis C virus genotype 1, 4, or 6 infection: a randomized trial. Ann Intern Med. 2015;163:1-13.
17. Forns X, Lee SS, Valdes J, et al. Glecaprevir plus pibrentasvir for chronic hepatitis C virus genotype 1, 2, 4, 5, or 6 infection in adults with compensated cirrhosis (EXPEDITION-1): a single-arm, open-label, multicentre phase 3 trial. Lancet Infect Dis. 2017;17:1062-1068.
18. Kwo PY, Poordad F, Asatryan A, et al. Glecaprevir and pibrentasvir yield high response rates in patients with HCV genotype 1-6 without cirrhosis. J Hepatol. 2017;67:263-271.
19. Asselah T, Alami N, Moreno C, et al. Ombitasvir/paritaprevir/ritonavir plus ribavirin for 24 weeks in patients with HCV GT4 and compensated cirrhosis (AGATE-I Part II). Health Sci Rep. 2018. Submitted
20. Asselah T, Hezode C, Qaish RB, et al. Ombitasvir, paritaprevir, and ritonavir plus ribavirin in adults with hepatitis C virus genotype 4 infection and cirrhosis (AGATE-I): a multicentre, phase 3, randomised open-label trial. Lancet Gastroenterol Hepatol. 2016;1:25-35.
21. Hezode C, Asselah T, Reddy KR, et al. Ombitasvir plus paritaprevir plus ritonavir with or without ribavirin in treatment-naive and treatment-experienced patients with genotype 4 chronic hepatitis C virus infection (PEARL-I): a randomised, open-label trial. Lancet. 2015;385:2502-2509.
22. Ruane PJ, Ain D, Stryker R, et al. Sofosbuvir plus ribavirin for the treatment of chronic genotype 4 hepatitis C virus infection in patients of Egyptian ancestry. J Hepatol. 2015;62:1040-1046.
23. Babatin MA, Alghamdi AS, Albenoussa A, et al. Efficacy and safety of simprevir or daclatasvir in combination with sofosbuvir for the treatment of hepatitis C genotype 4 infection. J Clin Gastroenterol. 2018;52:452-457.
24. Kohli A, Kapoor R, Sims Z, et al. Ledipsavir and sofosbuvir for hepatitis C genotype 4: a proof-of-concept, single-centre, open-label phase 2a cohort study. Lancet Infect Dis. 2015;15:1049-1054.
25. Asselah T, Bourgeois S, Pianko S, et al. Sofosbuvir/velpatasvir in patients with hepatitis C virus genotypes 1-6 and compensated cirrhosis or advanced fibrosis. Liver Int. 2018;38:443-450.
26. Schnell G, Tripathi R, Beyer J, et al. Hepatitis C virus genotype 4 resistance and subtype demographic characterization of patients treated with ombitasvir plus paritaprevir/ritonavir. Antimicrob Agents Chemother. 2015;59:6807-6815.

27. Koletzki D, Dumont S, Vermeiren H, Fevery B, De Smet P, Stuyver LJ. Development and evaluation of an automated hepatitis C virus NS5B sequence-based subtyping assay. Clin Chem Lab Med. 2010;48:1095-1102.

28. Murphy DG, Willems B, Deschenes M, Hilzenrat N, Mousseau R, Sabbah S. Use of sequence analysis of the NS5B region for routine genotyping of hepatitis C virus with reference to C/E1 and 5’ untranslated region sequences. J Clin Microbiol. 2007;45:1102-1112.

29. Krishnan P, Beyer J, Mistry N, et al. In vitro and in vivo antiviral activity and resistance profile of ombitasvir, an inhibitor of hepatitis C virus NS5A. Antimicrob Agents Chemother. 2015;59:979-987.

30. Katoh K, Asimenos G, Toh H. Multiple alignment of DNA sequences with MAFFT. Methods Mol Biol. 2009;537:39-64.

31. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 2003;52:696-704.

32. Guindon S, Lethiec F, Duroux P, Gascuel O. PHYML online—a web server for fast maximum likelihood phylogenetic inference. Nucleic Acids Res. 2005;33:W557-W559.

33. Hasegawa M, Kishino H, Yano T. Dating of the human-ape split- ing by a molecular clock of mitochondrial DNA. J Mol Evol. 1985;22:160-174.

34. Kearse M, Moir R, Wilson A, et al. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647-1649.

35. Tripathi RL, Krishnan P, He Y, et al. Replication efficiency of chimeric replicon containing NS5A-5B genes derived from HCV-infected patient sera. Antiviral Res. 2007;73:40-49.

36. Welzel TM, Bhardwaj N, Hedskog C, et al. Global epidemiology of HCV subtypes and resistance-associated substitutions evaluated by sequencing-based subtype analyses. J Hepatol. 2017;67:224-236.

37. Panasiuk A, Flisiak R, Mozer-Lisewska I, et al. Distribution of HCV genotypes in Poland. Przegl Epidemiol. 2013;67:11-16, 99-103.

38. Nkuize M, Mulkay JP, Moreno C, et al. Ethnic epidemiological profiles and antiviral therapy among patients infected with hepatitis C virus genotype 4: a multicenter study from Belgium. Acta Gastroenterol Belg. 2015;78:365-372.

39. Iles JC, Raghwani J, Harrison GL, et al. Phylogeography and epidemi history of hepatitis C virus genotype 4 in Africa. Virology. 2014;464-465:233-243.

40. El-Akel W, El-Sayed MH, El Kassas M, et al. National treatment programme of hepatitis C in Egypt: hepatitis C virus model of care. J Viral Hepat. 2017;24:262-267.

41. El Raziky M, Fathalah WF, El-Akel WA, et al. The effect of peginterferon alpha-2a vs. peginterferon alpha-2b in treatment of naive chronic HCV genotype-4 patients: A Single Centre Egyptian Study. Hepat Mon. 2013;13:e10069.

42. Hezode C, Hirschfield GM, Ghesquiere W, et al. Daclatasvir plus peginterferon alfa and ribavirin for treatment-naive chronic hepatitis C genotype 1 or 4 infection: a randomised study. Gut. 2015;64:948-956.

43. Zhou N, Hernandez D, Ueland J, et al. NS5A sequence heterogeneity and mechanisms of daclatasvir resistance in hepatitis C virus genotype 4 infection. J Infect Dis. 2016;213:206-215.

44. Yakoot M, AbdO AM, Abdel-Rehim S, Helmy S. Response tailored protocol versus the fixed 12 weeks course of dual sofosbuvir/daclatasvir treatment in Egyptian patients with chronic hepatitis C genotype-4 infection: a randomized, open-label, non-inferiority trial. EBioMedicine. 2017;21:182-187.

45. Crespo J, Calleja JL, Fernandez I, et al. Real-world effectiveness and safety of oral combination antiviral therapy for hepatitis C virus genotype 4 infection. Clin Gastroenterol Hepatol. 2017;15:945-949 e1.

46. Abergel A, Metivier S, Samuel D, et al. Ledipasvir Sofosbuvir for 12 weeks in patients with hepatitis C genotype 4 infection. Hepatology. 2016;64:1049-1056.

47. Camus G, Han B, Asselah T, et al. Resistance characterization of ledipasvir and velpatasvir in hepatitis C virus genotype 4. J Viral Hepat. 2015;22:134-143.

48. Waked I, Shiha G, Qaqish RB, et al. Ombitasvir, paritaprevir, and ritonavir plus ribavirin for chronic hepatitis C virus genotype 4 infection in Egyptian patients with or without compensated cirrhosis (AGATE-II): a multicentre, phase 3, partly randomised open-label trial. Lancet Gastroenterol Hepatol. 2016;1:36-44.

49. Technivie [package insert]. North Chicago, IL: AbbVie Inc.; 2017. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/207931s007s009lbl.pdf. Accessed January 6, 2017.

50. Viekirax [package insert]. North Chicago, IL: AbbVie Inc.; 2017. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/003839/WC500183997.pdf. Accessed September 21, 2017.