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Helicobacter suis Is Associated With Mortality in Parkinson's Disease

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Helicobacter pylori has been implicated in the pathogenesis of Parkinson's disease (PD). Its eradication, in a randomized placebo-controlled trial, improved PD hypokinesia. Helicobacter species zoonosis might explain excess mortality from PD and non-Hodgkin lymphoma in livestock, but not arable, farmers. Indeed, Helicobacter is causally-associated with gastric lymphoma. We have previously shown that the relative-frequency, H. suis to H. pylori, was 10-times greater in 60 PD-patients than in 256 controls. We now go on to evaluate the pathological significance of H. suis, detected in gastric-biopsy DNA-extracts by ureA-based species-specific qPCR, validated by amplicon sequencing. The methodology had been cross-validated by a carR-based PCR. The pathological significance is put in context of H. pylori detection [urea-breath-test (UBT) with biopsy-culture, and, if negative, PCR], and the potential reservoir in pigs. Here, we explore, in these 60 PD-patients, associations of H. suis status with all-cause-mortality, and with orthostatic cardiovascular and blood profiling. H. suis had been detected in 19 of the 60 PD-patients on one or more occasion, only two (with co-existent H. pylori) being UBT positive. We found that the hazard-of-death (age-at-diagnosis- and gender-adjusted) was 12 (95% CI 1,103) times greater (likelihood-ratio test, P = 0.005) with H. suis-positivity (6/19) than with negativity (2/40: one lost to follow-up). UBT-values did not influence the hazard. H. suis-positivity was associated with lower standing mean-arterial-pressure [6 (1, 11) mmHg], H. pylori-positivity having no effect. The lower total lymphocyte count with H. pylori-positivity [−8 (−1, −14) %] was not seen with H. suis, where T-cell counts were higher [24 (2, 52) %]. Regarding the potential zoonotic reservoir in the UK, Helicobacter-like-organism frequency was determined in freshly-slaughtered pigs, nature ascertained by sequencing. Organisms immunostaining for Helicobacter, with corkscrew morphology typical of non-H. pylori Helicobacter, were seen in 47% of 111 pig-antra. We conclude that H. suis is associated with all-cause-mortality in PD and has a potential zoonotic reservoir.

Keywords:all-causemortality,Parkinson'sdisease,*Helicobactersuis*,*Helicobacterpylori*,pigreservoir

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INTRODUCTION

In the one randomized, double-blind, placebo-controlled trial (RCT) of the effect of biopsy-proven Helicobacter pylori eradication on the facets of Parkinson's disease (PD), we found sustained, clinically relevant improvement in objectively-measured hypokinesia (1, 2), providing 1b level causative evidence (3). Indication specificityof the improvement following H. pylori eradication is inferred from a surveillance study of antimicrobial exposure for any indication in PD (4). There is a lack of birth cohort effect for H. pylori anti-urease-antibody in PD (5), as in gastric cancer and peptic ulcer where causal links withH.pyloriaregenerallyaccepted.Danishpopulationregisters show increased prescription of anti-Helicobacter drugs in the 5 years prior to PD diagnosis (6).

A single case of eradicating a corkscrew-shaped non-Helicobacter pylori Helicobacter (NHPH) has been described in a breath-test-positive PD-patient with antral gastritis (7). Biopsy-proven eradication in this cachectic patient, who had been wheelchair-bound for over a year, resulted in a U-turn in brady/hypokinesia, mastering previously neglected computing skills, and normalization of body mass index. Clinical relapse with return of breath-test positivity had occurred 14 weeks after giving a first-line anti-H. pylori regimen, but the lasting improvement followed exhibition of quadruple therapy containing oxytetracycline and bismuth. Indeed, subsequent susceptibilitytestingofH.suisisolatesfromsowssuggestsrelative intrinsic insensitivity to amoxicillin and metronidazole, greater intrinsic susceptibility to tetracycline (8). Repeat biopsy in the PD-patient, 11 months after the quadruple therapy showed almost complete resolution of gastritis, with no organisms (7).

Subsequently, we found the relative-frequency of H. suis to H. pylori, to be 10-times greater in PD, when comparing gastric biopsies from 60 PD-patients with those from 256 routine gastroenterology patients (9) (H. suis frequency was standardized against that of H. pylori to avoid it being construed as true prevalence.) Results of the species-specific ureAB gene-cluster based RT-PCR employed were confirmed by sequencing the amplicon. The PCR detection method was cross-validated against an independent carR-based species-specific RT-PCR (10). Gastric-biopsy histopathology had not detected corkscrew Helicobacter in any of the 60 PD-patients (9). Improvement in hypokinesia following eradication of H. pylori in such "low density" (2, 11) indicates that detection of Helicobacter at PCR-only level is clinically important.

Zoonosis might explain the increased proportional mortality from PD among livestock farmers (but not in arable farmers) compared with all decedents, reported in a study of 26 USA states (12). Systematic review and meta-analysis of agrochemical usage have not resolved this difference between livestock and arable farming (13–15). An infective explanation is credible in a disease where neuroinflammation is an early feature (16) and peptic ulcer prodromal (11): NHPH is a candidate. However, gastric cancer mortality (17) was not increased in livestock farmers (12). Here, we go back to audit mortality and available clinical observations, including blood profile and cardiovascular measures, subsequent to NHPH detection in the 60 PD-patients (9), with a view to hypothesis-generation and future hypothesis-testing.

There may be a substantial reservoir for NHPH zoonosis: historically prevalence estimates in pigs at slaughter age in Europe, North and South America and Asia are around 60% (18–24). We assess the frequency of corkscrew Helicobacter-like organisms in a small sample (111) of UK pigs, using immunohistochemistry, backed-up by 16S rRNA gene amplicon sequencing (10 pigs) and shotgun metagenome sequencing (1 pig). In patients with gastric disease, the prevalence of NHPH, based on histopathology of gastric biopsy, has been estimated at between 0.2 and 6%, depending on geographical distribution. Low infection load, compared to H. pylori (25), militates against detection by [^{13}C]urea-breath-test (UBT) and even histology.

Evidence points to facets of PD having different, not necessarily co-incident, drivers (26). Eliminating one aetiopathogenic marker might allow another to come to the fore. In the PD-patients audited here, frequency of H. suis on molecular microbiology was significantly greater where H. pylori had been eradicated (9). Similarly, the presence of H. pylori and small-intestinal-bacterial-overgrowth (SIBO) are inversely related in PD (27). We, thus, consider pathological associations of H. suis in the context of H. pylori and SIBO.

METHODS

Audit Plan

Table 1 details the audit plan. It accesses routinely available data from patients with "clinically-definite" idiopathic parkinsonism (PD) (31), diagnosed at a clinic incorporating specialist gastrointestinal expertise. Sixty PD-patients were eligible on the basis that archived microbial DNA-extracts from their gastric biopsies had been included in a service re-evaluation of NHPH detection (with Gastrointestinal Reference Unit, Public Health England) (9). Helicobacter suis had been targeted since it was the most frequently reported NHPH in humans and a species-specific assay (ureA-based species-specific qPCR, validated by amplicon sequencing) was available (9).

Patients Audited

These were all of the PD-patients attending our National outpatientclinic,whohadundergonegastroduodenalendoscopy over the decade before the service evaluation (9). The Gastrointestinal Reference Unit held archived microbial DNA-extract from all. **Table 2** gives status for H. pylori and H. suis, based on first archived DNA-extracts. H. suis was present in 16 of the 60 PD-patients, H. pylori in 17, both species in 3. Twenty PD-patients had follow-up microbial DNA extracts from gastric biopsy in the archive. H. pylori was not detected at a follow-up biopsy in any of the 17 initially positive patients. However, 8/20 follow-up DNA-extracts were H. suis-positive, 5 remaining positive (4 despite an H. pylori eradication regimen) and 3 becoming positive de novo (after such a regimen). Thus, H. suis was detected in 19/60 patients on at least one occasion.

TABLE1 | Audittopicsandinformationrequired.

Topic	Informationrequired
Demographic	Age,gender,height,weight,time-since-diagnosis, anti-parkinsonianmedication.
Survival	Datesofanydeathsandcauseshownondeath certificate,ascertainedfromrelatives.
Cognitivefunction	Mini-mentalstateexaminationscores(28),with cut-pointof<27/30forcognitiveimpairment*.
Depression	Beck'sHopelessnessscalescore(29),withcut-points formild(4□8/20),moderate(9□14)andsevere(>14) depression.
Bloodprofiling	Fullbloodcounts,leucocytesubsetcounts,serum haematinics(ferritin,folate,B12),homocysteine,and gastricautoantibodies.
Orthostaticcardiovascular monitoring	Pulseratesandbloodpressures:lying,standing (immediate,1and3min)andpost-exercise.
Lactulosehydrogen breathtest	Statusaccordingtowhethermetermanufacturer's diagnosticcut□point(20ppmincrement)(30) exceededin2consecutivereadings□.
Nature/extentlifetime exposuretopigsandraw porcineproducts	HistorytakenwhenH.suis-positivityfed-back followingservicere-evaluation□□.

*Nonehadcognitiveimpairmentattimeofbiopsy.
□Testfollowed24hdeprivationofdairy-products/medicinallactulose,andbreakfastofa mugofclearfluid.Breathhydrogenmeasured(Gastrolyser,MicroMedicalLtd.,Rochester, UK)beforeandafter(15minintervalsfor2h)25Glactulose.UsedinclinicasSIBOscreen: if positive, fluid/fiber intake and use of maintenance bulk/osmotic laxatives encouraged. (Usingglucosesubstratewouldriskmissingdistal SIBO,sinceitisabsorbedproximally).
□□Optionofre-biopsyofferedatthattimetothosereportedaspositive.

TABLE2 | FrequencyofH.suisandH.pylori infirstarchivedmicrobial DNA-extractfromgastricbiopsyin60PD-patientswithandwithoutknown exposuretoanti-H.pylori therapy[basedonBleacheretal.(9)].

Frequency	Eradicationcategory	PercentPD-patientswith *Helicobacter* species(no.with species/totalno.incategory)
H.suis	Previousexposureto anti-H.pylori therapy	58(11/19)
	Noknownexposure	12(5*/41)
	Total	27(16/60)
H.pylori**	Previousexposureto anti-H.pylori therapy	0(0/19)
	Noknownexposure	41(17/41)
	Total	28(17/60)

*3DNA-extractsalsopositiveforH.pylori.
**H. pylori status for each period defined by UBT-, culture- or molecular microbiology-positivity.Whereculture-negative,biopsiestestedusingPCRstargeting16SrRNA(primer pair HP1/HP2) and vacA (Vac3624F/Vac3853R) genes. UBT not positive, without co-existingH.pylori,inanyH.suis-positivepatient.

Histopathology and Immunohistochemistry

Histological assessment was performed on 10% formalin-fixed, para□n-embedded samples. Samples were oriented on cellulose acetate strips. Sections, 4 µm-thick, were cut perpendicular to mucosal surface, and stained with haematoxylin-eosin and immunostain with antibody generated against whole H. pylori lysate (VENTANA anti-Helicobacter pylori SP48 Rabbit Monoclonal Primary Antibody, Roche), using an automated slide-stainer (BenchMark XT, Roche). Slides were examined by light microscope at magnification ×4 to ×100. Preliminary testing showed that both the comma-shaped H. pylori-like and corkscrew organisms took up the immunostain.

Since human infection with NHPH is described as characteristicallysparseandpatchy(25),previoushistopathology was subjected to service re-evaluation. Further sections were taken from archived wax-embedded initial or follow-up gastric biopsies from (i) the 6 patients with the highest density of H. suis DNA found (2 of whom were H. pylori culture-positive); (ii) 2 H. pylori culture-positive, but H. suis PCR-negative, patients, and (iii) 2 without evidence of Helicobacter (on UBT, histopathology, culture, or PCR).

One hundred and one pig stomachs (from 8 di□erent UK farms) were sourced from an abattoir, immediately after slaughter, over a 2 month period, when both the team and pig stomachs available. On the first day, 10 stomachs were placed individually in sealed plastic bags. On the 2 subsequent days, stomachs were grouped by sealed bag according to farm. Ten old sow stomachs were sourced from the same abattoir on a separate occasion. Stomachs were opened, one at a time, along the major curvature. Contents were carefully removed manually, to retain as much mucus as possible. For the first 10 stomachs, a strip of mucosa, ⅓ 3 cm wide, was dissected o□ the underlying muscleineachparsoesophogeal,cardia,fundusandantrum,and mounted in sequence on a cellulose acetate strip (Sartorius AG, Goettingen, Germany) using a sterile needle. Immunostaining results were used to determine the optimal sampling site for the subsequent 100 stomachs. Loaded acetate strips were all fixed in formalinwithin4hofslaughter.Anyenlargedlymphnodes,lying along the lesser curvature of the first 10 stomachs, were dissected out and fixed.

Molecular Microbiology

Caecal-content samples were collected in sterile containers from the first 10 pigs, to ascertain Helicobacter status, and, where gastric load of corkscrew organisms high, H. suis status (see **Appendix**) (32, 33).

Statistical Analysis

Cox proportional-hazards models were used to assess which factors were associated with all-cause mortality. As with any mortality study, age and gender are a priori candidates for cofounding, and thus were included with no regard as to their statistical significance. Due to the limited number of events (deaths), apart from H. pylori infection and SIBO, which were deemed relevant to the aetiopathogenesis, no other covariates were considered because of the likelihood of over fitting. The proportional-hazard assumption was tested using an hypothesis-testing approach (34), as implemented in Stata 13s estat, phtest command. For all models fitted, the global test of the proportional-hazard assumption was not significant indication thatthisassumptionwasreasonable.Timeofentryintothestudy was the date of presentation to clinic, when date of diagnosis of PD was noted if previous. Time of exit was date of death, or, if still alive, date of analysis. H. suis □status□ (as evaluated

September2013)wasconsideredasafixedcovariateregardlessof date: a positive result being defined as molecular microbiology-positivity in any gastric biopsy from an individual. H. suis was considered as a fixed covariate regardless of date, because, at the time of this audit, non-invasive screening methodology to aid definition of presence/absence was not available. In contrast, H. pylori and SIBO status were each considered as time-varying covariates (35), constant within a specified period: (i) prior to any H. pylori eradication course; (ii) after that course and until any subsequent course; (iii) after any second course. Whilst all three are chronic, there are clear guidelines for establishing H. pylori and SIBO status (**Tables 1**, **2**). Adjustment was made for the potential confounding variables, age at the beginning of each period, and gender.

Independent associations of the two Helicobacter species and SIBO with blood profiling and cardiovascular measures were assessed in mixed e□ects linear regression models, with blood or cardiovascular measures as continuous outcome variables, and adjustment for age and gender. Where necessary, log transformation was used to obtain an approximately symmetric distribution. A random intercept for each patient was included, toaccountforserialclinicalmeasurements.H.suisstatusand,for each period, H. pylori and SIBO status were fitted as fixed e□ects.

RESULTS

Table 3 defines the PD-patients in whom association of H. suis withmortalityandmorbidityisstudied.Atthetimeofanalysis,it wasnotdeterminedwhetherone(H.suis-negative)oftheoriginal 60 patients was alive.

Mortality Associated With *H. suis*

Age of decedents at diagnosis of PD had a mean of 59 (data interval 43, 76) years, that of survivors being similar [58 (41, 76) years]. The proportions of males were also similar: 3/8 and 30/51, respectively. The H. suis-positive had a mean age at PD diagnosis of 57 (38, 76) years, the H. suis-negative 58 (41, 75) years,correspondingproportionsofmalesbeing13/19and20/40.

Crude all-cause mortality was greater in those with H. suis (log-rank test, p = 0.006), with 6/19 of those positive at any time during follow-up having died, compared with only 2/40 of the rest. No deaths were attributed to gastric malignancy or peptic ulceration. Causes were other malignancy (3), infection (4, with dementia in 1), and dementia (1). **Table 4** shows that the estimated hazard ratio of death during follow-up for the H. suis-positive was 12.1 [95% confidence interval (CI) 1.4, 103.1] (Cox proportional-hazards model, adjusted for age-at-diagnosis and gender: likelihood ratio test, p ≤ 0.005, neither age or gender reaching statistical significance at 0.05 level). UBT-values did not influence hazard.

Figure 1 plots the Kaplan□Meier estimate of the survival functionforthetwoconditions,H.suis-positivityand-negativity. The relatively short time-scale over which the deaths in the H. suis-positive occurred is striking. The first death in the H. suis-positive group was 3½ years after diagnosis of PD, the subsequent 5 losses occurring by 12 years, that is, on average, at 21 monthly intervals. In contrast, the first death in the H. suis-negative was not until 10½ years after diagnosis, the second at 14½ years.

TABLE3 | Characteristicsatstartofauditperiodorearliestrecordedafter.

Characteristic		Mean(95%data interval)*(*n*=59)**
Demographic	Age(years)	63(47,80)
	Gender(male/female)	33/26[a]
	Height(m)	1.69(1.52,1.86)
	Weight(kg)	77(51,102)
	Time-since-diagnosis(years)	4(0,16)[b]
	Anti-parkinsonianmedication*** (no/yes)	18/41[a]
	Medicationincludeslevodopa (no/yes)	27/14[a]
Bloodcellcounts	Hemoglobin(g/L)	138(116,160)
	Redcellcount(10^{12}/L)	4.6(3.8,5.3)
	Totalwhitecellcount(10^9/L)	6.3(3.8,12.0)[b]
	Neutrophils(10^9/L)	3.9(1.5,8.0)[b]
	Lymphocytes(10^9/L)	1.7(0.7,2.8)
	Plateletcount(10^9/L)	236(154,436)[b]
	Meanplateletvolume(fL)	10.2(7.4,13.0)
Haematinics& homocysteine	Serumferritin(μg/L)	93(7.4,374)[b]
	Serumfolate(μg/L)	408(131,985)[b]
	SerumB12(ng/L)	443(104,1030)
	Serumhomocysteine(μmol/L)	14.4(7.4,32.5)[b]
Gastricautoantibodies	Anti-intrinsicfactor(□ve/+ve)	41/17[a]
	Anti-parietalcell(□ve/+ve)	45/13[a]
Meanarterialpressure	Lying(mmHg)	94.4(65.9,112.8)
	Immediatestanding	95.3(67.8,122.8)
	1min	96.9(68.3,125.4)
	3min	96.6(68.5,125.3)
	Post-exercise	101.6(69.5,133.6)
Pulse	Lying	67.2(48.3,86.0)
	Immediatestanding(/min)	76.8(56.4,97.1)
	1min	77.4(55.8,99.0)
	3min	75.9(53.9,97.8)
	Post-exercise	87.7(64.2,111.3)
SIBO	Hydrogenbreath-teststatus (□ve/+ve)****	22/29[a]

*Exceptwheredenotedbysuperscriptletter:[a]Counts;[b]Centiles:50(2.5,97.5)th.
**n=48forautoantibodies,51forSIBO.
***Anti-parkinsonian medication used: amantadine, cabergoline, levodopa-combination, pramipexole, selegiline, rasagiline, trihexyphenidyl (low dose). Levodopa was combined with extracerebral dopa-decarboxylase inhibitor ± catechol-O-methyl transferase inhibitor,asevenlyspacedaspracticabletoavoidiatrogenicfluctuationsinperformance. Nonewerereceivingalevodopacombinationasmonotherapy.
****Fortytwopatientshydrogen-breath-testpositiveatsomepointduringauditperiod.

TABLE4 | EstimatedhazardratiosforCoxproportional-hazardsmodel.

Characteristic	Hazardratio(95%C.I.)	*p-value*
H.suis(positivity)	12.1(1.4,103.1)	0.02
Gender(female)	0.3(0.1,1.3)	0.1
Ageatdiagnosis(peryear)	1.04(0.95,1.15)	0.4

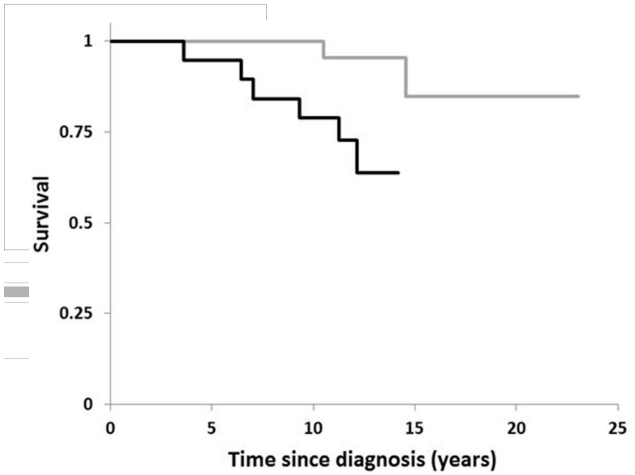

FIGURE1 | Comparisonof survivalinPDbyHelicobactersuisstatus. Kaplan-Meierplotfor19PD-patientswithH.suisdetectedinDNAextract fromgastricbiopsy(blackline)comparedwiththatforthe40inwhombiopsy wasnegativeforH.suis(grayline).

H. pylori-positivity was not associated with mortality. Any association with SIBO-positivity could not be assessed since 5/8 deaths had occurred before hydrogen breath testing was used routinely in clinic.

Cognition and Affect in Relation to *H. suis* Status

Fourteen of the 59 patients traced developed cognitive impairment (fall in mini-mental score to <27/30) whilst attending clinic or were diagnosed as having dementia after last seen there. The observed number of deaths in those with and without incident cognitive impairment (2 and 6, respectively) was not significantly different from number expected were death and cognitive impairment unrelated (log-rank test, p = 0.8). Cognitive impairment occurred in 8/19 H. suis-positive patientsand6/40H.suis-negative:oddsforbeingH.suis-positive tended to be higher [2.8 (95% CI 0.8, 9.5), p = 0.1] in those who developed cognitive impairment.

The majority of the PD-patients (82%) were classified as having at least mild depression (≥4/20) at some time during

TABLE5 | IndependentassociationsoftwoHelicobacter speciesandSIBOwith(A)totalwhitecellandsubsetcountsand(B)cardiovascularmeasures.

A

Cellcount	*H.pylori*-positivity(*n*=16/60)		*H.suis*-positivity(*n*=17/60)		LHBT-positivity(*n*=29/51)	
	Sizeeffect Mean(95%CI)%	*p*-value	Sizeeffect Mean(95%CI)%	*p*-value	Sizeeffect Mean(95%CI)%	*p*-value
Whitecellcount	2(−4,9)	0.5	6(−7,22)	0.4	−16(0,−30)	0.05
Neutrophil	6(−4,16)	0.3	4(−14,25)	0.7	−23(−1,−40)	0.04
Lymphocyte	−8(−1,−14)	0.02	12(−4,32)	0.1	−8(−26,13)	0.4
T-cell(CD3+)	0(−10,10)	0.9	24(2,52)	0.04	−8(−30,22)	0.6
CD4+T-cell	−3(−12,8)	0.6	20(−1,46)	0.06	9(−16,43)	0.5
CD8+T-cell	−1(−11,11)	0.9	38(2,87)	0.04	−26(−51,11)	0.2
B-cell(CD19+)	−3(−19,16)	0.7	21(−10,61)	0.2	6(−30,59)	0.8
Natural-killer(CD16+CD56+)	−4(−17,12)	0.6	−12(−33,16)	0.4	40(−4,106)	0.08

B

Measurement	*H.pylori*-positivity		*H.suis*-positivity		LHBTpositivity	
	Sizeeffect Mean(95%CI)	*p*-value	Sizeeffect Mean(95%CI)	*p*-value	Sizeeffect Mean(95%CI)	*p*-value
Meanarterialpressure(mmHg)						
Lying	0(−2,2)	0.9	−4(−9,1)	0.08	3(−5,10)	0.5
Standingimmediate	−1(−3,1)	0.5	−6(−11,−1)	0.03	4(−4,11)	0.4
1min	0(−2,2)	0.8	−6(−11,−1)	0.03	2(−6,10)	0.7
3min	0(−2,2)	0.8	−6(−11,−1)	0.02	2(−6,10)	0.6
Post-exercise	1(−3,2)	0.7	−6(−12,0)	0.07	4(−5,14)	0.4
Pulse(/min)						
Lying	0(−1,2)	0.8	1(−4,5)	0.8	−7(−13,−1)	0.03
Standingimmediate	0(−2,2)	0.9	0(−5,5)	0.99	−9(−16,−3)	0.003
1min	1(−2,3)	0.5	0(−5,5)	0.9	−9(−16,−2)	0.008
3min	1(−1,3)	0.5	0(−5,5)	0.9	−7(−14,−1)	0.03
Post-exercise	0(−3,2)	0.8	0(−7,6)	0.9	0(−9,8)	0.9

follow-up. There was no evidence of association between mortalityanddepressionscore[estimatedhazardratio0.95(0.83, 1.10), adjusted for age, gender and H. suis, p = 0.5]. The odds of being H. suis-positive was weakly (p = 0.1) related to the worst depressionscorerecordedperindividual,4/5patientswithsevere depression (score >14/20) being H. suis-positive (p = 0.05).

Differential Effect of *H. suis* Status on Blood Profile

Table 5A illustrates the differential effect of H. suis on blood indices. H. suis-positivity was associated with a markedly higher (by 24%) T-cell count, through an effect on both CD4+ and CD8+ subsets. This contrasts with a lower lymphocyte count with H. pylori (8%), and a lower neutrophil count (23%) but numerically much higher natural killer cell count (40%) with SIBO.

Mean hemoglobin concentration and red cell and platelet counts were not associated with Helicobacter species or SIBO status. Mean platelet volume (10.2 fL) was toward the upper end of the reference range, larger with H. pylori-positivity [by 0.56 (0.07, 1.04) fL, p = 0.02]. Serum folate was lower [32 (15, 45)%, p = 0.001] with H. pylori-positivity. No other association with haematinics or with serum homocysteine was identified. Helicobacter-positivity, in general, was associated with anti-intrinsic factor gastric autoantibody, both H. pylori- and H. suis-positivity contributing to the effect [odds ratio 11 (1, 144) & 8 (1, 91), p = 0.06 & 0.09, respectively], only H. suis-positivity tending to be associated with parietal cell antibody [odds ratio 5 (1, 36), p = 0.1].

TABLE6 | PresenceanddensityofHelicobacter onimmunostainingin111UK pigsatslaughter,(A)bygastricregionandinanyenlargedlymph-nodesininitial pigs(1–10),and(B)inantraofpigs(1–101),andadultsows(102–111).

A

Pigno.	Anatomicalsite				
	Para-oeshageal	Cardia	Fundus	Antrum	Lymph-node
1	0	0	0	0	n/a
2	0	++	a	+++	n/a
3	0	+	0	++	0
4	0	+	a	++	0
5	0	a	a	+++	n/a
6	0	a	0	a	n/a
7	0	a	0	+++	n/a
8	0	0	0	0	0
9	0	+	++	a	0
10	0	+	+	+++	0

B

Pigno.	No.pigswith*Helicobacter*-likeorganismineach densitycategory					Totalno.
	0	a	+	++	+++	
1–10	2	2	0	2	4	10
11–101	38	12	15	14	12	91
102–111	3	2	1	2	2	10
Total	43	16	16	18	18	111

0 = no Helicobacter; + = individual organisms, or small groups, covering <1/3 mucosal surface;+++=largegroupsoforganismsonsurfaceandupperpitsof>2/3mucosal surface;++=intermediatedensity(36,37).

[a]Positively stained "cytoplasmic dust" within follicles only, n/a, no enlarged lymph-nodeseen.

Differential Effect of *H. suis* Status on Cardiovascular Measures

Table 5B illustrates differential effect of H. suis on mean arterial pressure and pulse rate. With H. suis-positivity, only, mean arterial pressure was lower, and this by a clinically-relevant amount (6 mmHg), for all standing measurement times. Lying and post-exercise values were numerically lower by a similar amount. With SIBO, only, lying and standing pulse rates were lower (8 beats/min), an effect lost on exercise.

Previous Exposure to Pigs and Porcine Products in the *H. suis* Positive

Information on exposure to potential sources of H. suis was available in 11 of the 19 H. suis-positive patients. Of the five with exposure, two had daily contact with pigs (whilst growing up on farms), two had occasional contact (maximum once every month, during childhood), and one had worked in a butcher's shop as a youth. Otherwise, there was no recollection of contact with pig(s), handling raw pig products (other than in a domestic setting) or eating raw pig products.

Histopathology and Immunohistochemistry in Patients

Presence of Helicobacter was confirmed in immuno-stained sections from H. pylori positive "control" patients, absence confirmedinnegativecontrols.NoHelicobacterstainingwasseen in sections from 4 of the 6 patients with the highest abundance of H. suis DNA. However, in the 2 others, who had evidence of both Helicobacter species, irregularly-shaped immuno-stained Helicobacter-like organisms (HLOs) were attached to the mucosal surface. In one of these, most HLOs were thicker than usual for H. pylori and tended to bank-up in irregular bundles, the rest being of typical H. pylori morphology. In the other, the HLOs also tended to bank-up, but were smaller, more homogenous.

Histopathology, Immunohistochemistry and Molecular Microbiology in Pigs

Table 6A shows detection and density of immuno-stained corkscrew organisms, by gastric region sampled, in the 10 initial pig stomachs. Antrum was evidently the optimal sampling site. Enlarged lymph-nodes, found along the lesser curvature of stomach in 5 pigs, were immuno-stain-negative, despite 4 of them having obvious gastric Helicobacter. **Table 6B** compares frequency of detection and density of HLOs in antra from the 91 subsequent pigs (number 11–101) and from 10 adult sows (102–111). Immunostained corkscrew

organisms were seen in 47% of pig antra. There was no difference in detection frequency between pigs and adult sows.

In the 101 subsequent pigs, chronic inflammation was ubiquitous (mild 34%, moderate 61%, severe 5%) and more severe in presence of HLOs. Active inflammation was associated with eosinophils in 99%, with neutrophils in only 10%. Neutrophil infiltration was associated with cryptitis (all but one case), but not with HLOs. Lymphoid aggregates were found in 41% (low density in 95%, medium in rest). Lymphoid follicles were found in 73%, in medium or high density in two-thirds, and more evident in presence of HLOs. Erosive changes were present in two cases, both without HLOs. Mucosal morphology was confirmed as antral in type in 88 of the 91 subsequent pigs, being body-type in one, mixed body/antral in 2.

16S rRNA gene community profiles indicated that Helicobacter-specific sequences were detected in 6 of the 10 caecal-content samples. Metagenomic sequencing of caecal-content from a pig (no. 2), with pangastritis associated with corkscrew organisms and severe antral HLO colonization, confirmed the presence of H. suis (raw data: http://www.ebi.ac.uk/ena/data/view/PRJEB25966). Seven contigs representing 0.13% of the estimated H. suis genome were obtained following de novo assembly. When dataset reads were mapped against an available draft assembly (ADGY01), over 4,000 reads, covering 25% of the genome, mapped.

DISCUSSION

The much greater relative frequency, H. suis to H. pylori, in gastric biopsies from PD patients (38), than in biopsies from routine gastroenterology patients, might be an incidental finding oflittledirectaetio-pathogenicconsequencetoPD.ExcessH.suis couldbeamarkeroftheimmunedysfunctioninPD(38).Itcould also represent expansion of previously hidden infection into a niche left by H. pylori eradication (9). Indeed, we report 3 further PD-patients becoming H. suis-positive following exposure to H. pylori eradication therapy: thus three-quarters of those with H. suis had undergone H. pylori eradication. Contrary to this, the 12-fold increase in all-cause mortality associated with H. suis-positivity suggests it is a malign influence in PD, or a surrogate for one. The sample size is small, yet the magnitude of the effect warrants further investigation, including (as with any observational finding) independent replication. In contrast, H. pylori, although associated with increase in deaths from gastric cancer, is not associated with increased all-cause mortality in the general population (17) or (current audit) in PD. However, there is no information on impact of untreated H. pylori infection on all-cause mortality in PD.

Our survey of 111 pig stomachs confirms that there is a porcine reservoir for gastric corkscrew organisms in the UK, in line with studies in other countries. Immunostaining on all biopsies, backed-up by 16S rRNA profiling on caecal content from a sample of pigs, confirmed presence of Helicobacter. Metagenomic sequencing on caecal content from one pig confirmed presence of H. suis.

NHPH infection in humans is well documented (25, 39–42) with animal contact and rural living as risk factors (43–45). Moreover, human NHPH strains have been linked directly, by molecular methods, to infected companion animals (46). Analagously, H. suis provides a potential zoonotic candidate to explain the increased PD-specific proportionate mortality ratio in livestock farmers compared with non-farmers (12). In the 6 million decedents studied over a decade (1984–1993) in 26 US states, farmers were predominantly white male (42,857 farming livestock, 191,308 crops), and PD-specific mortality was 19% higher in these white male livestock farmers, 14% lower in crop farmers. In our study, less than half of the H. suis-positive PD-patients questioned had direct exposure to pigs or raw porcine products. No study has directly compared PD prevalence according to life-time abstinence, or not, from pork/exposure to pigs. Although the prevalence of PD is low in Arab populations of Israel, Tunisia, Lybia, and Saudi Arabia, it is high in specific rural Israeli Jewish populations (47): potential confounders include ascertainment, smoking, and genetics. Using the converse comparator, those engaged in or retired from an occupational group exposed to pigs and/or raw porcine products, is a practical option. There may, of course, be human-adapted NHPH strains, transmitted (as with most H. pylori infections) by close human contact in childhood and persisting. Indeed, the atypical HLO morphology seen here in two patients, who tested positive for both H. pylori and H. suis, might represent human adaptation.

It is well recognized that NHPH, as well as H. pylori, are associated with gastric MALT (a non-Hodgkin) lymphoma, and their eradication with its cure (48, 49). Were H. suis causally linked with PD, populations exposed to it might also be at risk of MALT lymphoma. Despite proportionate mortality from malignant neoplasms being less in farmers overall, white male livestock farmers, unlike crop farmers, had greater than expected mortality from non-Hodgkin lymphoma (17%), and acute lymphoid (63%) and myeloid (19%) leukaemias (12). The highest risk of deaths from non-Hodgkin lymphoma or leukemia was in the North Central US, top ranking states in cattle and pig farming, as compared with Northeast, South and West (50). In Canada, incident cases of non-Hodgkin lymphoma in male farm residents (total 1,262 studied) were increased in those exposed to pigs (when ≥13 head), but not to cattle (51), whereasinFinnishfarmersincidentcases(750)wereincreasedin neither group (52). No information is given about PD and non-Hodgkin lymphoma as comorbidities in the US livestock farmers (12). Lymphoma had not been implicated in any of the deaths reported here.

Clinical and laboratory observations, in line with all-cause mortality, revealed a profile for H. suis distinctive from that for H. pylori. Helicobacter suis was associated with lower blood pressure, whereas, outside PD, H. pylori has been linked weakly (if at all) to hypertension (53). Although orthostatic hypotension is a major problem in PD, we do not suggest that it is directly causal of the excess mortality associated with H. suis. However, it might be a biomarker of the causal pathway. Small intestinal bacterial overgrowth, common in PD from presentation (27) was associated here with a lower pulse rate. Low blood pressure and pulse are in keeping with PD dysautonomia (54).

Whilst confirming that H. pylori is associated with lymphopenia in PD (38), we associate H. suis with a higher circulating T-cell count: there appears to be a differential homeostatic redistribution of T-cells. Compared with H. pylori gastritis, NHPH gastritis in man is relatively mild, with less polymorhonuclear and lymphocytic infiltration. Helicobacter has been proposed as a trigger for autoimmune gastritis, but gastric atrophy, usually mild to moderate, was found in only 17% of PD patients with biopsy-confirmed H. pylori (2). Here, both Helicobacter species were associated with the more specific biomarker of autoimmune gastritis, serum intrinsic factor antibody (55). Regarding our third candidate driver, SIBO, we replicate its association with lower blood neutrophil and higher natural-killer cell counts (27). We previously associated lower neutrophils with tremor, higher natural-killer counts with rigidity and brady/hypokinesia. Indeed, there are biological gradients of these measures of PD facets on the respective leucocyte subset counts. We have also described a platykurtic distribution of serum folate in PD (38). Folate concentrations above reference range may be explained by bacterial production in SIBO (56), whilst lower concentrations are linked here with H. pylori but not H. suis. A lower ascorbic acid in gastric juice and higher pH, consequent on the H. pylori gastritis, might impact on dietary folate absorption (57, 58). Platelet volume was higher with H. pylori, as described in upper gastrointestinal endoscopy patients (59): whether due to occult blood loss or compensated immune thrombocytopenia is unknown. It was not higher with H. suis.

Regarding co-morbidities of PD, H. pylori positivity has been weakly linked to cognitive impairment where prevalence is not high (60–62) and, here, the odds for incident cognitive impairment tended to be higher with H. suis-positivity. Helicobacter pylori eradication has been associated with a decreased risk of progression of dementia (assessed by escalation within a set anti-dementia medication schedule) in a large database study (63) and with lower mortality and improved cognition and function in small open studies (64, 65). Similarly, the severe depression seen here in H. suis-positive PD patients might respond better to anti-depressant schedules were H. suis eradicated.

In summary, this is the first indication that NHPH infection may be associated with increased all-cause mortality in PD, a disease where peptic ulcer is prodromal (11). With respect to morbidity, it appears that H. suis is not entirely a H. pylori look-alike, having different immuno-inflammatory and orthostatic cardiovascular outcomes. Differences may reflect H. suis having jumped from macaques to find a niche in domesticated pigs, thereby providing a source for zoonosis, whereas H. pylori seems as old as anatomically modern humans (66). With better screening tools (such as reliable molecular detection in stool), prospective study of the effect of proven NHPH eradication on facets of PD would become practicable. At present, there is just the one case report on NHPH eradication in PD, but this is encouraging (wheelchair-bound to maintaining independent mobility during 4 years follow-up on stable anti-parkinsonian medication) (7). That PD manifests differently between patients, and the predominance of facets changes within patient over time, suggest an interplay of different drivers and mediators (26). Halting one disease driver may allow another to emerge, as might be the case in the reciprocal relationship between H. pylori and H. suis. A blanket gut microbiome signature approach, where clusters of co-existing organisms are related to presence or absence of a disease, may be too blunt an instrument to unravel the natural history of this slowly evolving chronic disease. Candidature of NHPH in the PD-specific mortality of livestock (but not arable) farmers (12) provides an alternative to the longstanding, but unsubstantiated, hypothesis on agrochemical usage. Moreover, our study suggests that there is a reservoir of human-adapted H. suis, remote in time, space, and person from livestock farming. Zoonosis may prove a vital clue, but the hypothesis generated encompasses human to human transmission.

ETHICS STATEMENT

Thisisanauditofclinicaloutcomeinpatientsandassuchitdoes not require ethics committee approval.

AUTHOR CONTRIBUTIONS

RD, SD, AC, and CW designed the study and wrote and revised the draft manuscript and subsequent manuscripts. AN, RE, AS, IB, and DT participated in design and coordination of the work and helped to draft and revise the manuscript. AA and RT contributed to reviewing the literature. AA performed the necropsies under supervision of AN. RE was responsible for the molecular microbiology. AS was responsible for the histology with immunostaining in humans and pigs. MI was responsible for performed the immunology. All authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could beconstruedasapotentialconflictofinterest.

APPENDIX

Methods

Molecular Microbiology (continued)

Caecal-content DNA was extracted (ExtractMaster Fecal DNA Extraction Kit, Epicentre, Madison, US).

Microbial community composition was assessed by sequencing 16S rRNA gene amplicons. Extracted DNA was amplified with V4 and V5 region universal primers (U515F: 5'-GTGYCAGCMGCCGCGGTA and U927R: 5'-CCCGYCAATTCMTTTRAGT) (39). Fusion primers consisted of the Illumina overhang forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and reverse (5'-GTCTCGTGGGCTCGGAGATGTGTAATAAG AGACAG) adapter. Amplification with FastStart HiFi Polymerase (Roche, Diagnostics Ltd, UK) used these cycling conditions: 95 ˚C for 3min; 25 cycles of 95 ˚C for 30s, 55 ˚C for 35s, 72 ˚C for 1min; followed by 72 ˚C for 8min. Amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). Each sample was then tagged with a unique pair of indices and the sequencing primer, using Nextera XT v2 Index kits, and 2x KAPA HiFi HotStart ReadyMix with these cycling conditions: 95 ˚C for 3min; 12 cycles of 95 ˚C for 30s, 55 ˚C for 30 s, 72 ˚C for 30 s; followed by 72 ˚C for 5 min. Index-tagged amplicons were purified using 0.8 volumes of magnetic beads. Sample concentrations were measured using the fluorescence-based Quantifluor assay (Promega). Concentrations were normalized before pooling samples, each of which would be subsequently identified by its unique index combination. Sequencing was performed on an Illumina MiSeq with2×300base-readsaccordingtoIllumina's(Cambridge,UK) instructions. Sequence reads were processed by the microbiome-helper pipeline (https://github.com/mlangill/microbiome_ helper/). Essentially paired end-reads are merged, based on overlapping ends using PEAR (http://sco.h-its.org/exelixis/web/software/pear/), before data filtering for base-calling quality and amplicon length. Processed sequences are then classified using pick open reference OTUs process implemented in QIIME v1.9.1 (33) against Greengenes 16S rRNA gene database (http://greengenes.secondgenome.com/). For shotgun metagenome sequencing, an aliquot of DNA extract was fragmented and tagged with sequencing primers and indexes using Nextera XT library preparation kit, sequenced on an Illumina NextSeq with 2 x 150 base-reads. Helicobacter-specific reads were filtered from the dataset using kraken (http://ccb.jhu.edu/software/kraken) and subsequently assembled with Spades.