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Clinical significance and impact of gastric non-*Helicobacter pylori Helicobacter* species in gastric disease

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Summary

Background: Gastric non-*Helicobacter pylori Helicobacter* (NHPH) species naturally associated with animals have been linked with gastric disease in human patients.

Aim: The prevalence and clinical significance of zoonotic gastric NHPHs was determined in large and well-defined, *H. pylori*-negative, gastric patient populations.

Methods: Patients were retrospectively ($n = 464$) and prospectively ($n = 65$) included for gastric biopsy collection: chronic gastritis (CG), peptic ulcer disease and gastric MALT lymphoma, without identified aetiology. PCR and sequencing was performed for the detection of gastric *Helicobacter* species. Retrospectively, asymptomatic gastric bypass patients ($n = 38$) were included as controls. Prospectively, additional saliva samples and symptom and risk factor questionnaires were collected. In this group, patients with gastric NHPH infection were administered standard *H. pylori* eradication therapy and underwent follow-up gastroscopy post-therapy.

Results: In the retrospective samples, the prevalence of gastric NHPHs was 29.1%, while no gastric NHPHs were detected in control biopsies. In the prospective cohort, a similar proportion tested positive: 27.7% in gastric tissue and 20.6% in saliva. The sensitivity and accuracy for the detection of gastric NHPHs in saliva compared to gastric tissue was 27.8% and 69.8% respectively. Following eradication therapy, clinical remission was registered in 12 of 17 patients, histological remission in seven of nine and eradication in four of eight patients.

Conclusion: These findings suggest a pathophysiological involvement of NHPHs in gastric disease. Patients presenting with gastric complaints may benefit from routine PCR testing for zoonotic gastric NHPHs.

Freddy Haesebrouck and Christophe Van Steenkiste are shared senior authorship.

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1 | INTRODUCTION

Gastric *Helicobacter* species are Gram-negative, motile and fastidious bacteria that are able to colonise the stomach of humans and animals and may cause gastric disease.[1] *Helicobacter* (*H.*) *pylori* is by far the best documented and studied gastric *Helicobacter* species and has been associated with severe gastric disease in humans, such as gastritis, peptic ulcer disease (PUD), gastric adenocarcinoma and low-grade B-cell mucosa-associated lymphoid tissue (MALT) lymphoma.[2,3]

Since the first isolation of *H. pylori* from a human gastric biopsy in 1982,[4] many other gastric *Helicobacter* species have been identified in patients' stomachs.[1,5] These long, spiral-shaped gastric non-*Helicobacter pylori Helicobacter* (NHPH) species abundantly reside in animal hosts, including pigs, dogs and cats. *H. suis* is mainly pig-associated, while *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. heilmannii* s.s. and *H. ailurogastricus* are mainly dog- and cat-associated.[1,5] Except for *H. ailurogastricus*, these gastric NHPHs have all been described as zoonotic. The reported prevalence of gastric NHPH infections in symptomatic human patients ranges from 0.2% to 6%, depending on the geographical area and diagnostic methods,[1,6] with *H. suis* being most frequently detected.[5,7,8] Transmission of gastric NHPHs from an infected animal host to a human is suggested to occur through direct or indirect contact with pigs, dogs or cats, or, in case of *H. suis*, via consumption of raw or undercooked pork meat.[1,9,10–13] Gastric NHPH infection in humans has been anecdotally associated with the development of chronic gastritis (CG),[6] peptic ulceration[14] and low-grade B-cell gastric MALT lymphoma.[15–18] Furthermore, case reports demonstrate successful eradication of gastric NHPHs along with complete remission of the histopathological alterations after treatment with standard *H. pylori* eradication therapy.[15,19,20]

Limitations in diagnostics, and hence in obtaining accurate data on prevalence, and in understanding the pathogenicity of gastric NHPHs in humans, are the focal and patchy distribution, mainly in the gastric antrum, with fewer organisms infecting the stomach compared to *H. pylori*,[21] and the demanding strategy to cultivate these fastidious bacteria.[22] Indirect testing with urea breath tests, serum serological tests and stool antigen tests, regularly used in diagnostics for *H. pylori* infection, are not sensitive for the detection of gastric NHPHs[23] and the current most accurate test for the detection of gastric NHPHs is molecular testing combined with sequencing.[24] Therefore, the true prevalence of gastric NHPHs in humans, in particular gastric disease patients, is hypothesised to be higher than it is suggested in existing literature.[1,6]

To the best of our knowledge, no prevalence and pathological data for gastric NHPHs in large Western gastric disease patient cohorts are available. In this study, high quality, specific PCR testing and sequencing was performed in the most systematic way possible to estimate the prevalence in Western patients suffering from gastric disease, without identified aetiology. Furthermore, a prospective pilot study was performed to look into these NHPH-associated gastric pathologies, their reversibility following eradication therapy and potential risk factors.

2 | MATERIALS AND METHODS

2.1 | Study population

The predefined study populations included (1) patients suffering from CG based on historical oesophagogastroduodenoscopy (EGD) with gastric biopsies showing the histopathological diagnosis of non-atrophic CG, atrophic CG or intestinal metaplasia according to the Sydney classification,[25,26] also including patients presenting with lymphoid aggregates or follicles based on historical EGD gastric pathology specimens,[27] (2) patients suffering from unexplained PUD, negative for *H. pylori* infection, and without current/regular use of non-steroidal anti-inflammatory drugs (NSAIDs), oral steroids, tobacco use and alcohol abuse and (3) patients suffering from gastric MALT lymphoma confirmed by clinical investigation, histology and immunophenotyping.[28]

General inclusion criteria included (1) ambulatory adult males and non-pregnant females (2) being 18 years or older and (3) belonging to one of the aforementioned patient groups. Exclusion criteria included (1) history of, or current *H. pylori* infection as diagnosed through histological or immunohistochemical stains of gastric mucosal biopsies and/or serum serology testing, (2) current or recent (i.e. <4 weeks preceding EGD) use of NSAIDs according to the electronic patient record and (3) recent antibiotic use (only for the prospectively recruited cohort).

2.2 | Sample collection

Retrospective inclusion involved the collection of 605 formalin-fixed paraffin-embedded (FFPE) gastric biopsy samples of 464 different patients, dating from 2014 to 2020.

Controls included 38 FFPE historic gastric biopsy samples of asymptomatic patients planned for Roux-en-Y gastric bypass surgery also meeting the exclusion criteria, showing no evidence of gastric disease on pathology, and dating from 2016 to 2020.

Selection of retrospectively included patients was done by reviewing electronic patient records for the selected nomenclature codes.

Prospective inclusion started in January 2020 and ended in May 2022. After consent, gastric biopsy samples were collected, as well as a cytobrush of the gastric mucosa (Cook Medical G22174, ECB-5-180-3-S) and a buccal swab (MasterAmp buccal swab brushes, Epicentre). In total, two to five biopsy specimens from the gastric corpus and two to five from the antrum were pooled for analysis. To optimise the diagnostic yield for gastric NHPHs, a cytobrush was used on the mucosa of the corpus and antrum (as pointed out earlier by Debongnie et al[29]). Since buccal swabs are less invasive than gastric biopsies, the presence of gastric NHPHs was assessed in the saliva of patients and the sensitivity and accuracy of this test was determined, using gastric biopsy analysis as the gold standard. Patient recruitment was performed in two non-academic (AZ Maria Middelares, Ghent/AZ Groeninge) and one academic hospital (University Hospital Antwerp) in Belgium.

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This study was approved by the institutional review board of AZ Maria Middelares/AZ Groeninge and the University of Antwerp (B017201941026 and B67020111159 respectively) and conducted according to the principles of the Declaration of Helsinki. Participants gave their written consent, both for participation in the study and for collecting and processing personal data.

2.3 | Histopathology

Biopsy samples were snap-frozen or fixed in 4% formaldehyde solution for further processing. Sections of FFPE specimens were stained with haematoxylin and eosin. Sections were examined by either of two experienced gastrointestinal pathologists (blinded to all clinical data) and graded according to the updated Sydney classification for gastritis and gastropathy.[25] MALT lymphomas were graded according to recent clinical, histopathological and immunophenotypical guidelines.[28,30]

2.4 | Data collection

Electronic patient records were reviewed for patients' characteristics, symptoms, medication use and endoscopic features.

In the prospective cohort, information concerning recent use of proton pump inhibitors (PPI) and antibiotics (i.e. <4 weeks preceding EGD), occurrence of gastric complaints, exposure to animals, occupation, consumption of raw and cooked meat, country of birth and occurrence of gastric complaints in relatives was retrieved via a questionnaire.

2.5 | DNA extraction, PCR assays and sequencing

2.5.1 | DNA extraction

The retrospectively collected FFPE gastric biopsy samples (retrospective patient cohort and gastric bypass control group) were cut using a microtome (10 sections of 20 μm) and the sections were stored in nuclease-free 1.5 mL tubes. DNA contamination was prevented by decontaminating the microtome with ethanol and chloramine in between the cutting of each FFPE tissue sample. The sections were then pre-treated with xylene to remove the paraffin. Genomic DNA was extracted using the DNeasy Blood & Tissue kit (QIAGEN®) according to the manufacturer's instructions and the samples were stored at −20°C for PCR assay.

The DNA extraction from the fresh frozen, prospectively collected gastric biopsy samples was performed using the DNeasy Blood & Tissue kit (QIAGEN®) according to the manufacturer's instructions. The DNA samples were stored at −20°C for PCR assay.

The cytobrushes and saliva swabs were put into a pipet tip in a 1.5 mL tube to which 100 μL of PrepMan®Ultra solution (Applied Biosystems) was added. After centrifugation (1 min; 8000 rpm), the brush was removed and the samples were incubated in a heat block (10 min, 100°C) and centrifuged (3 min, 13,000 rpm). The DNA samples were stored at −20°C for PCR assay.

2.5.2 | *Helicobacter* genus-specific PCR assay

Helicobacter genus-specific PCR assays were performed using *16S rRNA* gene-based primers (Table S1). Each PCR reaction volume consisted of 20 μL containing $MgCl_2$ (Promega) (1.5 mM), 1× GoTaq® Flexi PCR buffer (Promega), deoxynucleotide triphosphates (dNTPs) (Bioline) (200 μM), forward primer (0.5 μM), reverse primer (0.5 μM), 1 U GoTaq® Flexi DNA polymerase (Promega) and 1 μL of the DNA sample. The protocol for PCR amplification was as follows: pre-incubation for 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C, followed by a final completion step for 5 min at 72°C. As a positive control, genomic DNA of the *H. suis* strain HS5 was used. For visualisation and analysis of the PCR assays, 5 μL of each PCR product was analysed through gel electrophoresis in 1.5% agarose (AGRMP-RO Roche, Merck KGaA) with Midori Green (NIPPON Genetics) in TBE buffer (VWR Life Science). GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific™ SM0323) was used as a weight marker. Images were acquired on a UV transilluminator (UVP PhotoDoc-it Imaging Systems, Fisher Scientific).

2.5.3 | *Helicobacter* species-specific PCR assays

PCR assays for *H. suis*, *H. bizzozeronii*, *H. felis*, *H. salomonis* and *H. pylori* were performed using urease gene-based species-specific primers. Species-specific primers for *H. heilmannii* s.s. and *H. ailurogastricus* were based on the *IceA* gene and *LpsA* gene respectively. An additional, more sensitive, PCR assay for *H. pylori* using species-specific primers based on the *glmM* gene was also performed (Table S1). All PCR assays were performed in 20 μL reaction volume, however, reaction mixtures differed between different species-specific PCRs and are described in the supplementary materials (Table S2). The protocols for PCR amplification for each species-specific PCR assay are included in Table S1. As positive controls, genomic DNA of *H. suis* HS5, *H. bizzozeronii* R1051, *H. felis* CS1, *H. salomonis* R1053, *H. heilmannii* s.s. ASB1, *H. ailurogastricus* ASB7 and *H. pylori* SS1 strains was used. For visualisation and analysis of the PCR assays, 5 μL of each PCR product was analysed through gel electrophoresis in 1.5% agarose (AGRMP-RO Roche, Merck KGaA) with Midori Green (NIPPON Genetics) in TBE buffer (VWR Life Science). GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific™ SM0323) was used as a weight marker. Images were acquired on a UV transilluminator (UVP PhotoDoc-it Imaging Systems, Fisher Scientific).

All samples were also tested in a PCR assay for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in order to evaluate the DNA integrity (details on the PCR assay can be found in Tables S1 and S2; results in Table S3).

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2.5.4 | Sequencing of PCR products positive in PCR assay

The PCR products of samples positive for genus-specific and/or species-specific PCR were sent to Eurofins Genomics® for bidirectional Sanger sequencing, in order to avoid false positive results and confirm the *Helicobacter* species present. Sequencing analysis of amplicons positive for *Helicobacter* genus-specific PCR allows discrimination between *H. suis*, canine and feline-associated gastric NHPHs as a group, and *H. pylori*. Sequence editing and assembly of the received amplicon sequences was done using BioNumerics® software (version 7.6.3, Applied Maths) and the contig sequences were subjected to the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) using the non-redundant nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed on June 27 2022). A cut-off value of 96% was used for average nucleotide identity as a threshold for species delineation.[31] A patient was considered positive when at least one of the samples originating from the patient was positive in at least one of the PCR assays, followed by a confirmatory sequencing result. Accordingly, this was the prerequisite for prospectively included patients to be administered eradication therapy.

2.6 | Eradication and follow-up endoscopy protocol in prospectively included patients positive for gastric non-*Helicobacter pylori Helicobacter* species

Prospectively included patients with a positive result for gastric NHPH presence were prescribed standard *H. pylori* triple eradication therapy. A follow-up EGD was scheduled for each patient who received eradication therapy. Timing of this EGD follow-up was 12–18 months post-therapy in the CG group and 8 weeks post-therapy in the PUD group. This timing was based on the estimated time window for regression of the histopathological features.[28,32]

2.7 | Immunohistochemistry

Immunohistochemical staining for CD3 and CD20 was done in 44 FFPE CG biopsy samples (nine positive for *H. suis*, 11 positive for *H. bizzozeronii*, 12 positive for *H. felis*, one positive for *H. salomonis* and 11 negative for gastric NHPH infection in PCR and sequencing analysis) in order to measure the density of T and B cells respectively. For the selection of these biopsy samples from the retrospective CG patient cohort, the aim was to include approximately 10 biopsies per most prevalent single gastric NHPH infection based on PCR and sequencing, as well as from negative samples, with similar distributions of non-atrophic CG/atrophic CG/intestinal metaplasia patients. Immunohistochemical staining for *Helicobacter* was done in 33 of the 44 FFPE CG biopsy samples, which were positive for gastric NHPH infection in PCR and sequencing analysis, in order to detect and visualise gastric NHPHs. See Appendix S1 for more protocol details.

2.8 | Statistical analysis

Results were expressed as 'mean ± SD or 'median (range)' for continuous variables and 'number (%)' for categorical data. In order to investigate the difference in prevalence of gastric NHPH infection (exposure variable) between gastric disease/CG/PUD (outcome variable) patients and the control group, χ^2 tests and Fisher's exact tests were performed where appropriate. This was not done for MALT lymphoma since the sample size was too small. In order to investigate differences in T- and B-cell densities associated with gastric NHPH infection, univariate linear regression was performed, and an ANOVA model was used in order to investigate their differences with respect to the different gastric NHPHs. To determine differences in the prevalence of gastric NHPHs in gastric tissue and/or saliva between the different types of CG, a Kruskal–Wallis rank-sum test was performed. Taking gastric biopsy analysis as a reference (since it is the gold standard for gastric NHPH detection), the sensitivity and accuracy of the saliva swabs was calculated. For risk factor and transmission route analysis, χ^2 tests and Fisher's exact tests were used where appropriate and corresponding odds ratios (ORs) together with the 95% confidence interval (CI) and *p*-value were calculated. For all statistical analyses, a $p \leq 0.05$ was considered statistically significant. All *p*-values were two sided. Statistical analyses were performed using R version 4.0.3.

3 | RESULTS

3.1 | Patient characteristics

In the retrospective cohort, 182 males and 282 females were recruited with a mean age of 60 ± 17.4 years. The majority suffered from CG (86%, *n* = 399), 11.9% had PUD (*n* = 55) and 2.2% had MALT lymphoma (*n* = 10). Epigastric pain was the most common symptom (29.3%). The prospective cohort included 34 males and 31 females with a mean age of 55 ± 16.7 years. This cohort also included mainly CG patients (84.6%, *n* = 55), 12.3% had PUD (*n* = 8) and 3.1% had MALT lymphoma (*n* = 2). Abdominal pain/discomfort was the most frequently reported symptom (55.4%). The asymptomatic control group included 14 males and 24 females with a mean age of 43 ± 13.2 years (Table 1).

3.2 | Retrospective patient cohort

3.2.1 | Prevalence of gastric *Helicobacter* species

Overall, gastric NHPHs were detected in 135 of the 464 retrospectively included patients (29.1%). In 118 patients (25.4%), a single

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TABLE 1 Sociodemographic factors and symptoms of the retro- and prospective patient cohorts and the gastric bypass control group.

	Retrospective cohort	Prospective cohort	Gastric bypass control group
Number of included patients (*n*)	464	65	38
Age (years ± SD)	60 ± 17.4	55 ± 16.7	43 ± 13.2
Sex (M/F)	182/282 (0.65)	34/31 (1.1)	14/24 (0.58)
Gastric histopathology			
CG (*n*, %)	399 (86%)	55 (84.6%)	NA
Non-atrophic CG	272 (68.2%)	30 (54.5%)	
Atrophic CG	5 (1.3%)	7 (12.7%)	
Intestinal metaplasia	122 (30.6%)	18 (32.7%)	
PUD	55 (11.9%)	8 (12.3%)	
MALT lymphoma	10 (2.2%)	2 (3.1%)	
Gastric symptom characteristics			
None	41 (8.8%)	3 (4.6%)	NA
Abdominal pain/discomfort	68 (14.7%)	36 (55.4%)	
Nausea	17 (3.7%)	21 (32.3%)	
Vomiting	15 (3.2%)	8 (12.3%)	
Heartburn	49 (10.6%)	23 (35.4%)	
Anorexia/weight loss	30 (6.5%)	0 (0%)	
Anaemia	46 (9.9%)	3 (4.6%)	
Bowel movement problems	35 (7.5%)	2 (3.1%)	
Epigastric pain	136 (29.3%)	2 (3.1%)	

Abbreviations: CG, chronic gastritis; F, female; M, male; MALT, mucosa-associated lymphoid tissue; NA, not applicable; PUD, peptic ulcer disease; SD, standard deviation.

species was detected, with the canine/feline species *H. bizzozeronii* and *H. felis* being the most prevalent species (10.1% and 8.8% of the study population respectively), followed by *H. suis* in 22 patients (4.5%). In 17 patients (3.7%), two or three different species were detected. In one case, two different species were detected in gastric biopsy samples taken at two different time points 3 years apart. In the 16 other cases, the different *Helicobacter* species were detected at the same time point. Focusing on the specific disease entities, gastric NHPHs were most frequently detected in PUD patients (32.7%), closely followed by CG patients (29.1%). The lowest frequency was observed in MALT lymphoma patients (10%). When looking into the subgroups of CG, gastric NHPHs were most frequently detected in atrophic CG patients (40%), followed by intestinal metaplasia (35.2%) and non-atrophic CG (26.1%) (Table 2).

Although patients were screened for a negative diagnosis of *H. pylori* at inclusion, the PCR assays for *H. pylori* combined with sequencing were positive in 54 patients (11.6%). These included 22 patients (4.7%) from whom biopsies were also positive for gastric NHPHs. In two of these patients, *H. pylori* was detected in a gastric biopsy sample taken at a different time point compared to the gastric biopsy sample in which the gastric NHPH species was detected (Table S4).

In the control group including 38 gastric bypass patients, no gastric *Helicobacter* species were detected. Based on these results, the prevalence of gastric NHPH infection was significantly higher in case of gastric disease ($p < 0.001$), as well as CG ($p < 0.001$) and PUD ($p < 0.001$) separately, compared to the control group.

3.2.2 | Revision of the original *Helicobacter* immunohistochemical stainings

To assess the diagnostic accuracy for detection of gastric NHPHs by the clinical pathologist in daily practice, the original *Helicobacter* immunohistochemistry (IHC) slides of the NHPH PCR-positive patients were revised (see Table S5). In total, 132 slides of 130 patients out of the 135 NHPH PCR-positive patients were retrieved. These data demonstrate that gastric NHPHs are missed in 19.7% of cases (26/132) by the clinical pathologist during routine *Helicobacter* IHC evaluation. Consequently, in 80.3% of cases, gastric NHPHs could truly not be found in the original IHC stainings, confirming that PCR and sequencing analysis is clearly the superior detection method for NHPH. Of note, also the original IHC slides of the *H. pylori* PCR-positive patients were revised and all depicted as true negatives (i.e. no missed cases by the clinical pathologist). These findings underline the very focal and not densely colonising distribution of gastric NHPHs compared with *H. pylori*.

3.2.3 | Immunohistochemistry for the detection of gastric non-*Helicobacter pylori Helicobacter* species in the human gastric biopsy samples

Immunohistochemical examination for the detection of gastric NHPHs using an antibody directed against *Helicobacter* was done in

TABLE 2 Prevalence of gastric non-*Helicobacter pylori Helicobacter* species per gastric disease patient group (retrospective cohort).

		CG (*n* = 399)				PUD (*n* = 55)	MALT lymphoma (*n* = 10)
	n/N (%)		Non-atrophic CG (*n* = 272)	Atrophic CG (*n* = 5)	Intestinal metaplasia (*n* = 122)		
Total gastric NHPH positive patients	135/464 (29.1%)	116/399 (29.1%)	71/272 (26.1%)	2/5 (40%)	43/122 (35.2%)	18/55 (32.7%)	1/10 (10%)
Single infections	118/464 (25.4%)	100/399 (25%)	61/272 (22.4%)	2/5 (40%)	37/122 (30.3%)	17/55 (30.9%)	1/10 (10%)
H. bizzozeronii	47/464 (10.1%)	42	23	1	18	5	0
H. felis	41/464 (8.8%)	33	27	0	6	7	1
H. suis	22/464 (4.5%)	18	8	1	9	4	0
H. salomonis	4/464 (0.9%)	4	2	0	2	0	0
H. heilmannii s.s.	1/464 (0.2%)	1	0	0	1	0	0
H. ailurogastricus	0/464 (0%)	0	0	0	0	0	0
Not further identified canine/feline associated gastric NHPH species	3/464 (0.6%)	2	1	0	1	1	0
Mixed infections	17/464 (3.7%)	16/399 (4%)	10/272 (3.7%)	0/5 (0%)	6/122 (4.9%)	1/55 (1.8%)	0/10 (0%)
H. suis + *H. bizzozeronii*	8/464 (1.7%)	8	5	0	3	0	0
H. suis + *H. felis*	3/464 (0.6%)	3	1	0	2	0	0
H. suis + *H. salomonis*	1/464 (0.2%)	0	0	0	0	1	0
H. bizzozeronii + *H. felis*	2/464 (0.4%)	2	2	0	0	0	0
H. felis + *H. salomonis*	1/464 (0.2%)	1	1	0	0	0	0
H. suis + *H. bizzozeronii* + *H. felis*	1/464 (0.2%)	1	0	0	1	0	0
H. bizzozeronii + *H. felis* + *H. salomonis*	1/464 (0.2%)	1	1	0	0	0	0

Abbreviations: CG, chronic gastritis; MALT, mucosa-associated lymphoid tissue; NHPH, non-*Helicobacter pylori Helicobacter*.

33 FFPE CG biopsy samples which were positive for a single gastric NHPH species in PCR and sequencing analysis. These included 11 biopsies positive for *H. bizzozeronii*, 12 biopsies positive for *H. felis*, nine biopsies positive for *H. suis* and one biopsy positive for *H. salomonis*. *Helicobacter* species with a spiral-shaped morphology, a characteristic typically described for gastric NHPHs allowing differentiation from *H. pylori*, were only found in one biopsy positive for *H. suis* and one biopsy positive for *H. felis*. Pictures of the detected gastric NHPHs are shown in Figure 1.

3.2.4 | Immunohistochemistry to measure T- and B-cell density in the human gastric biopsy samples

Immunohistochemical examination for T- and B-cell density using antibodies directed against CD3 and CD20, respectively, was done in 44 FFPE CG biopsy samples, including 33 positive and 11 negative for gastric NHPH infection in PCR and sequencing analysis. Although the median T-cell density in the samples positive for gastric NHPH infection was consistently higher than in the negative samples (1.66% [0.18–4.37] vs 0.97% [0.57–2.48]), statistical significance could not be reached ($p = 0.12$). The same was true for the average T-cell density associated with the different species when comparing them with each other ($p = 0.53$; Figure 2A).

The overall median B-cell density in the gastric NHPH-positive group was neither different from negative controls (0.14% [0.0018–7.22] vs 0.10% [0.025–2.62], $p = 0.49$), nor did the B-cell densities differ significantly between the different gastric NHPHs ($p = 0.42$). However, the average B-cell density for *H. felis*-positive samples tended to be non-significantly increased compared to other species and negative controls (Figure 2B).

3.3 | Prospective patient cohort

3.3.1 | Prevalence of gastric *Helicobacter* species in gastric tissue (biopsy sample/cytobrush)

Using PCR detection and sequencing methods, gastric NHPHs were detected in the gastric tissue of 18 out of 65 prospectively included patients (27.7%). These included 17 infections (26.2%) where a single species was detected, with the canine/feline species *H. bizzozeronii*

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FIGURE 1 Immunohistochemical staining for the detection of *Helicobacter* in human gastric biopsy samples. Detection of *Helicobacter* in a sample that was positive for *H. suis* in PCR and sequencing analysis, total magnification 1000× (A). Detection of *Helicobacter* in a sample that was positive for *H. felis* in PCR and sequencing analysis, total magnification 1000× (B). The black arrows indicate the location of the detected *Helicobacter* species (A,B).

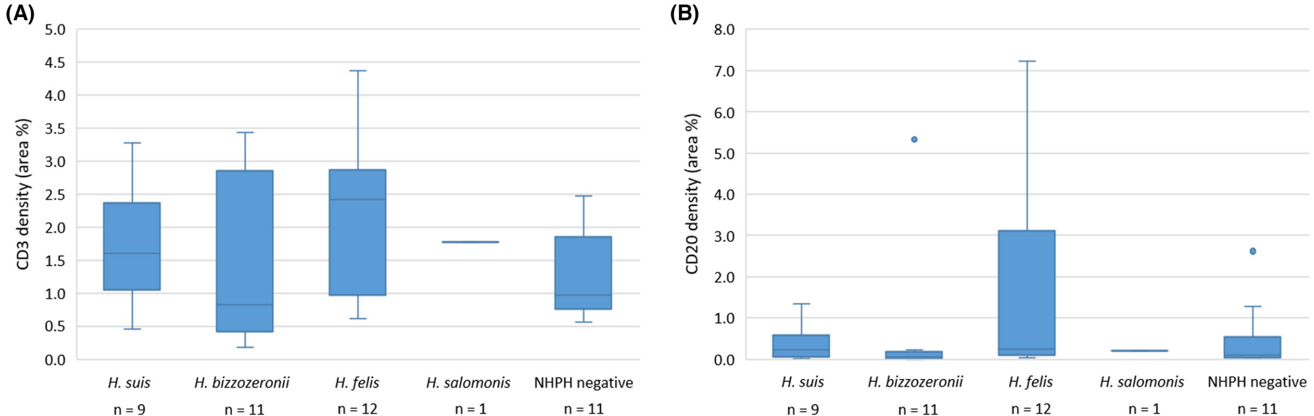

FIGURE 2 Immunohistochemistry for the detection of CD3 (T-cell marker) (A) and CD20 (B-cell marker) (B) in human gastric biopsy samples positive for different gastric NHPHs and negative for gastric NHPHs in PCR and sequencing analysis. No statistically significant differences were found. NHPH, non-*Helicobacter pylori Helicobacter*.

and *H. felis* being the most prevalent species (10.8% and 9.2% of the study population respectively), followed by *H. salomonis* in three patients (4.6%). Twelve of these single infections were detected in the biopsy sample and 5 in the cytobrush. One mixed infection (1.5%) was observed (*H. suis* [detected in the cytobrush] and *H. heilmannii* s.s. [detected in the biopsy sample]). Gastric NHPHs were detected in 29.1% of the CG patients, with the highest frequency in non-atrophic CG (36.7%), followed by intestinal metaplasia (22.2%) and atrophic CG (14.3%). One of two MALT lymphoma (50%) and one of eight PUD (12.5%) patients were also positive (Table 3). Despite the screening for a negative diagnosis of *H. pylori* at inclusion, the PCR assays for *H. pylori* combined with sequencing were positive in one patient (1.5%; single infection).

No statistically significant difference was observed in the prevalence of gastric NHPHs in the gastric tissue and/or saliva when comparing the different types of CG (non-atrophic CG, atrophic CG and intestinal metaplasia) ($p = 0.11$).

3.3.2 | Buccal swab as non-invasive tool to detect gastric non-*Helicobacter pylori Helicobacter* species in saliva

In the saliva samples, gastric NHPHs were detected in 13 of 63 of the prospectively included patients (20.6%). Similar to the gastric samples, the canine/feline species *H. bizzozeronii* and *H. felis* were most frequently detected (4.8% and 9.5% respectively), followed by *H. suis* and *H. salomonis* (both 1.6%). One mixed infection (1.6%) was present with simultaneous *H. bizzozeronii* and *H. salomonis* detection and in one saliva sample, co-infection with *H. felis* and *H. pylori* was detected (Table S6).

Ideally, the diagnosis of gastric NHPHs on tissue biopsy could be replaced by a non-invasive alternative. For that purpose, buccal swabs were collected from the patients to perform PCR assays combined with sequencing, and the results were compared with the gastric tissue samples as this is the gold standard. Compared to

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TABLE 3 Prevalence of gastric non-*Helicobacter pylori Helicobacter* species in human gastric biopsy samples and cytobrushes, per gastric disease patient group (prospective cohort).

		CG (*n* = 55)				PUD (*n* = 8)	MALT lymphoma (*n* = 2)
	n/N (%)		Non-atrophic CG (*n* = 30)	Atrophic CG (*n* = 7)	Intestinal metaplasia (*n* = 18)		
Total gastric NHPH-positive patients	18/65 (27.7%)	16/55 (29.1%)	11/30 (36.7%)	1/7 (14.3%)	4/18 (22.2%)	1/8 (12.5%)	1/2 (50%)
Single infections	17/65 (26.2%)	15	10	1	4	1	1
H. bizzozeronii	7/65 (10.8%)	6	4	0	2	0	1
H. felis	6/65 (9.2%)	5	3	1	1	1	0
H. suis	0/65 (0%)	0	0	0	0	0	0
H. salomonis	3/65 (4.6%)	3	2	0	1	0	0
H. heilmannii s.s.	0/65 (0%)	0	0	0	0	0	0
H. ailurogastricus	0/65 (0%)	0	0	0	0	0	0
Not further identified canine/feline associated gastric NHPH species	1/65 (1.5%)	1	1	0	0	0	0
Mixed infections	1/65 (1.5%)	1	1	0	0	0	0
H. suis + *H. heilmannii* s.s.	1/65 (1.5%)	1	1	0	0	0	0

Abbreviations: CG, chronic gastritis; PUD, peptic ulcer disease; MALT, mucosa-associated lymphoid tissue; NHPH, non-*Helicobacter pylori Helicobacter*.

the gastric tissue samples (gold standard), the sensitivity and accuracy of buccal swabs was 27.8% (5/18, Table S7) and 69.8% (44/63) respectively.

3.3.3 | Eradication therapy in patients positive for gastric non-*Helicobacter pylori Helicobacter* species

Eradication therapy was administered in 20 out of 24 patients positive for gastric NHPHs in the gastric tissue and/or saliva. In three cases, patients refused therapy and one was lost-to-follow-up. Specifications concerning gastric disease, gastric complaints and detected gastric NHPHs can be found in Figure 3. Clinical remission, defined as a disappearance of symptoms reported pre-therapy, was observed in 12 of these patients and five reported persistent gastric complaints (two reported no symptoms pre-therapy and clinical remission was thus not applicable, and 1 was lost-to-follow-up). A follow-up gastroscopy was performed in nine patients; seven of whom histological remission could be established, meaning that the initial gastric disease was no longer present after eradication therapy. In two patients, histological remission was not obtained. In four out of eight of these patients, eradication of the gastric NHPH species could be confirmed. No biopsy specimens were retrieved to check for eradication in the MALT lymphoma patient. In one patient without clinical remission, but with histological remission, the gastric NHPH species was no longer detected in the gastric tissue, but was detected in the saliva. In two other patients, another gastric NHPH species emerged in conjunction with the gastric NHPH species detected before eradication therapy. There was also one patient with clinical remission, but without histological remission, in whom another gastric NHPH species (*H. salomonis*) was detected after eradication therapy as opposed to before eradication therapy (*H. felis*). Detailed follow-up data per patient who received eradication therapy can be found in Table S8.

Different eradication therapy schemes were implemented depending on the known local microbial resistance[33] and the patient's allergies and tolerance. In eight cases, a PPI combined with amoxicillin and clarithromycin was administered, in three cases, sequential therapy with a PPI combined with amoxicillin and a PPI combined with clarithromycin and metronidazole and in one case, pantoprazole combined with bismuth subcitrate potassium, metronidazole and tetracycline. The MALT lymphoma patient received eradication therapy combined with rituximab monotherapy.

3.3.4 | Predisposing risk factors for infection and transmission route analysis

Information concerning possible infection risk factors and transmission routes of gastric NHPHs was prospectively collected via questionnaires. None of the explanatory variables assessed were significantly associated with a higher risk for gastric NHPH infection in our population (Table S9). The use of a PPI around the time of EGD did not seem to influence the probability to detect gastric NHPHs

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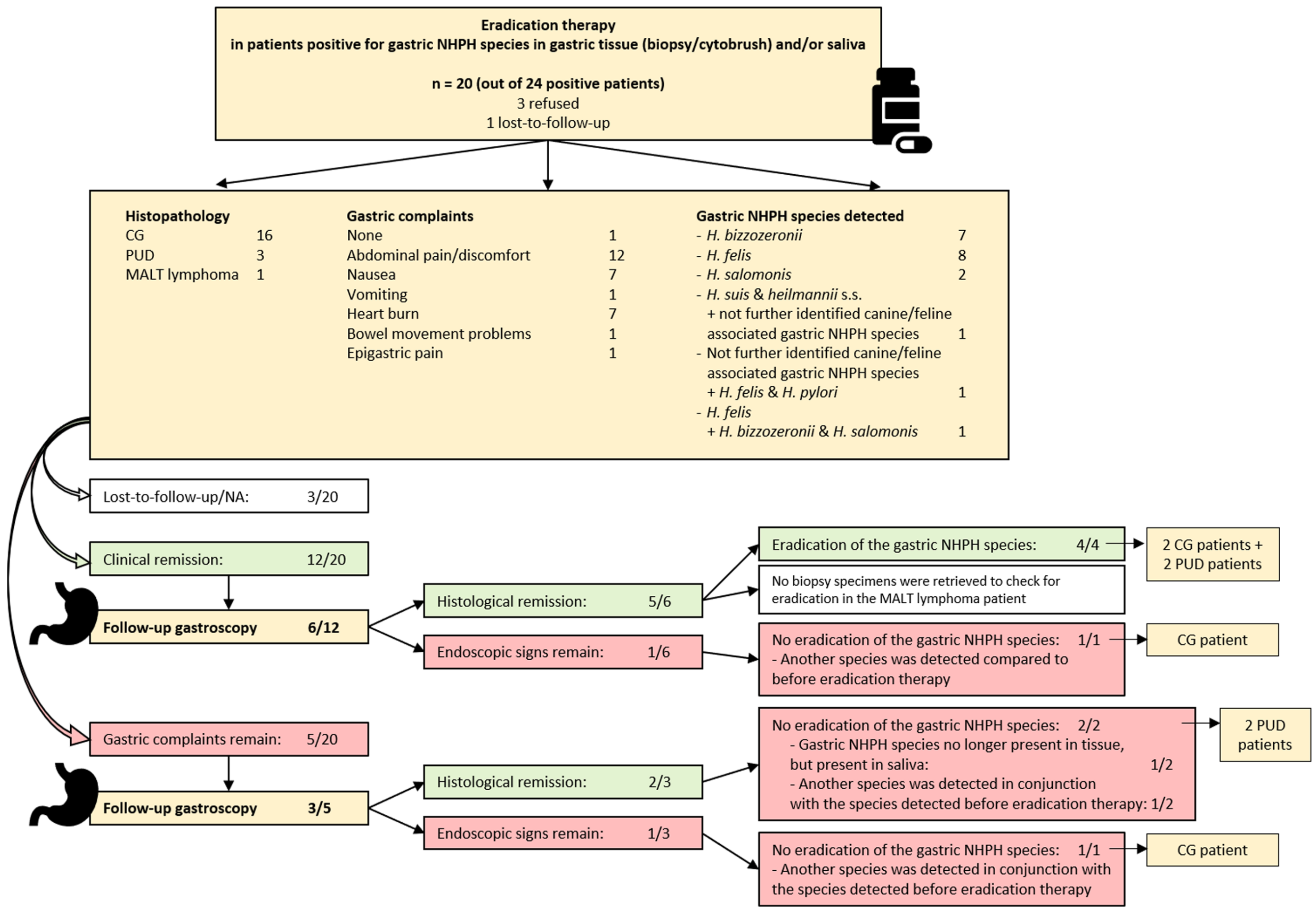

FIGURE 3 Flow chart of patients positive for gastric NHPH species in gastric tissue and/or saliva receiving eradication therapy and their follow-up data. CG, chronic gastritis; MALT, mucosa-associated lymphoid tissue; NA, not applicable; NHPH, non-*Helicobacter pylori Helicobacter*; PUD, peptic ulcer disease.

(OR = 0.86 (95% CI 0.30–2.55), $p = 0.79$), as reported for *H. pylori* in previous studies.[34]

4 | DISCUSSION

It has been hypothesised that porcine, canine and feline gastric NHPH infections may occur more frequently than currently believed in symptomatic, *H. pylori* negative CG, PUD and MALT lymphoma patients, in whom no definite diagnosis can be made in our current clinical practice, and that these *Helicobacter* species may be linked with gastric disease. In this study, we found high prevalence rates of gastric NHPHs in a Western, gastric patient population, namely 29.1% in the retrospective cohort and 27.7% in the prospective cohort. These numbers are in line with a study from Japan, reporting a 20% prevalence of gastric NHPHs in *H. pylori*-negative gastric disease patients, by means of PCR and immunohistochemical methods.[6] This is in contrast with previously reported prevalence rates of 0.2%–6% in unselected symptomatic patient populations.[1,6] Of note, in a pilot study conducted earlier in our laboratory, a prevalence of gastric NHPH infections of only 1% was detected in an ambulatory setting in 100 consecutive patients presenting with stomach ache (without further specifications—unpublished data). The higher prevalence rates detected in gastric disease groups may indicate a causal relationship between gastric NHPH infections and the development of CG, PUD and MALT lymphoma. The statistically significant association observed between gastric NHPH infection and gastric disease in the retrospectively collected samples (cfr. gastric bypass control group vs gastric disease group) further highlights a potential pathophysiological role for gastric NHPHs. Finally, the finding that no infections could be detected in the asymptomatic gastric bypass control group (acknowledging this was only a small group), 1% in the symptomatic non-selected group (earlier study—unpublished data) and 27.7%–29.1% in the highly selected groups, reinforces this suggestion for a pathophysiological involvement. This is also consistent with the observation that bacterial host jumps usually lead to an increase in disease severity, while coevolution between a bacterium and its natural host generally results in less severe pathogenicity.[35]

In contrast to previous studies, the canine/feline gastric NHPHs *H. bizzozeronii* and *H. felis* were the most prevalent species in our

cohort, and not *H. suis*, which is mainly pig-associated.[7,8] This might be explained by the regular and closer contact with domestic cats and dogs, more than with pigs. Indeed, frequent and intense contact with pets has been shown to be an important risk factor for gastric NHPH infections.[1,12,13,36] In this study, however, no statistically significant association between animal contact and gastric NHPH infection could be derived from the obtained questionnaires. One bias could have been the long latency between infection and detection (i.e. lead time bias). Indeed, gastric *Helicobacter* species persist for longer periods of time in the stomach in murine models[37] and cause lifelong infections of the host, and hence, contact with animals more early in life, for example, during childhood, may still be detected at a later stage of age. It is possible that our questionnaire was not comprehensive enough to capture such data. The detection of mixed infections with dog- and cat-associated gastric NHPHs in four patients in this study further highlights the importance of dogs and cats as gastric NHPH reservoirs for humans. Indeed, several different gastric NHPHs often co-reside in the stomach of dogs and cats,[1] which may increase simultaneous transmission of multiple gastric NHPHs from pets to humans.

Using murine models, CD4-positive T cells have been shown to be essential in the development of gastric mucosal hypertrophy and nodular hyperplasia during experimental gastric NHPH infection.[38] Furthermore, lymphoid follicles in murine gastric NHPH infection have been found to be composed of B cells, CD4-positive T cells and dendritic cells.[39] T-helper (Th) 1 cells prime IFN-γ, involved in the formation of gastric lymphoid follicles in gastric NHPH infection, while CD4-positive T cells would promote their expansion. Also, T cells have been shown to contribute in vitro in low-grade B-cell MALT lymphomas.[40] To the best of our knowledge, this study investigated for the first time the changes of T- and B-cell densities in the gastric NHPH-positive human patients in order to study the host bacterial immune response. Our results showed that T cells were increased in CG samples on IHC, although not significantly. The association between T-cell infiltration and gastric pathology development needs to be further investigated, preferably in larger trials.

Interestingly in this study, *H. felis* and *H. bizzozeronii* were the only two species observed in MALT lymphoma patients. This might be explained by the election of a stronger immune response by these species compared to other gastric NHPH species. Indeed, *H. felis* was associated with the highest T- and B-cell density and a slight increase in B-cell density was observed in *H. bizzozeronii*-positive patients. Further research on T- and B-cell immunophenotyping with flow cytometry is needed to confirm these findings.

The accurate pathological detection of *H. pylori* is essential for managing infected patients. Among various diagnostic methods, histology and IHC play a pivotal role. In this study, however, IHC for the detection of gastric NHPHs proved an insensitive tool for the diagnosis of gastric NHPHs, since only two out of 33 gastric immunostainings were concordant with the PCR and sequencing analysis. Currently, no commercial specific immunostainings for NHPHs are available, and hence, immunostainings were performed with a commercial *Helicobacter* genus-specific antibody generated from an immunogen prepared from heat-treated cells of *H. pylori*. In addition, the distribution of gastric NHPHs is more patchy and focal and their colonisation density is lower than with *H. pylori*,[21] possibly leading to false-negative results due to sampling error and making histology and immunostaining a less suited tool for accurate detection compared with molecular techniques. Furthermore, while all included patients were regarded *H. pylori* negative based on the current standard practice, *H. pylori* DNA was detected in 11.6% of the retrospectively included patients using PCR. This finding suggests that some *H. pylori* infections might be missed in the hospital. One confounding factor could be the use of PPIs possibly leading to decreased sensitivity of histology, whereas PCR techniques are less prone to that (cfr. further in the discussion section).[34] Taken together, performing molecular techniques on gastric biopsies is the most accurate and valuable method for the detection and identification of gastric NHPHs, as well as for *H. pylori*.[24,34,41,42]

In this study, we investigated whether the sampling of saliva might be feasible as a non-invasive technique for the diagnosis of gastric NHPHs. Smet et al[43] pointed out that *H. suis* DNA could be detected in buccal swabs obtained from slaughtered pigs and De Cooman et al[44] showed the presence of *H. suis* DNA in the oral cavity of pork carcasses. Moreover, gastric NHPHs have been detected in the saliva of dogs[45] and *H. pylori* has been isolated from the oral cavity of human patients.[46] Unfortunately, from our analysis, it was shown that analysis of saliva by PCR and sequencing was neither sensitive (sensitivity = 27.8%) nor accurate (accuracy = 69.8%). Hence, other non-invasive alternative methods for the detection of gastric NHPHs have to be investigated.

In the prospectively recruited patients, the use of cytobrushes was tested as a potential method to optimise the diagnostic yield of gastric NHPH infections. Since gastric NHPHs have a focal and patchy distribution, the use of cytobrushes could be potentially interesting as they allow sampling of a more extensive area. However, the number of gastric NHPH-positive samples was lower in the cytobrushes than in the gastric biopsy samples. This might be caused by the fact that gastric NHPHs often reside deep in the gastric pits and parietal cells rather than in the mucus, which may have hampered their capture by cytobrush. A combination of both sampling methods may be appropriate as in our study, several gastric NHPH infections would have been missed if only one sampling technique would have been used.

The use of PPIs has been suggested to reduce sensitivity and specificity of *H. pylori* diagnosis due to a decrease in bacterial load and urease activity, in particular when performing standard histology and rapid urease tests.[47,48] A recently published (although in vitro) study indeed demonstrated that acid suppressants, and especially a potassium competitive acid blocker, may damage NHPHs within parietal cell intracellular canaliculi,[49] challenging the previous in vivo findings of Bazin et al.[34] The Maastricht IV/Florence Consensus Report for the management of *H. pylori* infection recommends that, if possible, PPIs should be stopped 2 weeks

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before a diagnostic test is performed, including histology, culture, rapid urease test, urea breath test or stool antigen test.[47] This was, however, not applied in this study and we estimate this did not impact the results we obtained. First, an elegant study of Bazin et al[34] showed that PCR methods have the best performance for *H. pylori* detection in gastric samples, independently of previous PPI treatment. Second, the frequency of gastric NHPH detection in our cohort did not differ regardless of previous PPI treatment at the time of endoscopy or not, confirming the earlier findings that PPI use should not be discontinued for gastric *Helicobacter* species PCR analysis.

In the gastric NHPH-infected patients who received eradication therapy, we were able to show both clinical and histological remission, again pointing to a pathophysiological role of these gastric NHPHs. This is in line with previous case reports.[20,50,51] However, the true efficacy of eradication therapy for gastric NHPH infections cannot be deducted from this study due to an insufficient sample size and non-randomised set-up. Intrinsic, decreased susceptibility and acquired antimicrobial resistance has already been reported in *H. suis* and pet-associated gastric NHPHs,[52,53] which may confound the eradication and potential histological and clinical remission. Furthermore, strict compliance analysis with the sometimes difficult-to-tolerate antibiotic combination was not performed and may have influenced the outcomes. In addition, since molecular analysis on gastric biopsies is the gold standard, the success of eradication therapy can only be appraised by follow-up endoscopy, which is not often obtained in patients with complete clinical remission.

Nevertheless, this study is unique since patients (both in the retrospective and prospective cohort) were highly selected according to stringent, predefined criteria in a very systematic manner, eliminating as many confounding variables as possible, including potential *H. pylori* infection. Furthermore, the retrospective study lists the largest cohort ever published on gastric NHPHs and included a negative control group. Moreover, the inclusion of a prospective patient cohort provides data on clinical and histological improvement/cure after NHPH eradication with antibiotics and PPIs.

5 | CONCLUSION

Gastric NHPH infections were frequently detected in gastric patient populations. Eradication of the gastric NHPHs resulted in clinical and histological improvement, pointing to a pathophysiological role of these bacteria. In contrast to existing literature, *H. bizzozeronii* and *H. felis* showed a higher prevalence than *H. suis*. As we have demonstrated that saliva testing and IHC are not accurate enough for gastric NHPH diagnosis, patients presenting with gastric complaints refractory to empirical treatment may benefit from routine PCR testing for zoonotically important gastric NHPHs. These results should be confirmed in a large multicentre prospective validation study.

AUTHOR CONTRIBUTIONS

Emily Taillieu: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Chloë De Witte:** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); project administration (equal); writing – review and editing (equal). **Heiko De Schepper:** Resources (equal); writing – review and editing (equal). **Wouter Van Moerkercke:** Resources (equal); writing – review and editing (equal). **Sophie Rutten:** Data curation (equal); investigation (equal); methodology (equal); resources (equal); validation (equal); writing – review and editing (equal). **Stijn Michiels:** Data curation (equal); investigation (equal); methodology (equal); resources (equal); validation (equal); writing – review and editing (equal). **Yuna Arnst:** Data curation (equal); investigation (equal); methodology (equal); resources (equal); validation (equal); writing – review and editing (equal). **Sofie De Bruyckere:** Data curation (equal); investigation (equal); methodology (equal); resources (equal); validation (equal); writing – review and editing (equal). **Sven Francque:** Resources (equal); writing – review and editing (equal). **Frauke van Aert:** Data curation (equal); writing – review and editing (equal). **Christophe George:** Resources (equal); writing – review and editing (equal). **Emma Callewaert:** Data curation (equal); writing – review and editing (equal). **Tiene Callewaert:** Data curation (equal); writing – review and editing (equal). **Glenn Vanneste:** Resources (equal); writing – review and editing (equal). **Erik Vanderstraeten:** Resources (equal); writing – review and editing (equal). **Nina Van Heddegem:** Data curation (equal); writing – review and editing (equal). **Margaux Vansteelant:** Data curation (equal); writing – review and editing (equal). **Koen Chiers:** Methodology (equal); supervision (equal); writing – review and editing (equal). **Freddy Haesebrouck:** Conceptualization (equal); funding acquisition (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Christophe Van Steenkiste:** Conceptualization (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to disclose.

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