Peri-implantitis-associated methanogens: a preliminary report

Souad Belkacemi, Anthony Mazel, Delphine Tardivo, Patrick Tavitian, Grégory Stephan, Giancarlo Bianca, Elodie Terrer, Michel Drancourt & Gérard Abd El-Hamam

Methanogens have already been described in periodontitis but not in peri-implantitis. Thirty peri-implantitis samples and 28 control samples were collected from 28 consenting peri-implantitis patients. PCR-sequencing of the 16S rRNA gene was used as a broad-spectrum screening method and results were further confirmed by real-time quantitative PCR targeting the mcrA genes. Results showed a methanogen community dominated by Methanobrevibacter oralis in 31/58 (53%) samples including 16/28 (57%) control samples and 15/30 (50%) peri-implantitis samples. Methanobrevibacter massiliense was detected in 5/58 (8.6%) samples including 3/28 (1%) control samples and 2/30 (6.7%) peri-implantitis samples. The prevalence of M. oralis & M. massiliense did not significantly differ in peri-implantitis and control samples (exact Fisher test, P = 0.61 and P = 0.67, respectively). Further ponderation of the methanogen load by the real-time quantitative PCR for actin human gene again indicated non-significant difference (Wilcoxon-Mann-Whitney test, P = 0.48 and P = 0.40, respectively). These data show that the prevalence of methanogens does not differ in peri-implantitis lesions and healthy sites, when individuals are their own control. These data do not allow assigning a specific pathogenic role to methanogens in peri-implantitis; methanogens rather are part of the commensal and healthy flora of the oral cavity.

Peri-implantitis is an inflammatory disease characterized by the destruction of soft and hard tissues and the formation of pockets around osteo-integrated dental implants. Contrasting with metagenomics studies which failed to detect any methanogen in peri-implantitis lesions, methanogen-targeting molecular investigations detected DNA sequences specific for the methanogens Methanobrevibacter oralis, Methanobrevibacter smithii, Methanomassiliicoccus luminyensis, "Methanobacterium congolense/curvum" and Methanobrevibacter massiliensis in parodontitis pockets. M. oralis, an oral cavity inhabitant, has been more frequently found in parodontitis pockets in association with anaerobes and its role was analyzed as moderate. Yet, studies relying on the simple, un-quantified molecular detection of sequences leaved unsatisfied the relative role of these methanogens in the pathogenesis of peri-implantitis. We hypothesized that the relative load of methanogens among the total flora in peri-implantitis lesions, would give an indication on the relative role of methanogens in the pathogenesis of peri-implantitis as previously showed for periodontitis.

Patients and Methods

Twenty-eight patients diagnosed with peri-implantitis were prospectively included in this study from January 2016 to June 2016. This study was approved by the clinical research ethics committee of the IFR 48 of the University of Aix-Marseille approved (protocol No. 2016–011). All methods were performed in accordance with the relevant guidelines and regulations. The study protocol was explained to each patient and all patients signed an information document and an informed consent document. All patients were interviewed in order to collect their medical and dental history including the date of implantation. An intra-oral examination was performed to measure bleeding on probing (BOP), probing depth (PD), the surfaces with presence of plaque (%), the presence of pus, the number of implants affected by the peri-implantitis, the presence of mobility and the vertical resorption of the supporting bone. In addition, a radiographic status and a 3D radiography were realized. Patient was then diagnosed with peri-implantitis when the following criteria were met: (1) vertical resorption of the supporting bone ≥2 mm or (2) probing depth ≥5 mm or (3) bleeding or (4) pus or (5) motility of the implant according to the literature. Subgingival dental plaque samples were collected from all peri-implantitis pockets of each individual with sterile Gracey curettes (Hu-Friedy, Rotterdam, Netherlands) and placed into

1Aix-Marseille Univ, IRD, MEPHI, IHU Méditerranée-Infection, Marseille, France. 2UFR Odontologie, Aix-Marseille Université, Marseille, France. 3Private practice Marseille France, Marseille, France. Correspondence and requests for materials should be addressed to M.D. (email: michel.drancourt@univ-amu.fr)
tubes containing 1 mL of the SAB anoxic transport medium for methanogens composed of 0.1 g/L: MgCl2, 0.2 g/L; KH2PO4, 0.2 g/L; KCl, 0.1 g/L; CaCl2, 3 g/L; NaCl, 1.15 g/L; Na2HPO4, 1 g/L; ascorbic acid, 1 g/L; uric acid, 1 g/L; glutathione, pH 7.5 with 0.3/0.6 g/L; KO2. Negative control subgingival dental plaque samples were collected from healthy gingival sites with another curette of Gracey sterile and places in the same medium. Each sample collected was suspended in 1 mL transport medium. After homogenization, a 250-μL aliquot was shaken with 0.3 g of acid-washed beads (≤106 mm; Sigma, Saint-Quentin-Fallavier, France) in a FastPrep-24 5G™ instrument (MP Biomedical Europe, Illkirch, France) at a speed of 6.5 m/s (full speed) for 90 s. The supernatant was incubated overnight at 56 °C with 180 μL lysis buffer and 25 μL proteinase K (20 mg/mL) in the Qiagen EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France) and total DNA was then extracted using a kit Nucleospin® Nucleospin® Tissue kit (MACHEREY-NAGEL, Duren Germany; REF: 74095250). Extraction DNA realize after adding 200 μL B3 buffer, 10 μL synthetic plasmid and incubated for 60 minutes at 70 °C. DNA was then washed with 200 μL BW buffer and 600 μL B5 buffer (MACHEREY-NAGEL). After centrifugation for 1600 g, the DNA pellet was eluted in 100 μL of EB buffer (heated to 70 °C) (MACHEREY-NAGEL). As for PCR amplifications, negative controls consisting of sterile DNA-free water were introduced in all the manipulations. We have performed a specific amplification of the external synthetic plasmid to check for inhibitor absence in PCR. In the first broad-spectrum screening step, methanogen DNA was tentatively detected using PCR-sequencing of the methyltransferase M reductase (mcrA) gene (primers mcrAluF 5′-GTTGTTGMGATCCACCATGAYGACG-3′ and mcrAluR 5′-TTTATCSTGATTTWGRTAGTTT-3′) and the 16S rRNA gene (primers 16SrDNA SDArch033aS15-F 5′-CACCCGACCCTAGGC-3′ and 16SrDNA SDArch0958aA19-R 5′-CCTGAGTMTCAATATT-3′) as previously described1. The PCR program was 94 °C for 30 s, 58 °C for 1 min for an annealing step, followed by 30 cycles of 10 min at 72 °C for amplifying the 16S rRNA gene; and 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s followed by extension of 10 min at 72 °C for amplifying the mcrA gene. PCR product sequences were analyzed with the ChromaPro program, version 1.7: sequence similarity values were determined by BLAST program in the online analysis platform from NCBI (blast.ncbi.nlm.nih.gov).

The availability of data and materials. The datasets generated and/or analyzed during the current study are available within the manuscript.

Results

Eight men and twenty women aged between 35 and 86 years diagnosed with peri-implantitis were prospectively included in the study. One control sample and one peri-implantitis sample were collected in each patient, except for patient no. 24 in whom three peri-implantitis samples were collected; giving a total of 28 control samples and 30 peri-implantitis samples. A total of 10 individuals had generalized bleeding while 18 had localized bleeding. The pocket depths ranged from 4 to 12 mm (x: 7.14; σ: 1.99). The number of implants affected by peri-implantitis ranged from 1 to 7 (x: 2.64; σ: 1.80). In four implants diagnosed with peri-implantitis, pus was observed. The vertical resorption of the supporting bone (% of implant length) ranged from 29% to 100% (100% in only one case) (x: 54.64; σ: 17.69) (Table 1). In all PCR experiments, negative controls remained negative and the positive amplification of the external synthetic plasmid control demonstrated the absence of PCR inhibitors. Using methanogen-specific PCR amplification, 31/58 samples (53.4%) were positive for M. oralis with
100% gene sequence similarity with the reference 16S rRNA and mcrA gene sequences; comprising 16/30 (53%) peri-implantitis positive samples and 15/28 (53.6%) control samples. Further, 5/58 (8.6%) samples were positive for *M. massiliense* with 100% 16S rRNA and mcrA gene sequence similarity with reference sequences9 including 2/30 peri-implantitis samples and 3/28 control samples (Table 2). Altogether, methanogens were detected in 36/58 (62%) samples including 17/30 (57%) peri-implantitis samples and 19/28 (67.8%) control samples. Further *M. oralis* and *M. massiliense*-targeted PCR amplifications confirmed all the negative and positive results obtained by using methanogen-specific PCR amplifications. There was no statistically significant difference in the prevalence of methanogens in the peri-implantitis and control samples (*M. oralis*, *P* = 0.61; *M. massiliense*, *P* = 0.67; all methanogens, *P* = 0.45).

Further detection of actin by qPCR extrapolated into 1.15E+01–9.29E+05 cells/sample (x̄: 7.71E+04, σ: 1.89E+05) (Tables 2 and 3). Actin measurement based deriving the load of host cells which calculated to be (x̄: 1.04E+05, σ: 2.33E+05) in the peri-implantitis samples and (x̄: 4.86E+04, σ: 1.23E+05) in the control specimen for positive sample. Methanogen DNA was detected in 19/28 (67%) control samples with an average number of cells of (x̄: 1.42E+04, σ: 4.94E+04) and in 17/30 (60%) in the peri-implantitis sample with an average number of cells of (x̄: 8.03E+05, σ: 2.53E+06). The mean load ratio of *M. oralis* or *M. massiliense* in the peri-implantitis samples was not significantly different than in the control samples (*P* = 0.48 and *P* = 0.4, respectively).

| Patient | Sample | Sex | BOP | PD (mm) | Surfaces with presence of plaque (%) | Presence of pus | Number of implants affected by periimplantitis | Presence of mobility | Vertical resorption of the supporting bone (% of implant length) |
|---------|--------|-----|-----|---------|-------------------------------------|----------------|-----------------------------------------------|---------------------|-----------------------------------------------|
| 1       | P1     | f   | Generalized | 8 | 50 | No | 4 | No | 48 |
| 2       | P2     | f   | Generalized | 7 | 100 | Yes | 2 | No | 70 |
| 3       | P3     | f   | Generalized | 7 | 25 | No | 1 | No | 51 |
| 4       | P4     | m   | Localized | 4 | 100 | No | 2 | No | 37 |
| 5       | P5     | m   | Localized | 6 | 100 | No | 7 | No | 46 |
| 6       | P6     | f   | Localized | 4 | 50 | No | 2 | Yes | 29 |
| 7       | P7     | f   | Generalized | 7 | 50 | No | 3 | Yes | 69 |
| 8       | P8     | f   | Localized | 8 | 25 | Yes | 1 | No | 51 |
| 9       | P9     | f   | Localized | 5 | 25 | No | 2 | No | 51 |
| 10      | P10    | m   | Localized | 4 | 25 | No | 1 | No | 46 |
| 11      | P11    | f   | Localized | 7 | 100 | Yes | 2 | No | 71 |
| 12      | P12    | f   | Localized | 12 | 25 | No | 1 | Yes | 100 |
| 13      | P13    | f   | Localized | 10 | 50 | No | 5 | No | 52 |
| 14      | P14    | f   | Generalized | 9 | 100 | No | 2 | Yes | 74 |
| 15      | P15    | f   | Localized | 6 | 100 | No | 1 | Yes | 74 |
| 16      | P16    | m   | Generalized | 5 | 100 | Yes | 6 | Yes | 35 |
| 17      | P17    | f   | Localized | 7 | 25 | Yes | 7 | No | 67 |
| 18      | P18    | f   | Localized | 8 | 100 | Yes | 6 | No | 60 |
| 19      | P19    | m   | Generalized | 7 | 100 | Yes | 7 | No | 57 |
| 20      | P20    | f   | Localized | 10 | 100 | Yes | 2 | No | 81 |
| 21      | P21    | f   | Localized | 6 | 50 | No | 1 | No | 50 |
| 22      | P22    | m   | Localized | 6 | 100 | Yes | 2 | No | 50 |
| 23      | P23    | f   | Localized | 9 | 100 | No | 2 | No | 40 |
| 24      | P24, P25, P26 | f | Generalized | 10 | 100 | No | 4 | No | 85 |
| 25      | P27    | m   | Localized | 6 | 100 | No | 2 | No | 36 |
| 26      | P28    | m   | Generalized | 7 | 100 | Yes | 2 | No | 35 |
| 27      | P29    | f   | Generalized | 9 | 50 | No | 4 | No | 46 |
| 28      | P30    | f   | Localized | 12 | 50 | No | 2 | No | 35 |

**Table 1.** Clinical data collected in patients with periimplantitis. BOP, bleeding on probing; PD, probing depth.

| Samples | Peri-implantitis | Healthy control |
|---------|------------------|-----------------|
|         | M. massiliense | Human cells load | M. massiliense/Actin ratio | M. massiliense load | Human cells load | M. massiliense/Actin ratio |
| 4       | 7.71E+04 | 2.82E-01 | 1.08E+02 | 8.87E+02 | 1.22E-01 |
| 7       | 6.31E+01 | 1.11E+02 | 5.08E+03 | 2.21E+02 | 2.30E+01 |
| 18      | N/A            | 3.33E+03 | 1.80E+04 | 1.85E-01 |

**Table 2.** Ratio *Methanobrevibacter massiliense* load/human cells load.
than 40%15. Here, lesions is not surprising as bias which occurs when controls become more like the cases about exposure than the general population 11.

Discussion

In this study, we detected the same prevalence of *M. oralis* and *M. massiliensis's* DNA in control samples as in the peri-implantitis samples. Both types of samples originated from the same patient, which means that every patient was also his own control. Using this strict methodology and appropriate negative controls which remained negative in all experiments, we observed that the load of both methanogens was not significantly different in peri-implantitis samples than in the control samples. These results agree with a previous study detecting *M. oralis* in 10 patients with aggressive periodontitis and in 10 periodontally healthy individuals 6. In a more recent study, *M. oralis* represented 82% of cloned methanogen DNA in patients and 70.1% in healthy individuals 6.

These results however differ from those previously reported using 16S rRNA gene clone library analysis, which failed to recover any methanogen sequences in peri-implantitis samples 3. We proposed that differences in the environment; oral microbiota and disease sites versus oral microbiota and healthy sites 8. We argue that during this infection, the microorganisms of the oral microbiota within the same oral cavity are not exposed to the data here reported. With the reserve of a potential overmatching bias, our analyses showed an absence of any difference in the prevalence and in the load of methanogens in peri-implantitis samples and control samples collected in the very same person. Furthermore; a study has shown that the environment in the sulcus depths of peri-implantitis is well adapted to the growth of obligate anaerobic bacteria which provides a specific signature during this infection, the microorganisms of the oral microbiota within the same oral cavity are not exposed to the same environment; oral microbiota and disease sites versus oral microbiota and healthy sites 5. We argue that

Table 3. Ratio *Methanobrevibacter oralis* load/human cells load. N/A, not available.
these controls are of better value than control samples collected in another individual, as previously reported in studies of peri-implantitis and in studies of periodontitis. The possibility that methanogens from diseased tissues did contaminate healthy tissues cannot be excluded in our study. This would obviously temperate the conclusions here reported.

Methanogens have been previously detected in another, closely related situation of periodontitis, and that their load was significantly associated with the severity of the periodontal lesions. The results here reported therefore mirror and extend previous data to a yet unexplored situation of peri-implantitis. Indeed, the microbial flora recovered from peri-implantitis lesions differs from that of periodontitis. The genus *Olsenella, Sphingomonas, Peptostreptococcus* and unclassified *Neisseriaceae* are more abundant in peri-implantites whereas the genus *Desulfovibrio* is lower. The methanogens, although unable to detect species, are more important in peri-implantitis than periodontitis but without significant difference. In these situations, it has been postulated that the particular metabolism of methanogens, which reduces CO$_2$ with H$_2$ as the electron donor for methanogenesis, favors the growth of pathogenic bacteria in anaerobic gingival pockets. Also, methanogens including *M. oralis* and *M. smithii* have been detected and cultured in anaerobe abscesses, including several cases of life-threatening brain abscesses and in one case of muscular abscess. These clinical observations along with experimental data in *M. oralis* challenged mice undoubtedly indicate that methanogens are opportunistic pathogens (Table 4).

Here, we observed that the methanogen load but not the sole detection of methanogens, was not significantly associated with peri-implantitis. This observation differs from that drawn from a previous comparative study of peri-implantitis sites, healthy implant sites and healthy sites. In latter study, authors found a significant difference in the prevalence of methanogens in peri-implantitis sites (48%) and in healthy implants (16%) and natural teeth (8%). Nevertheless, we argue that detecting the presence of methanogens alone is not sufficient to diagnose peri-implantitis, which is probably due to their universal presence in the oral cavity, producing an unavoidable contamination of samples. Only a strict quantification of those methanogens would allow us to overcome this issue. Indeed, we previously made exactly the same observation as for periodontitis.

Further studies are warranted to ensure that the observations here reported are not restricted to our center. Indeed, other populations with other geographical and nutritional backgrounds may exhibit different repertoires of methanogens. Cumulative studies will refine our knowledge of the potential role of methanogen in mixed infection with anaerobes in the pathology of peri-implantitis, guiding therapeutic proposals. In any case, peri-implantitis is a complex pathology which definite diagnosis does not rely on the detection of any single microorganism.

**References**

1. Pérez-Chaparro, P. J. et al. The Current Weight of Evidence of the Microbiologic Profile Associated with Peri-Implantitis: A Systematic Review. *J. Periodontol.* 87, 1295–1304 (2016).
2. Heuer, W. et al. Metagenomic analysis of the peri-implant and periodontal microflora in patients with clinical signs of gingivitis or mucositis. *Clin. Oral Investig.* 16, 843–850 (2012).
3. Koyanagi, T. et al. Comprehensive microbiological findings in peri-implantitis and periodontitis. *J. Clin. Periodontol.* 40, 218–226 (2013).

**Table 4.** List of studies reporting the tentative detection of methanogens in diseased and healthy subjects. + Detection; − No detection; nt, not tested.
4. Vianna, M. E., Conrads, G., Gomes, B. P. F. A. & Horz, H. P. Identification and Quantification of Archaea Involved in Primary Endodontic Infections. J. Clin. Microbiol. 44, 1274–1282 (2006).

5. Huynh, H. T. T., Pignoly, M., Drancourt, M. & Aboudharam, G. A new methanogen “Methanobrevibacter massiliensis” isolated in a case of severe periodontitis. BMC Res. Notes 10 (2017).

6. Matarazzo, F., Ribeiro, A. C., Feres, M., Faverti, M. & Mayer, M. P. A. Diversity and quantitative analysis of Archaea in aggressive periodontitis and periodontally healthy subjects: Archaea in periodontitis and health. J. Clin. Periodontol. 38, 621–627 (2011).

7. Huynh, H. T. T., Pignoly, M., Nakanga, V. D., Drancourt, M. & Aboudharam, G. The Repertoire of Archaea Cultivated from Severe Periodontitis. Plos One 10, e0121565 (2015).

8. Tamura, N., Ochi, M., Miyakawa, H. & Nakazawa, F. Analysis of Bacterial Flora Associated with Peri-implantitis Using Obligate Anaerobic Culture Technique and 16S rDNA Gene Sequence. Int. J. Oral Maxillofac. Implants 28, 1521–1529 (2013).

9. Faverti, M. et al. Prevalence and microbiological diversity of Archaea in peri-implantitis subjects by 16S ribosomal RNA clonal analysis: Archaea in peri-implantitis subjects. J. Periodontal Res. 46, 338–344 (2011).

10. Bringuier, A., Khaleda, S., Richet, H., Aboudharam, G. & Drancourt, M. Real-Time PCR Quantification of Methanobrevibacter oralis in Periodontal: Table 1 J. Clin. Microbiol. 51, 993–994 (2013).

11. Milliron, M., Diallo, A. & Raoul, D. Gut microbiota and malnutrition. Microb. Pathog. 106, 127–138 (2017).

12. Khelaifa, S. et al. Aerobic culture of methanogenic archaea without an external source of hydrogen. Eur J. Clin. Microbiol. Infect. Dis. 35, 985–991 (2016).

13. Maruyama, N. et al. Intraindividual variation in core microbiota in peri-implantitis and periodontitis. J. Clin. Rep. 4, 560 (2014).

14. Khelaifa, S., Garibal, M., Robert, C., Raoul, D. & Drancourt, M. Draft Genome Sequencing of Methanobrevibacter oralis Strain RM01, Isolated from the Human Intestinal Microbiota. Genome Announc. 2, e00737–14 (2014).

15. Li, C. L. et al. Prevalence and molecular diversity of Archaea in subgingival pockets of periodontitis patients. Oral Microbiol. Immunol. 24, 343–346 (2009).

16. Wacholder, S., Silverman, D. T., McLaughlin, J. K. & Mandel, J. S. Selection of controls in case-control studies. II. Types of controls. Am. J. Epidemiol. 135, 1029–1041 (1992).

17. Drancourt, M. et al. Evidence of Archaea Methanogens in Brain Abscess. Clin. Infect. Dis., Off. Publ. Infect. Dis. Soc. Am. 65, 1–5 (2017).

18. Nakanga, V. D., Lotte, R., Roger, P. M., Drancourt, M. & Ruiny, R. Methanobacterium smithii and Bacteroides thetaiotaomicron cultivated from a chronic paravertebral muscle abscess. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 22, 1008–1009 (2016).

19. Ferrari, A., Brusa, T., Rutili, A., Canzi, E. & Biavati, B. Isolation and characterization of “Methanobrevibacter oralis” sp. nov. Curr. Microbiol. 29, 7–12 (1994).

20. Lepp, P. W. et al. Methanogenic Archaea and human periodontal disease. Proc. Natl. Acad. Sci. USA 101, 6176–6181 (2004).

21. Yamabe, K. et al. Distribution of Archaea in Japanese patients with periodontitis and humoral immune response to the components. FEMS Microbiol. Lett. 287, 69–75 (2008).

22. Vianna, M. E., Holtgrew, S., Seyfarth, I., Conrads, G. & Horz, H. P. Quantitative Analysis of Three Hydrogenotrophic Microbial Groups, Methanogenic Archaea, Sulfate-Reducing Bacteria and Acetogenic Bacteria, within Plaque Biofilms Associated with Human Periodontal Disease. J. Bacteriol. 190, 3742–3755 (2008).

23. Horz, H. P., Seyfarth, I. & Conrads, G. M. Analysis of 16S rRNA gene suggests a novel lineage of Archaea phylogenetically affiliated with Thermoplasmatales in human subgingival plaque. Anaerobe 18, 373–377 (2012).

24. Mansfield, J. M., Campbell, J. H., Bhargari, A., Jesiowskis, A. M. & Vickerman, M. M. Molecular Analysis of 16S rRNA Genes Identifies Potentially Pathogenic Bacteria and Archaea in the Plaque of Partially Erupted Third Molars. J. Oral Maxillofac. Surg. 70, 1507–1516 (2012).

25. Belay, N., Johnson, R., Raisch, D., Consyn de Macario, E. & Daniels, L. Methanogenic Bacteria from Human Dental Plaque. Appl. Environ. Microbiol. 66, 600–603 (1999).

26. Brusa, T., Conca, R., Ettorre, A., Ferrari, A. & Pecchioni, A. The presence of methanobacteria in human subgingival plaque. J. Clin. Periodontal. 14, 470–481 (1987).

27. Langendijk-Genevaux, P., Kukla, M., Sandmeier, H., Meyer, I. & van der Hoeven, J. S. Identification of Desulfovibrio vulgaris sp. nov. and Desulfovibrio strain NY682, oral sulfate-reducing bacteria involved in human periodontal disease. Int. J. Syst. Evol. Microbiol. 51, 1035–1044 (2001).

28. Robichaux, M., Weisel, M. & Boopathy, R. Methanogenic Activity in Human Periodontal Pocket. Curr. Microbiol. 46, 0053–0058 (2003).

Acknowledgements
The authors acknowledge the expertise of Olga Cusack in editing the manuscript.

Author Contributions
E. K. M.D., G.A. conceived the study and designed the methodology. S.B., A.M., D.T., P.T., G.S., G.B. performed the experiments and E.T., G.A. and M.D. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Additional Information
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