Genetic relatedness of \textit{Staphylococcus aureus} isolates obtained from cystic fibrosis patients at a tertiary academic hospital in Pretoria, South Africa

T. Goolam Mahomed\textsuperscript{1}, M. M. Kock\textsuperscript{1,2}, R. Masekela\textsuperscript{3}, E. Hoosien\textsuperscript{4} & M. M. Ehlers\textsuperscript{1,2}

Cystic fibrosis (CF) is an inherited recessive disease that affects mucociliary clearance in the lung, allowing it to be colonised with bacteria such as \textit{Staphylococcus aureus}. To survive in the CF lung \textit{S. aureus} adapts both phenotypically and genotypically, through various mechanisms. In this study, multiple specimens were collected from the participants and were processed routinely and were additionally cultured in chromogenic media. Multiplex PCR assays were employed to detect methicillin resistance and selected virulence and quaternary ammonium compound (\textit{qac}) genes. Genetic relatedness of the \textit{S. aureus} was determined using \textit{agr}, \textit{SCCmec} and \textit{spa} typing as well as pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Thirty-three \textit{S. aureus} isolates were isolated, of which 51\% (17/33) were methicillin resistant \textit{S. aureus} (MRSA). The virulence and \textit{qac} genes were more prevalent in MRSA than the methicillin sensitive \textit{S. aureus} (MSSA) isolates. The PFGE analysis showed nine distinct pulsotypes while MLST showed eight sequence types. All the STs detected in this study, except for ST508 have been previously isolated from CF patients according to the literature. This study showed a genetically diverse \textit{S. aureus} population with a high prevalence of virulence genes among the MRSA isolates from the CF clinic.

Cystic fibrosis (CF) is an inherited disease, which affects one in 2000 Caucasians, one in 12000 people of mixed ancestry and one in 34000 Africans in South Africa\textsuperscript{1}. This recessive disease is caused by mutations in the \textit{CFTR} gene, a gene that encodes for a chloride channel protein, i.e. \textit{CF transmembrane conductance regulator} (CFTR) protein\textsuperscript{2}. As a result of mutations in the \textit{CFTR} gene, the \textit{CFTR} protein is dysfunctional, causing impaired mucociliary clearance\textsuperscript{2}. The mucus layer in CF patients is thus without adequate defence systems and colonisation of the airways by pathogens (e.g. bacteria, fungi and viruses) often occurs\textsuperscript{2,3}. \textit{Staphylococcus aureus} is one of the most frequently reported bacterial pathogens to infect the CF lung, especially in young children\textsuperscript{4}.

By undergoing phenotypic and genotypic adaptations, \textit{S. aureus} is able to persist in the CF lung\textsuperscript{5}. One such mechanism by which \textit{S. aureus} adapts to the CF lung is through genomic rearrangements, with mobile genetic elements and phage mobilisation being key players\textsuperscript{6}. This movement of genetic material may affect virulence traits by either interrupting the expression of virulence genes (e.g. $\beta$-haemolysin gene) or by equipping \textit{S. aureus} with virulence genes, such as the Panton-Valentine leukocidin (PVL) toxin\textsuperscript{5,6}.

The PVL toxin is one of the most widely studied virulence factors in \textit{S. aureus} and studies have shown that it can cause inflammation in the lung (using rabbit models)\textsuperscript{7–9}. However, observational studies reporting on the PVL toxin in CF patients found no association between the PVL toxin and clinical outcome and as such its role in CF is still unclear\textsuperscript{10,11}. Another important virulence mechanism of \textit{S. aureus}, especially in CF patients, is biofilm formation\textsuperscript{5}. The \textit{S. aureus} biofilm is multilayered and is often composed of glycocalyx forming part of a slime

\textsuperscript{1}Department of Medical Microbiology, Faculty of Health Sciences, Prinshof Campus, University of Pretoria, Pretoria, 0001, South Africa. \textsuperscript{2}Tshwane Academic Division (TAD), National Health Laboratory Service, Pretoria, 0001, South Africa. \textsuperscript{3}Department of Maternal and Child Health, School of Clinical Medicine, University of KwaZulu-Natal, Durban, 4041, South Africa. \textsuperscript{4}Ampath Laboratory, Highveld Office Park, Centurion, 0157, South Africa. Correspondence and requests for materials should be addressed to M.M.E. (email: marthie.ehlers@up.ac.za)
Staphylococcus aureus can form biofilms based on two conditions: (i) polysaccharide intracellular antigen (PIA)-dependent formation and (ii) PIA-independent formation. Polysaccharide intracellular (PIA)-dependent formation is produced from the products of the ica locus. The ica locus is induced by the staphylococcal respiratory response regulator protein (SrrAB) and can also be induced by glucose, osmolarity, temperature or antibiotics. The ica locus can be controlled by the reversible inactivation of the insertion sequence IS256. In PIA-independent biofilm formation, Protein A (Spa) is essential. The virulence of S. aureus is aided by methicillin resistance; patients infected with MRSA strains have a lower lung function than patients infected with MSSA strains. Methicillin resistance is conferred by the mecA gene, which can be found on a mobile genetic element known as the staphylococcal cassette chromosome mec (SCCmec) element.

In South Africa, there is very limited data regarding both MSSA and MRSA isolated from the CF lung. Thus, the aim of this study was to determine the molecular characteristics and genetic relatedness of S. aureus isolates using multiplex PCR assays and genotyping methods, such as SCCmec, spa and agr typing, followed by pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) on selected isolates.

Materials and Methods

This study was conducted at a CF clinic in Pretoria from October 2013 to May 2014, with ethical approval from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (343/2013). Informed consent (and assent, for participants over the age of seven) was obtained from each participant as well as from his/her parent/guardian (when under the age of eighteen).

As per routine, cough swabs and/or spontaneously expectorated sputum specimens were collected from participants and processed by the Diagnostic Laboratory of Tshwane Academic Division (TAD), National Health Laboratory Service (NHLs) using culture (mannitol salt agar for S. aureus) and the VITEK®2 automated system (bioMérieux, France) for identification. Additionally, a nasal swab (ESwab LQ Amies Pernasal Flocked Applicator (Copen, USA)) was collected from each participant and along with the remaining portions of the routine specimens were cultured on ChromID MRSA/Chrom ID S. aureus bi-plate (bioMérieux, France) and incubated (Digital Oven, Scientific Engineering (Pty) Ltd, Roodepoort, South Africa) at 37°C, up to 72 h to obtain pure colonies. The identification of all presumptive S. aureus isolates was confirmed using the MALDI Biotyper (Bruker Daltonics, USA).

Genomic DNA was extracted using the ZR Fungal/Bacterial DNA Miniprep (Zymo Research Corporation, USA) kit. Multiplex-polymerase chain reaction (M-PCR) assays were used to: (i) detect the prevalence of selected antibiotic resistance and virulence genes, such as mecA, responsible for methicillin-resistance; icaA/B, involved in biofilm formation; the insertion sequence 256 (IS256), associated with control of the ica locus and biofilm formation and the lukS/F-PV genes, which encodes for PVL toxin, (ii) detect genes associated with quaternary ammonium resistance (qac) i.e. qacA/B, qacC, qacG, qacH and qacJ genes, (iii) SCCmec type of the MRSA isolates and (iv) agr type of S. aureus isolates. All multiplex PCR assays were performed using the Qiagen Multiplex PCR master mix (Qiagen, Germany) according to the manufacturer’s instructions (Supplementary Table S1). The VITEK®2 automated system (bioMérieux, France) was used to determine the antimicrobial susceptibility of the MRSA isolates, isolated from both routine analysis and chromogenic media (using M100 document of the CLSI guidelines for 2013/2014).

Using the Smal enzyme and S. aureus subsp. aureus ATCC® 12600™ (as reference marker), PFGE of the S. aureus isolates was performed as previously described. Electrophoresis was performed using the Rotaphor system (Biotek, Germany) on a 1.2% gel for 25 h (at 14°C) at an angle of 120° with a field strength of 6 V/cm and an increasing pulse time (linear) from 5 sec to 40 sec. The gel was stained with 1 L ethidium bromide solution [250 µL of ethidium bromide (10 mg/mL stock (Sigma-Aldrich, USA)) digitally captured using a transilluminator (DigiDoc-It, UVP, LCC, USA) and stored. GelCompar II (Applied Maths, Belgium) was used to analyse the banding patterns and construct a dendrogram, showing the percentage of relatedness by means of the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) methods. In this study, a similarity coefficient of ≥80% was used to assign pulsotypes to the S. aureus isolates. A major pulsotype was defined as having more than five isolates within the cluster and a minor pulsotype was defined as less than five isolates within a cluster. Spa typing was performed as previously described using the TakaRa Taq™ (Clontech Laboratories Inc., Japan) according to the manufacturer’s instructions and the gel image was analysed as with PFGE (using a similarity coefficient of ≥80%).

Based on the PFGE and spa typing analysis, representative isolates were sent for MLST (twelve isolates) and spa sequencing (ten isolates), which were performed as previously described. The assembled spa sequences were analysed using the DNA Gear Software (Open source software available at w3.ualg.pt/~hshah/DNAGear) to assign spa types. Assembled MLST sequences for each locus were compared to the sequences in the S. aureus database (saureus.mlst.net) and each sequence was assigned an allelic number. The allelic profile was compared to the other profiles in the S. aureus database and a sequence type was generated.

Results

A total of 19 participants were enrolled in this study from patients attending the CF clinic. The population was predominantly male and had a median age of seven (ranging from one to 40 years old). Eleven of 19 participants were colonised with S. aureus, with the number of isolates obtained per participant ranging from one to six. Five of the eleven participants (45%) tested positive for S. aureus from routine analysis and chromogenic media (two participants with MSSA only and three participants with both MRSA and MSSA) and six of the eleven participants (55%) were culture positive for S. aureus from chromogenic media only (one participant with MSSA only, one participant with both MRSA and MSSA and four participants with MRSA only).

In total 33 S. aureus isolates were collected, ten from routine analysis and an additional 23 using the chromogenic media. All isolates were confirmed as S. aureus by MALDI-tof [MALDI Biotyper (Bruker Daltonics, Billerica,
MA). The participants showed an almost equal distribution of MRSA and MSSA isolates, with 51% (17/33) being mccA positive. In both the MRSA and MSSA isolates, the icaA/B gene (biofilm formation) was most prevalent, followed by the qac gene. No qacG and qacH genes were detected. Figure 1 shows the prevalence of the virulence and qac genes in the MRSA and MSSA isolates.

The SCCmec typing of the MRSA isolates showed that 53% (9/17) were SCCmec type I and 47% (8/17) were SCCmec type IV. No other SCCmec types were detected. The agr type I was the dominant agr type.

The antibiotic susceptibility of the MRSA isolates was determined using the VITEK®2 automated system (bioMérieux, France). Two of the 17 MRSA isolates (12%) were susceptible to all antibiotics, 41% (7/17) were resistant to benzylpenicillin only, 23% (4/17) were resistant to two antibiotics (benzylpenicillin and another antibiotic) and 23% (4/17) were resistant to eleven antibiotics i.e. were multidrug resistant (Supplementary Table S2).

Pulsed field gel electrophoresis showed two major pulsortypes (A and C) and seven minor pulsortypes (see Fig. 2). Two isolates (SA21 and SA22) were untypeable using spa typing, while the remaining isolates clustered into three groups with four outliers (Supplementary Fig. S2).

As can be seen in Fig. 2, two sequence types dominated, ST15 [27% (9/33)] and ST45 [21% (7/33)]. The distribution of the spa types of the ST15 strains (all MSSA) was as follows: (i) four isolates were t465 (three agr type II and one agr type IV) and (ii) five isolates were t715 (two agr type II and three agr type IV). The distribution of the spa types of the ST45 strains (all agr type I) was as follows: (i) four isolates were t465 (two SCCmec type I isolates, one SCCmec type IV isolate and one MSSA isolate), (ii) one isolate was t715 (MSSA) and (iii) two isolates were untypeable (SCCmec type IV).

**Discussion**

The prevalence of S. aureus in this clinic was relatively high, occurring in 58% (11/19) of the participants. The prevalence of MRSA was 42% (8/19) and the prevalence of MSSA was 37% (7/19). In Europe and the USA, the prevalence of MSSA (15% to 56%) is usually higher than MRSA (2% to 23%)7. The prevalence of MSSA in this study was within the previously reported range; however, the prevalence of MRSA was much higher than the previously reported range and was also higher than the MSSA prevalence observed in this study. The reason for the higher prevalence of MRSA among these participants is unclear as the last reported prevalence of MRSA (2007) in this clinical setting was 23%26. However, as participants attending the clinic only have biannually follow-up visits, the increased prevalence may possibly be due to the acquisition of MRSA circulating in the community.

The most prevalent virulence gene was the icaA/B gene [82% (14/17) of MRSA isolates and 25% (4/16) of MSSA isolates), which encodes for biofilm formation (polysaccharide-dependent). The prevalence of the icaA/B gene was higher in the MRSA isolates than the MSSA isolates, although biofilm formation has been known to occur in both MSSA and MRSA strains, especially in CF patients14. The prevalence of the IS256 gene was much lower than that of the icaA/B gene [59% (10/17) of the MRSA isolates and 19% (3/16) of the MSSA isolates]. The IS256 gene has been shown to be associated with biofilm formation (it controls the ica locus) and increased invasiveness, the lower prevalence of IS256 can be seen as a positive indicator of better outcomes for these patients13. The prevalence of the lukS/F-PV genes (encoding for the PVL toxin) were higher in the MRSA isolates than MSSA isolates, which is congruent with previous studies7. The PVL toxin has been associated with more severe disease in CF patients15.

The quaternary ammonium compound (QAC) resistance genes showed a high prevalence with the qacC gene being the most common gene detected in both MRSA [13/17 (76%)] and MSSA [10/16 (63%)] isolates. This higher prevalence of the qacC gene is unusual, typically the qacA/B gene is more prevalent (than the other qac genes) among the staphylococci18. However, the (low) prevalence of the qacG, qacH and qacJ genes is expected as these genes are have a low prevalence in staphylococci18. Even though the qac genes are known to confer resistance to QACs, the resistance is found to be negligible if the QACs are used according to the manufacturer’s instructions15. However, the high prevalence of the qac genes is worrisome as these genes are associated with efflux pump mediated resistance and have the ability to confer resistance to antibiotics, such as aminoglycosides and β-lactams15,24. It has been observed that there is an association between biocide (QAC) resistance and antibiostic resistance24. According to the literature, a possible reason for this association is the close proximity of the
qac genes and antibiotic resistance genes on mobile genetic elements, such as plasmids; in one instance the qacC gene was found on the same plasmid as the genes encoding for aminoglycoside (aacA-aphD), β-lactam (blaZ) and trimethoprim (dfrA) resistance in staphylococci.

Several of the mecA positive isolates showed an oxacillin-susceptible phenotype when the VITEK®2 automated system (bioMérieux, France) was used for antimicrobial susceptibility testing. Strains which show this phenomenon are referred to as oxacillin-susceptible MRSA (OS-MRSA) and are often overlooked during routine testing.

The CF participants in this study showed a genetically diverse S. aureus population for both MRSA and MSSA isolates, with most participants being infected with more than one S. aureus strains. The collection and processing of the nasal swab together with the remainder of the sputum specimen and/or throat swab, using chromogenic media, from the participants may have contributed to the increased isolation of S. aureus, which may explain the isolation of multiple strains per participant. In most instances, these strains belonged to the same sequence type. However, for patient 3 (P3), two different sequence types were detected and clustered according to sampling location, ST30 was detected from the nasal swabs (upper airways) and ST8 was detected from sputum specimens (lower airways). Additionally, ST30 was detected from a throat swab, suggesting that colonisation of the upper airways may precede colonisation of the lower airways. Several studies have been conducted which compare the bacteria isolated from the upper and lower airways in CF patients. These studies all suggested that the upper airways may act as reservoirs for the lower airways and the bacteria present in the upper airways may translocate to the lower airways through micro-aspiration or post-nasal drip. The PFGE dendrogram (Fig. 2), showed two sequences types, ST15 [27% (9/33)] and ST45 [21% (7/33)], that predominated. Sequence type 15 (ST15) is rarely associated with MRSA and is found to be associated with MSSA, as was seen in this study where all ST15 isolates were MSSA. Sequence type 45 on the other hand is associated with MRSA, which is similar to...
the findings of this study, where 71% (5/7) of the ST45 isolates were MRSA. The SCCmec types of the ST45 MRSA isolates in this study were either type I or type IV. Both these strains have been detected previously with ST45-MRSA-I being detected sporadically in Hong Kong and ST45-MRSA-IV being an epidemic strain that is also known as the Berlin Epidemic Strain, WA MRSA-75 or USA600-MRSA-IV. Additionally, this study showed a high prevalence of SCCmec type I (53%) in the MRSA isolates. Typically a low prevalence of SCCmec type I has been observed in countries such as the USA, however the results from this study correlated with a study conducted in Italy (2004/2005) which showed a prevalence of 49.5%. This high prevalence of SCCmec type I, is of particular note in the South African context, where a study conducted in the same healthcare setting (in Pretoria) showed a prevalence of 3.1% and a study conducted on bacteremia in South Africa did not detect SCCmec type.

The agr type I was the most prevalent agr type [55% (18/33)] mostly associated with MRSA isolates [77% (13/17)]. Nastaly et al. (2010) had previously reported that 71% of all MRSA isolates belonged to agr type I, which is similar to the findings obtained in this study.

The predominance of ST15 and ST45 in CF patients was not unique to this setting; in Europe and the Czech Republic these STs predominate as well. Most of the STs identified in this study (ST1, ST8, ST20, ST30 and ST152), have previously been reported from CF patients in Europe. However, ST508 (a single locus variant of ST45) has not been isolated from CF patients previously; this is the first report in CF patients. This sequence type (ST508) had been previously detected in Africa, mostly from nasal swabs. However, in this study ST508 was detected from a throat swab.

While this study provided valuable insight into S. aureus in the CF lung, it did have several limitations. These limitations include that it was a single centre study (with a small number of participants) and only selected antibiotic resistance and virulence genes were detected.

Conclusion
This study showed a high prevalence of important virulence factors, such as biofilm formation and the PVL toxin. These factors were much higher in the MRSA population than in the MSSA population, along with the genes for quaternary ammonium compound resistance. This clinic showed a genetically diverse S. aureus population, with ST15 and ST45 (epidemic strains) dominating.

References
1. South African Cystic Fibrosis Association. The South African cystic fibrosis consensus document at <www.sacfr.co.za/consensus_doc_2012.pdf> (2012).
2. Gaspar, M. C., Couet, W., Olivier, J. C., Pais, A. A. C. C. & Sousa, J. J. S. (2013). Pseudomonas aeruginosa infection in cystic fibrosis lung disease and new perspectives of treatment: a review. Eur. J. Clin. Microbiol. Infect. Dis. 32, 1231–1252 (2013).
3. Folkesson, A. et al. Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. Nat. Rev. Microbiol. 10, 841–851 (2012).
4. Barley, M. et al. Annual Data Report 2016 Cystic Fibrosis Foundation Patient Registry. Cyst. Fibros. Found. Patient Regist. 1–94. at https://www.cff.org/Research/Research-Resources/Patient-Registry/2016-Patient-Registry-Annual-Data-Report.pdf (2016).
5. Goerke, C. & Wolz, C. Adaptation of Staphylococcus aureus to the cystic fibrosis lung. Int. J. Med. Microbiol. 300, 520–525 (2010).
6. Goerke, C. et al. Diversity of probiotics in dominant Staphylococcus aureus clonal lineages. J. Bacteriol. 191, 3462–3468 (2009).
7. Gosa, C. H. & Mullelbach, M. S. Review: Staphylococcus aureus and MRSA in cystic fibrosis. J. Cyst. Fibros. 10, 298–306 (2011).
8. Diep, B. A. et al. Polymorphonuclear leukocytes mediate Staphylococcus aureus Panton-Valentine leukocidin-induced lung inflammation and injury. Proc. Natl. Acad. Sci. 107, 5587–5592 (2010).
9. Ma, X., Chang, W., Zhang, C., Zhou, X. & Yu, F. Staphylococcal Panton-Valentine leukocidin induces pro-inflammatory cytokine production and nuclear factor-kappa B activation in neutrophils. PLoS One 7, 1–11 (2012).
10. Mellelbach, M. S. et al. Multicenter observational study on factors and outcomes associated with various methicillin-resistant Staphylococcus aureus types in children with cystic fibrosis. Am. Am. Thorac. Soc. 12, 864–871 (2015).
11. Heltshe, S. L. et al. Outcomes and treatment of chronic methillin-resistant Staphylococcus aureus differs by Staphylococcal cassette chromosome mec (SCCmec) type in children with cystic fibrosis. J. Pediatric Infect. Dis. Soc. 4, 225–231 (2015).
12. Archer, N. K. et al. Staphylococcus aureus biofilms: Properties, regulation, and roles in human disease. Virulence 2, 445–459 (2011).
13. Koskela, A., Nilsdotter-Augustinsson, Å., Persson, L. & Söderquist, B. Prevalence of the agr operon and insertion sequence IS258 among Staphylococcus epidermidis prosthetic joint infection isolates. Eur. J. Clin. Microbiol. Infect. Dis. 28, 655–660 (2009).
14. McClure, J. A. et al. Novel multiplex PCR assay for detection of the Staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methillin-susceptible from -resistant Staphylococci. J. Clin. Microbiol. 44, 1141–1144 (2006).
15. Smith, K., Gemmell, C. G. & Hunter, I. S. The association between biocide tolerance and the presence or absence of qac genes among hospital-acquired and community-acquired MRSA isolates. J. Antimicrob. Chemother. 61, 78–84 (2008).
16. Boye, K., Bartels, M. D., Andersen, I. S., Møller, J. A. & Westh, H. A new multiplex PCR for easy screening of methillin-resistant Staphylococcus aureus SCCmec types I-V. Clin. Microbiol. Infect. 13, 725–727 (2007).
17. Shopsin, B. et al. Prevalence of agr specificity groups among Staphylococcus aureus strains colonizing children and their guardians. J. Clin. Microbiol. 41, 456–459 (2003).
18. McDougal, L. K. et al. Pulsed-field gel electrophoresis typing of Oxacillin-Resistant Staphylococcus aureus isolates from the United States: Establishing a national database. J. Clin. Microbiol. 41, 5113–5120 (2003).
19. Centers for Disease Control and Prevention. Unified pulsed field gel electrophoresis (PFGE) protocol for Gram-positive bacteria at <www.cdc.gov/hai/pdfs/labSettings/Unified_PFGE_Protocol.pdf> (2012).
20. PulseNet. Standard operating procedure for PulseNet PFGE of Listeria monocytogenes. 1–11 at <http://www.cdc.gov/pulsenet/PDF/listeria-pfge-protocol-508c.pdf> (2013).
21. Petrano, G. et al. Characteristics of Escherichia coli sequence type 131 isolates that produce extended-spectrum β-lactamas: Global distribution of the H30-Rx sublineage. Antimicrob. Agents Chemother. 58, 3762–3767 (2014).
22. Schmitz, F. et al. Typing of methillin-resistant Staphylococcus aureus isolates from Dusseldorf by six genotypic methods. J. Med. Microbiol. 47, 341–351 (1998).
23. Larsen, J. et al. Multilocus sequence typing scheme for Staphylococcus aureus: Revision of the gmk locus. J. Clin. Microbiol. 50, 2538–2539 (2012).
24. Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J. & Spratt, B. G. Multilocus sequence typing for characterization of methillin-resistant and methillin-susceptible clones of Staphylococcus aureus. J. Clin. Microbiol. 38, 1008–1013 (2000).
Author Contributions
T.G.M. contributed to the study protocol writing, specimen collection, experimental procedures, data collection, data analysis and writing of the manuscript. M.K.K. contributed to the study design, protocol development, data collection, data analysis and writing of the manuscript. R.M. contributed to the clinical data, data collection, data analysis and writing of the manuscript. E.H. contributed to the study protocol writing, specimen collection, experimental procedures, data collection, data analysis and writing of the manuscript. M.M.E. contributed to the conceptualisation of the study, study design, protocol development, data collection, data analysis and writing of the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-30725-x.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/. 

© The Author(s) 2018