REVIEW ARTICLE
The zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: a systematic review and meta-analyses of the evidence

L. A. WADDELL[1,2]*, A. RAJIĆ[3], K. D. C. STÄRK[4] AND S. A. McEWEN[1]

[1] *University of Guelph, Department of Population Medicine, Guelph, ON, Canada*
[2] *Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, ON, Canada*
[3] *Agriculture and Consumer Protection Department, Food and Agriculture Organization, Rome, Italy*
[4] *Royal Veterinary College, North Mymms, Hertfordshire, UK*

SUMMARY

This systematic review–meta-analysis appraises and summarizes all the available research (128 papers) on the zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*. The latter has been debated for a century due to pathogenic and clinical similarities between Johne's disease in ruminants and Crohn's disease (108 studies) in humans and recently for involvement in other human diseases; human immunodeficiency virus (HIV) infection (2), sarcoidosis (3), diabetes mellitus type 1 (T1DM) (7) and type 2 (3), multiple sclerosis (5) and Hashimoto's thyroiditis (2). Meta-analytical results indicated a significant positive association, consistently across different laboratory methods for Crohn's disease [odds ratio (OR) range 4·26–8·44], T1DM (OR range 2·91–9·95) and multiple sclerosis (OR range 6·5–7·99). The latter two and the thyroiditis hypothesis require further investigation to confirm the association. Meta-regression of Crohn's disease studies using DNA detection methods indicated that choice of primers and sampling frame (e.g. general population *vs.* hospital-based sample) explained a significant proportion of heterogeneity. Other epidemiological studies demonstrated a lack of association between high-risk occupations and development of Crohn's disease. Due to knowledge gaps in understanding the role of *M. paratuberculosis* in the development or progression of human disease, the evidence at present is not strong enough to inform the potential public health impact of *M. paratuberculosis* exposure.

Key words: Crohn's disease (CD), diabetes, Johne's disease, meta-analysis, multiple sclerosis *Mycobacterium avium* ssp. *paratuberculosis*, paratuberculosis, systematic review, thyroiditis.

INTRODUCTION

The zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis* has been debated for a century since it was first suggested that Crohn's disease (CD) is pathologically and clinically analogous to Johne's disease [1–3]. Research on this association has increased over the last 30 years, from the first isolation of *M. paratuberculosis* in a CD patient by Chiodini *et al.* and the subsequently published review of evidence supporting the potential association in 1989 [4, 5]. A detailed history of this research can be found in a number of papers and books on this topic [1, 6]. More recently *M. paratuberculosis* has been hypothesized to play a role in the development or progression of a number of other human diseases including HIV infection, sarcoidosis, diabetes mellitus

* Author for correspondence: Ms. L. A. Waddell, Public Health Agency of Canada, 160 Research Lane, Unit 206, Guelph, Ontario N1G 5B2, Canada.
(Email: lisa_waddell@phac-aspc.gc.ca)

type 1 (T1DM) and type 2 (T2DM), multiple sclerosis and Hashimoto's thyroiditis mainly on the basis of immune dysfunction hypotheses [7–11].

These chronic diseases have no known cure and together they affect several hundred million people globally. CD is a chronic debilitating idiopathic gastroenteritis that affects 1–2 million people worldwide [6, 12]. Similarly diabetes mellitus (T1DM, an autoimmune condition where insulin is not produced by the pancreas and T2DM, a condition where cells do not respond to normal levels of insulin) affects 382 million people worldwide [13]. Multiple sclerosis is a complex autoimmune disease of the central nervous system that affects an estimated 1·3 million people worldwide [8, 14]. Hashimoto's thyroiditis (chronic lymphocytic thyroiditis) is an autoimmune disease in which the thyroid gland is attacked by a variety of cell- and antibody-mediated immune processes and affects 7·2–36 million people worldwide [15]. Sarcoidosis is an unexplained abnormal collection of inflammatory cells in organs that usually resolves without medical intervention except in rare cases where it can become a chronic condition; it is estimated to affect 0·07–2·9 million people worldwide [16].

Many of the hypotheses on the potential zoonotic role of *M. paratuberculosis* draw on pathological and clinical similarities (analogy) between Johne's disease in ruminants and CD in humans, and *M. paratuberculosis*' ability to cause natural infection in non-ruminants, e.g. dogs, rabbits, non-human primates [1, 17]. The evidence for *M. paratuberculosis*' role in human disease to date points to multiple interacting factors such as dysregulation of the immune system (e.g. genetic predisposition, immune-mediated tissue injury) and environmental exposures, such as diet and exposure to pathogens or changes in the normal gut microflora that when combined may cause the disease [1, 6]. Molecular mimicry has also been suggested to have a role in the pathogenesis of CD, T1DM and multiple sclerosis, proposing that an infectious agent like *M. paratuberculosis* has antigenic elements similar to the host that can induce autoimmunity [8].

This systematic review (SR) tabulates the global evidence and presents meta-analytical summaries of the current knowledge underpinning the public health question, 'Is *M. paratuberculosis* zoonotic?' [18, 19]. This SR updates a previous SR covering primary research until the end of 2005 [20] and evaluates agreement or changes in research knowledge published over the last 9 years [6, 21–23]. The scope of this SR is extended beyond potential association between

M. paratuberculosis and CD, and includes all human diseases that have been linked with *M. paratuberculosis*. To the best of our knowledge no other SR has evaluated evidence on *M. paratuberculosis* as an infectious disease candidate for multiple human diseases.

The objectives of this SR/meta-analysis (MA) are: (1) to identify and characterize the research investigating an association between human diseases and *M. paratuberculosis*, (2) to estimate the strength of the association(s) and agreement across epidemiological studies using various detection methods and, (3) evaluate strengths, weaknesses, and gaps in current evidence. Knowledge gaps and future research priorities to address public health questions are highlighted in the Discussion.

METHODS

Protocol

An *a-priori* developed and pre-tested SR protocol (Supplementary material: Appendix 1) included the study question, sub-questions, definitions, procedure for literature search, study inclusion/exclusion criteria and checklists for conducting relevance screening, basic characterization, methodological assessment and data extraction on relevant primary research, following the general principles of SR methodology [18, 24, 25]. All primary research investigating the zoonotic potential of *M. paratuberculosis* and human disease, and published in English or French languages was analysed. This included case-control studies investigating associations between *M. paratuberculosis* and human disease, studies looking at the association between Johne's disease in ruminants and human disease and molecular epidemiology studies examining the relatedness of human and animal isolates of *M. paratuberculosis*. Relevant expertise within our SR research team included *M. paratuberculosis*, food safety, risk assessment, epidemiology, library, and synthesis research (e.g. SR and MA) methods.

Search strategy

The original search strategy by Waddell *et al.* [20] was simplified and updated based on recent improvements in electronic bibliographic databases and the number of databases was reduced based on the results of unpublished research on search sensitivity and specificity [26]. The following search algorithms were implemented on 27 September 2013 in three databases, Pubmed, Scopus and Current Contents:
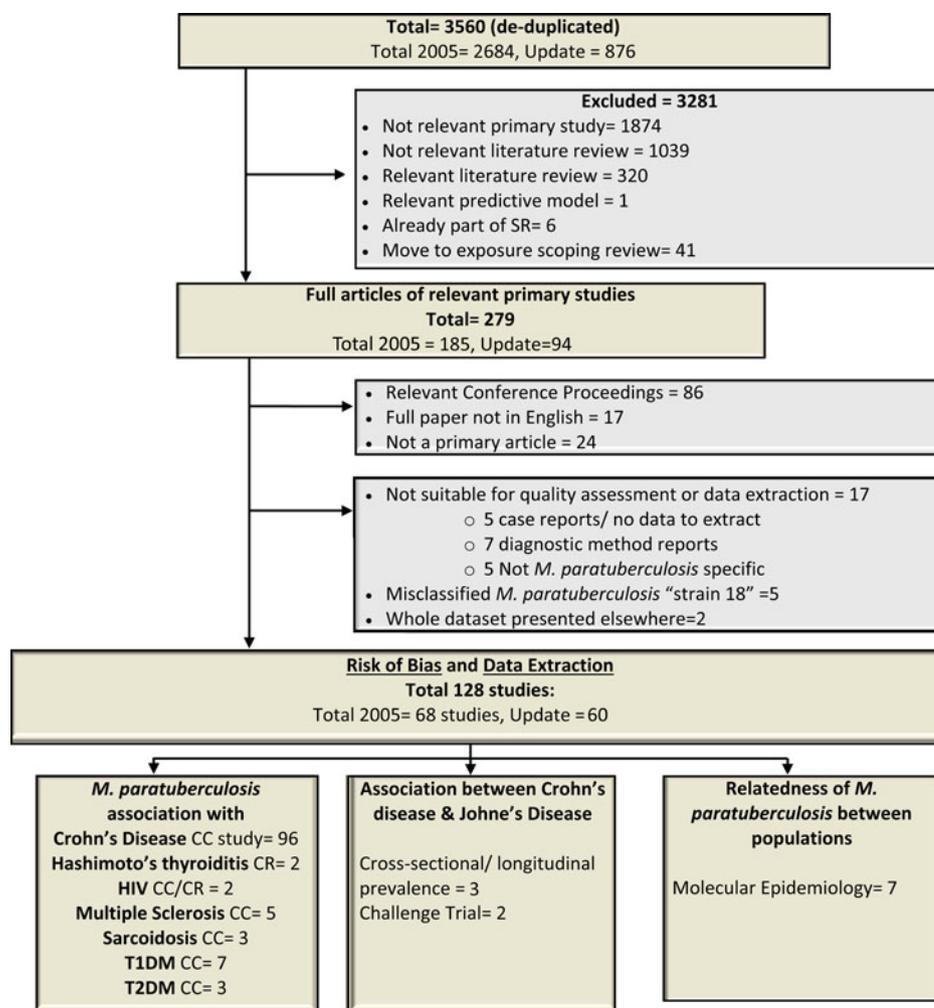
Fig. 1. Flow chart describing the movement of 3560 citations, the exclusion of 3432 and the inclusion of 128 studies through the systematic review process. HIV, Human immunodeficiency virus; T1DM/T2DM, diabetes mellitus type 1/type 2; CC, case-control study; CR case report or case series.

((johne disease) OR (johne's disease) OR (johne* disease) OR paratuberculosis OR (mycobacterium paratuberculosis) OR (mycobacteria AND crohn*)) AND ((crohn* disease) OR (crohn disease) OR (crohn's disease) OR (inflammatory bowel disease) OR (inflammatory bowel diseases) OR diabetes OR sarcoidosis OR lymphadenitis OR immunocompromis* OR (AIDS) OR (Blau syndrome) OR (multiple sclerosis) OR thyroiditis)

For research on CD, publication date was set from 1 January 2005 onward without any other restrictions or filters as this aspect was already investigated in the previous SR covering research to 2005 [20]. For all other diseases we did not impose any restrictions. Citations retrieved from all databases were imported into reference management software Procite v. 5.0.3 (ISI Researchsoft, 1995) and de-duplicated. Search verification was twofold; first, reference lists of review articles [6, 27–31] and three SRs published on *M. paratuberculosis* [21–23] were searched by hand. Second we identified all citations relevant to this SR during relevance screening for a scoping review on sources of *M. paratuberculosis* being conducted at the same time to identify any potentially missed citations. Any potentially relevant unique citation identified by search verification was added to the SR at the relevance screening stage and processed through all SR tools as appropriate.

Abstract and article-level relevance screening and study characterization

Through initial abstract-based screening (Fig. 1) by two reviewers working independently, all potentially relevant primary research in English or French investigating the zoonotic potential of *M. paratuberculosis*

was identified. Non-primary research studies (e.g. narrative reviews) or primary research studies outside of the scope were intentionally excluded. All citations deemed relevant at the abstract-based screening level were procured as full articles. Abstract screening was conducted by two independent reviewers using standardized and pre-tested checklists. Reviewer agreement ($\kappa \geqslant 0.8$) was evaluated using 30 abstracts. Conflicts were resolved through consensus between respective reviewers and if this was not possible, by a third team member.

Risk of bias, GRADE and data extraction

Full paper assessment using a direct modification of the risk of bias and Grading of Recommendations Assessment, Development and Evaluation (GRADE) criteria endorsed by the Cochrane collaboration was undertaken on each study and GRADE criteria were summarized for groups of studies contributing to each MA [32–35]. The risk-of-bias assessment aims to assess the internal validity of the study which informs of the GRADE criteria [33, 34]. GRADE criteria are summarized across groups of similar studies to indicate the level of confidence in the current evidence [35]. The 1- to 4-star grading system indicates: high confidence that the effect estimate is close to the true effect (****); moderate confidence in the effect estimate, but future studies may be substantially different (***); limited confidence in the estimate of effect, the true effect may be substantially different (**); and very little confidence in the estimate of effect, the true effect is likely to be substantially different (*) [36–38]. The data extraction tool (Supplementary material, Appendix 1) aimed to efficiently capture the pertinent information and results needed for MA from each study. Both steps were conducted by two reviewers working independently and conflicts were resolved by consensus.

Study management and data analysis

All SR stages, i.e. relevance screening, risk-of-bias assessment and data extraction, were conducted in Microsoft Excel 2010 (Microsoft Corp., USA). The data were analysed descriptively [Stata v. 13 (StataCorp., USA)], followed by random-effects MA of selected subgroups of data in Comprehensive Meta-analysis Software v. 2 (Biostat, USA), which employs the method of moments weighting procedure [39]. Heterogeneity, or across study variation, was quantified by Higgins' I^2 [40]. Meta-regression was conducted

in Stata v. 13 using the random-effects maximum likelihood (REML) weighting procedure and changes in T^2, an estimate of τ^2 or true standard variance across studies was used to quantify the amount of heterogeneity that was explained by covariates in the model [41]. Where appropriate, homogenous MAs with more than 10 lines of data were evaluated for publication bias by Begg's and Egger's [42, 43] tests. If publication bias was detected, Duval & Tweedie's trim-and-fill method was used to estimate the potential impact on the estimate and conclusions of the MA [44]. Results with high heterogeneity are still reported in this review as we believe that they are more informative than reporting a range of results. However, we caution readers to use heterogeneous results carefully as the source of heterogeneity is unknown and future research may significantly change the results of the MA.

RESULTS

Description of relevant studies

The updated electronic search yielded 876 citations after de-duplication and Figure 1 shows the progression of papers through the SR process. A total of 128 articles, 60 added in this update 2005–2013, are included and summarized in this SR (Supplementary Appendix 2, list of included studies). All studies were published in English and originated from the USA/Canada (30%), Europe (52·6%), Asia (9·8%), Australia and New Zealand (6·0%) and the Middle East (0·8%). The top three publishing countries were: USA (25·6%, $n = 34$), Italy (18·8%, $n = 25$, 16 from a single research group) and UK (12%, $n = 16$). The first relevant study was published in 1980; however, the majority (65%) of the studies summarized in this SR were published since 2000. Studies were mainly observational (95%) and case control was the most common study design (87%). Patients with CD were the focus of 80% of studies, with *human immunodeficiency virus* (*HIV*) (1·6%), sarcoidosis (2·4%), T1DM (5·5%), T2DM (2·4%), multiple sclerosis (5·5%), Hashimoto's thyroiditis (1·6%) and human (disease not reported in a few prevalence surveys and molecular epidemiology studies) (3·9%) also captured in this SR.

Studies were categorized in three main groups; (1) those that evaluated the association of *M. paratuberculosis* with human disease, (2) the association between Johne's disease in ruminants and human disease (mainly CD), and (3) molecular epidemiology studies investigating the relatedness of *M. paratuberculosis* isolates from different species to evaluate evidence for strain sharing and zoonotic potential. There were

116 studies that contributed 163 lines of data summarized in Tables 1–4 on the association of *M. paratuberculosis* with CD (96 studies), HIV (2), sarcoidosis (3), T1DM (7), T2DM (3), multiple sclerosis (5), Hashimoto's thyroiditis (2). Five studies with varying study designs and objectives looked at evidence for an association between Johne's disease and CD including two cross-sectional studies examining high exposure occupations and CD [45, 46], one case series that tested newly diagnosed CD patients over 5 years for *M. paratuberculosis* [47] and two challenge trials that investigated whether *M. paratuberculosis* isolates from humans could produce Johne's disease in goats [48, 49]. Seven molecular epidemiology studies looked at the relatedness of *M. paratuberculosis* isolates from a variety of species including humans [50–56].

M. paratuberculosis association with CD

One hundred and eight studies examined the association between *M. paratuberculosis* bacteria or antigen with CD, and of these 96 were case-control studies. Sixty-seven case-control studies directly detected the presence of *M. paratuberculosis* from human samples; faeces, blood, or intestinal tissue using culture [confirmed by staining, histology and polymerase chain reaction (PCR)], PCR (single and nested) or hybridization. Of these 67 studies, 18 were excluded from summarization and analysis because they did not have any *M. paratuberculosis*-positive results (17 studies) or they had 100% positive samples in both groups (1) [57–74]. There were 15 studies (19 lines) that reported detection of live *M. paratuberculosis* by culture with an overall odds ratio (OR) of 8·44 [95% confidence interval (CI) 4·51–15·83, $I^2 = 42\%$; Table 1]. Similarly, across 40 studies (45 lines) that detected *M. paratuberculosis* DNA (44) or RNA (1) in samples by PCR and hybridization the overall OR of 5·24 (95% CI 3·33–8·25, $I^2 = 83\%$) showed a heterogeneous positive association. Investigating reasons for this heterogeneity by subgroup analysis of PCR primers and using meta-regression for selected explanatory variables indicated that the origin of the sampling frame (hospital, clinic, general population or isolate library) accounted for a significant proportion (33%) of heterogeneity. Substantial variation in OR by different detection methods was observed across studies, particularly in PCR primers used (many developed in-house); the results are synthesized by primer in Table 1. Several primers yielded non-significant results across several studies. No other

a-priori selected explanatory variable accounted for the heterogeneity, including choice of control group (healthy, other intestinal disease or ulcerative colitis), *M. paratuberculosis* detection methods (direct PCR, nested PCR, hybridization), sample type (faeces, blood or intestinal tissue: biopsy *vs.* resected), sample state (fresh, frozen, paraffin embedded), primer size, GRADE (down grade or no grade change) or risk of bias (low, unclear, high) designation. In direct detection studies, 27 reported a significant positive association, 15 a positive non-significant association, five a negative non-significant association, and two a significant negative association. A further 17 did not isolate any *M. paratuberculosis* and one reported 100% samples positive; these studies could not assess association. Since most studies were secondary-based case-control designs, confidence in this evidence is moderate to weak (GRADE ** to ***).

Thirty-eight studies (54 lines of data) used evidence of immune response by ELISA or immunoblot and/or examined differing results in lymphocyte or cytokine assays in CD patients compared to controls. Similar to the results for detection of *M. paratuberculosis*, 16 ELISA studies (22 lines) used a number of antigens, demonstrated high heterogeneity between studies and had an overall OR of 4·26 (95% CI 2·45–7·38, $I^2 = 81\%$; Fig. 2). The two studies (four lines) that reported results from sodium dodecyl sulfate (SDS) immunoblot had an overall OR of 25·01 (95% CI 12·55–49·85, $I^2 = 43\%$; Table 2). Four other studies reported significant associations with reactivity to *M. paratuberculosis* [75–78], four reported no significant association [79–82] and one reported contradictory associations between children and adults with CD [83] (Table 2). Three studies were excluded from this summary because no reaction to *M. paratuberculosis* occurred [63], only CD patients were tested [84] and results were not reported separately for CD and ulcerative colitis patients [85]. Eleven studies (14 lines) examined differences in lymphocyte proliferation or cytokine production in response to *M. paratuberculosis* between CD patients and controls (Table 3). A significant association between CD patients and controls was reported in two of four studies using lymphocyte proliferation where peripheral blood lymphocytes (PBL) were suppressed and there was increased suppression cell function in one study [86] and higher T-cell proliferation in the other for CD patients compared to controls [87]. Eight studies examined cytokine production, three found no association and the remaining five reported lower IFN-γ and higher IL-4, IL-2, TNF-α in CD

Table 1. *Summary of findings: association of direct detection of* Mycobacterium paratuberculosis *and Crohn's disease in humans*

Population: Humans
Comparison: Crohn's disease patients compared to ulcerative colitis, non-inflammatory bowel disease and healthy human controls
Outcome: Prevalence or concentration of *Mycobacterium avium* ssp. p*aratuberculosis*
Study design: Case-control ($n = 66$), cross-sectional ($n = 1$)

Studies grouped by *M. paratuberculosis* detection method		OR (95% CI) from a MA/a single study value	Hetero-geneity I^2	Number of observations/ trials/ studies	Weight of evidence (GRADE)	Comments
Test	Primers					
Culture and staining		8·44 (4·51–15·83)	42%	2080/19/15	***	15/18 studies captured was included in this MA. Excluded studies failed to culture *M. paratuberculosis* or had 100% positive samples. Study laboratory methods direct staining (1), culture (7), culture (10) confirmed by PCR (4) and nested PCR (6)
IS*900* PCR and hybridization		5·24 (3·33–8·25)	83%	7192/45/40	**	40/58 studies reporting the identification of *M. paratuberculosis* DNA in samples were included in the MA and meta-regression. 18 studies excluded for having no positive samples: 3 hybridization, 8 PCR and 6 nested PCR and 1 nested PCR study that had 100% positive samples
IS*900* PCR	[IS1, IS2]	7·82 (0·33–183·08)	0%	59/1/1	*	Study from Japan
	[SF214, SR289]	1·86 (0·55–6·34)	0%	561/1/1	*	Study from New Zealand/general population sample
	[1, 2]	7·74 (1·11–53·92)	0%	92/3/3	**	3 small studies from USA and UK
	[150C, 921]	4·71 (1·66–13·37)	0%	187/3/3	*	3 studies from Europe and India. Results downgraded due to one or more studies with a high risk of bias
	[MapF, MapR]	1·66 (0·37–7·49)	0%	74/1/1	*	Study from Italy
	[P3N, P4N]	50·48 (6·74–378·06)	0%	1293/1/1	*	A cross-sectional study from Germany
	[p89, p92]	15·88 (5·11–49·38)	41%	254/3/3	**	Three studies from Italy, same research group
	[P90, P91]	4·51 (1·94–10·47)	52%	444/6/6	**	4 USA and 2 UK
	[PBT5, PBT12]	0·99 (0·25–3·92)	0%	178/1/1	*	Large study with samples from USA and Denmark
	[TDB3, TDB4]	4·00 (0·36–45·05)	0%	39/1/1	*	Study from New Zealand
	Not stated	17·11 (0·69–425·04)	0%	27/1/1	*	Study from Ireland

Table 1 (*cont.*)

Population: Humans
Comparison: Crohn's disease patients compared to ulcerative colitis, non-inflammatory bowel disease and healthy human controls
Outcome: Prevalence or concentration of *Mycobacterium avium* ssp. p*aratuberculosis*
Study design: Case-control (*n* = 66), cross-sectional (*n* = 1)

Studies grouped by *M. paratuberculosis* detection method		OR (95% CI) from a MA/a single study value	Hetero-geneity I^2	Number of observations/ trials/ studies	Weight of evidence (GRADE)	Comments
Test	Primers					
Nested IS*900* PCR	[Liz1, Liz2] [AV1, AV2]	9·80 (4·54–21·18)	78%	941/9/9	**	Studies from Canada, Australia, UK, Germany, China and Italy
	[P90, P91] [AV1, AV2]	0·65 (0·21–2·00)	63%	868/4/4	**	Studies from Canada, Spain and USA
	[P90, P91] [TJ1, TJ2]	0·33 (0·01–13·08)	0%	260/1/1	*	Study from USA
	[PTB1, PTB2] [PTB5, PTB12]	4·70 (0·59–37·78)	0%	192/1/1	*	Study from Denmark
	[TJ1, TJ2]	5·03 (1·07–23·88)	0%	223/2/1	*	Study from Australia
	[S1, R3] [S2, R1]	5·90 (0·78–44·70)	0%	88/1/1	*	Study from Italy. This study also investigated an F57 target [F57/R57, F57rn] with complete agreement at the patient level with the IS*900* target.
	Not stated	8·73 (1·29–59·17)	0%	200/1/1	*	Study from Italy
Hybridization		15·33 (2·87–81·86)	73%	253/4/4	**	Heterogeneous due to 1 study with a high OR. USA (3) and Italy (1)
Meta-regression of PCR and hybridization studies	Sample: hospital	7·07 (4·27–11·70)				The sampling frame origin accounted for 33% of the between study heterogeneity in the 40 PCR and hybridization studies
	Sample: clinic	1·05 (0·34–3·19)				
	Sample: general population	1·05 (0·22–4·91)				
	Sample: library	9·72 (3·63–26·47)				

OR Odds ratio; CI, Confidence interval; MA, meta-analysis; PCR, polymerase chain reaction; I^2, measure of heterogeneity.
GRADE: GRADE Working Group grades of evidence (see explanations): **** high quality: very confident the true effect lies close the estimate of effect;
*** moderate quality: moderate confidence the true effect is likely close to the estimate of effect, but is possibly substantially different; ** low quality: limited confidence in the estimate of effect, the true effect may be substantially different; * very low quality: very little confidence in the estimate of effect, the true effect is likely substantially different from the estimate of effect.
The potential association of *M. paratuberculosis* with Crohn's disease was investigated in 67 studies by culture, PCR or hybridization to identify *M. paratuberculosis* in samples (tissue, blood, faeces). Forty-nine out of 67 studies provided usable data and are included in the meta-analyses presented in this table. The 18 excluded either had no *M. paratuberculosis* isolated or 100% of samples were positive. The methodological soundness of included studies was poor [case-control (66) and cross-sectional (1)] and there was a lot of unexplained heterogeneity in the PCR and hybridization studies. Many studies did not detect a significant association between *M. paratuberculosis* and Crohn's disease; however, the MA results were significant for both the culture and PCR results. Due to the heterogeneity and low methodological quality of the included studies, future research will likely alter the estimates from this summary-of-findings table.

Table 2. *Summary of findings: association of* Mycobacterium paratuberculosis *and Crohn's disease in humans by ELISA*

Population: Humans
Comparison: Crohn's disease patients compared to ulcerative colitis, non-inflammatory bowel disease and healthy human controls
Outcome: Positive immune reaction to *M. paratuberculosis* or evaluation of lymphocyte, T-cell proliferation or cytokine levels.
Study design: Case-control

Studies grouped by *M. paratuberculosis* detection method		OR (95% CI)/results of statistical test as indicated	Heterogeneity I^2	Number of observations/ trials/studies	Weight of evidence (GRADE)	Comments
Test	Target					
SDS immunoblot	p35^r, p36^r	25·01 (12·55–49·85)†	43%	629/4/2	*	2 studies: both had similar targets and methods. A third immunoblot study was excluded from the analysis because it failed to identify any reactors
ELISA	See forest plot	4·26 (2·45–7·38)†	81%	4319/22/16	**	17 studies reported the results of ELISA using various target antigens with general to very specific targets (PPA, PPD and recombinant proteins)
ELISA [79, 80]	ASCA	No correlation with MAP		167/2/2	*	2 studies looked at the correlation of ASCA with a patient reacting to a MAP specific protein for having a positive MAP PCR result and neither observed a correlation
ELISA [75, 81, 82]	PPA	1/3 studies reported significant elevation		483/4/3	*	2/3 studies that used a general PPA reported no difference in immune reaction between CD and controls. The third reported a significant elevation in CD and more significantly with human MAP antigens
ELISA [83]	PPD-Johnin	Gut lavage fluids: $P < 0.05$, CD had higher reaction Sera: $P < 0.05$, CD children had higher and CD adults had lower reactions.		136/2/1	*	1 study looked at differences in IgG and IgA reactions

Table 2 (*cont.*)

Population: Humans
Comparison: Crohn's disease patients compared to ulcerative colitis, non-inflammatory bowel disease and healthy human controls
Outcome: Positive immune reaction to *M. paratuberculosis* or evaluation of lymphocyte, T-cell proliferation or cytokine levels.
Study design: Case-control

Studies grouped by *M. paratuberculosis* detection method		OR (95% CI)/results of statistical test as indicated	Heterogeneity I^2	Number of observations/ trials/studies	Weight of evidence (GRADE)	Comments
Test	Target					
ELISA [81]	LAM, 35 kDa	Not significant		199/1/1	*	Only the significance level and direction was available in these 6 studies as most results were reported only in graphs
ELISA [81]	Lipid A	Not significant		199/1/1	*	
ELISA [76]	PPD: 14 kDa, AhpC, AhpD	$P < 0.05$: CD had higher reaction against 14 kDa		20/1/1	*	
ELISA [77]	PPD MAP a352 (34 kDa)	$P < 0.05$: IgG, $P < 0.001$ IgA CD had higher reactions		123/1/1	*	
ELISA [78]	PtpA[r] and protein kinase G[r]	$P < 0.01$: CD had higher reactions for both proteins		20/1/1	*	
ELISA [75]	rMoDd[r], rPepA[r]	$P < 0.001$ CD had higher reactions		94/1/1	*	

OR, Odds ratio; CI, Confidence interval; SDS, sodium dodecyl sulfate; [r] recombinant protein; † results of a random effect meta-analysis; ELISA, enzyme-linked immuno-sorbent assay; PPA, protoplasmic antigen; PPD, purified protein derivative; I^2, measure of heterogeneity; ASCA, anti-*Saccharomyces cerevisiae* antibody; MAP, *Mycobacterium avium* ssp. *paratuberculosis*; PCR, polymerase chain reaction; CD, Crohn's disease; LAM, lipoarabinomannan; AhpC, alkyl hydroperoxide reductase C; AhpD, alkyl hydroperoxide reductase D; PtpA, protein tyrosine phosphatase A.
GRADE: GRADE Working Group grades of evidence (see explanations): **** high quality: very confident the true effect lies close the estimate of effect; *** moderate quality: moderate confidence the true effect is likely close to the estimate of effect, but is possibly substantially different; ** low quality: limited confidence in the estimate of effect, the true effect may be substantially different; * very low quality: very little confidence in the estimate of effect, the true effect is likely substantially different from the estimate of effect.
The potential association of *M. paratuberculosis* with CD was investigated in 30 studies that used ELISA to determine if the immune response to a *M. paratuberculosis* antigen was different in CD compared to controls. All studies except one used blood samples as well as gut lavage samples. Target antigens ranged from non-specific PPA or LAM preparations to targeted pure recombinant proteins. Nineteen of the 30 studies provided usable data and are included in the meta-analyses presented in this table and Figure 2. The three studies excluded from this table had no reaction [63], analysed inflammatory bowel disease patients as one group [85] or did not test the control group [84]. The methodological soundness of included studies was poor (case-control), there was a lot of heterogeneity in the methods and the target antigens. Many studies individually did not detect a significant association between *M. paratuberculosis* and CD; however, overall the meta-analyses detected a significant association and the ELISA results were similar to those from culture and PCR studies. Due to the heterogeneity and low methodological quality of the included studies, future research will likely alter the estimates from this summary-of-findings table.

Table 3. *Summary of findings: association of* Mycobacterium paratuberculosis *and Crohn's disease in humans using lymphocyte/cytokine assays*

Population: Humans
Comparison: Crohn's disease patients compared to ulcerative colitis, non-Inflammatory bowel disease and healthy human controls
Outcome: Evaluation of lymphocyte and T-cell proliferation or cytokine production from lymphocytes.
Study design: case-control

Author (year)	Population CD	Control	Lymphocyte population	Antigens	Cytokine	Association
Antigen/mitogen induced proliferation of lymphocytes						
Olsen (2001) [76]	10	10	PBMC	AhpC, AhpD, 14 kDa, PPD-Johnin		No significant association
Rowbotham (1995) [136]	13	34	PBL	PPD-MAP		No significant association
Ebert (1991) [86]	33	55	PBL	MAP lysate		$P < 0.001$: lymphocyte proliferation lower in CD and ulcerative colitis sera compared to controls
Olsen (2009) [87]	7	18	T cells	PPD-MAP^r		$P < 0.025$: T-cell proliferation was higher in CD compared to ulcerative colitis and controls
Suppression of mitogen-induced proliferation of lymphocytes						
Dalton (1992) [137]	10	9	PBMC	PPD-MAP		No significant association
			LPL	PPD-MAP		No significant association
Ebert (1991) [86]	33	55	PBL	MAP lysate		$P < 0.001$: increased suppression cell function in CD and ulcerative colitis compared to controls
Antigen/mitogen-induced cytokine production from lymphocytes						
Olsen (2001) [76]	10	10	Whole blood	AhpC, AhpD, 14 kDa, PPD-Johnin	IFN-γ, IL-10	No significant association
Collins (2000) [129]	101	200	Whole blood	PPD-MAP	IFN-γ	$P < 0.01$: CD patients had lower IFN-γ levels than controls
	56	127	PBMC	PPD-MAP	IFN-γ, IL-5	$P < 0.01$: CD patients had lower IFN-γ levels than controls IL-5 no difference across groups.
Ren (2008) [138]	46	70	Whole blood	PPD-MAP	IL-4, IL-2, TNF-α and IFN-γ	$P < 0.05$: IL-4 and IL-2 were significantly higher in subjects that had *M. paratuberculosis* positive samples
Netea (2006) [139]	4	8	Cultured monocytes	MAP	IL-8, IL-1B, TNF-α	No significant association
Olsen (2009) [87]	11	23	PBMC	PPD-MAP^r	IFN-γ, IL-17, IL-4	No significant association
Sibartie (2010) [89]	45	66	PBMC	PPD-MAP	IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and IFN-γ	$P < 0.05$: TNF-α and IL-10 higher in CD patients. $P < 0.05$: IFN-γ supressed CD and ulcerative colitis compared to the healthy controls (also seen for *Listeria* and *Salmonella*)
Clancy (2007) [90]	63	127	Intestinal organ culture	MAP	TNF-α and IFN-γ, IL-2, IL-10 and IL-12	TNF-α higher in CD patients *vs.* ulcerative colitis ($P < 0.05$) or IBD ($P < 0.01$) and controls ($P < 0.0001$)

Table 3 (*cont.*)

Population: Humans
Comparison: Crohn's disease patients compared to ulcerative colitis, non-Inflammatory bowel disease and healthy human controls
Outcome: Evaluation of lymphocyte and T-cell proliferation or cytokine production from lymphocytes.
Study design: case-control

| Author (year) | Population | | Lymphocyte population | Antigens | Cytokine | Association |
	CD	Control				
Campos (2011) [88]	22	47	PBMC	PPD-MAP	IL-23, IL-10, TNF-α	$P < 0.05$: at 3 h IL-23 was higher in CD $P < 0.05$: at 3 days IL-10 was suppressed in CD ($P > 0.05$ at 7 days)

CD, Crohn's disease; PBMC, peripheral blood macrophage cells; AhpC, alkyl hydroperoxide reductase C; AhpD, alkyl hydroperoxide reductase D; PPD, purified protein derivative; PBL, peripheral blood lymphocytes; MAP, *Mycobacterium avium* ssp. *paratuberculosis*; LPL, lamina propria lymphocytes; IL, interleukin.

GRADE: GRADE Working Group grades of evidence (see explanations): **** high quality: very confident the true effect lies close the estimate of effect; *** moderate quality: moderate confidence the true effect is likely close to the estimate of effect, but is possibly substantially different; ** low quality: limited confidence in the estimate of effect, the true effect may be substantially different; * very low quality: very little confidence in the estimate of effect, the true effect is likely substantially different from the estimate of effect.

The potential association of *M. paratuberculosis* with CD was investigated in 11 studies that examined lymphocyte proliferation or cytokine production in response to *M. paratuberculosis*. Various cytokines were evaluated, most studies did not have extractable data, and data was displayed graphically in the papers. The methodological soundness of included studies was poor (case-control), there was a lot of heterogeneity in the methods and most are not directly comparable to each other. Many studies individually did not detect a significant difference in the immune reaction of CD samples *vs.* controls. Due to the heterogeneity and low methodological quality of the included studies, future research will likely alter the estimates and conclusions in this table.

Table 4. *Summary of findings: association of* Mycobacterium paratuberculosis *and diseases other than Crohn's disease in humans*

Population: Humans
Comparison: Humans with diseases: sarcoidosis, HIV, T1DM, T2DM, multiple sclerosis and Hashimoto's thyroiditis compared to healthy humans
Outcome: prevalence or concentration or immune reaction to *Mycobacterium avium* ssp. *paratuberculosis*

Disease	Prevalence or continuous results	Study design	Detection method	OR (95% CI) from a meta-analysis/a single study value/results of statistical test as indicated	Number of observations/ trials/ studies	Weight of evidence (GRADE)	Comments
HIV	P	Case-control	Culture-PCR	14·83 (0·74–295·97)	31/1/1	*	Compared whether mycobacterial isolates from HIV *vs.* pulmonary patients were likely to be *M. paratuberculosis*. Does not reflect risk of *M. paratuberculosis* in HIV patients
	P	Case report	PCR	Positive isolate	1/1/1	*	HIV patient with severe Crohn's disease symptoms. One or more tissue or blood samples were culture-PCR positive
Sarcoidosis	P	Case-control	PCR	No MAP	259/1/1	*	No *M. paratuberculosis* was detected in sarcoidosis patients or controls
	P	Case series	PCR	No MAP	18/1/1	*	No *M. paratuberculosis* was detected in sarcoidosis patients or controls
	P	Case-control	Immunoblot	17·55 (0·95–323·89)	57/1/1	*	Sarcoidosis patients had an insignificant increased odds of reacting to p36 compared to controls
	P	Case-control	Immunoblot	7·67 (0·81–72·26)	42/1/1	*	Sarcoidosis patients had an insignificant increased odds of reacting to MPTB65k compared to controls
T1DM	P	Case-control	PCR	2·91 (1·33–6·33)†	531/4/4	**	T1DM patients had increased odds of *M. paratuberculosis* detection compared to the controls. Heterogeneity $I^2 = 31\%$
	P	Case-control	ELISA	9·95 (5·75–17·24)†	1154/8/4	*	In all trials T1DM were more likely to react to ELISA targets [HbHA (2 trials), cell lysates, Gsd protein, anti-MAP IgG (2), fMptD and MptD] compared to controls. Heterogeneity $I^2 = 50\%$
	C	Case-control	ELISA	*t* test $P < 0.0001$	360/2/2	*	T1DM ELISA results showed a significantly higher reaction against MAP3738c, and MAP3733C antigen compared to the controls
	C	Case-control	ELISA	*t* test $P < 0.0002$	136/1/1	*	T1DM ELISA results showed a significantly higher reaction against Gsd protein compared to the controls

Table 4 (*cont.*)

Population: Humans
Comparison: Humans with diseases: sarcoidosis, HIV, T1DM, T2DM, multiple sclerosis and Hashimoto's thyroiditis compared to healthy humans
*Outcome*: prevalence or concentration or immune reaction to *Mycobacterium avium* ssp. *paratuberculosis*

Disease	Prevalence or continuous results	Study design	Detection method	OR (95% CI) from a meta-analysis/a single study value/results of statistical test as indicated	Number of observations/ trials/ studies	Weight of evidence (GRADE)	Comments
T2DM	C	Case-control	ELISA	No significant results $P > 0.05$	1030/9/3	*	T2DM patients ELISA results showed no significant difference in reactions (HbHA antigen (2), Gsd protein (2), MAP lysate (2), Anti-MAP IgG, fMptD and MptD) compared to the controls
	P	Case-control	PCR	No significant results $P > 0.05$	28/2/2	*	No MAP was detected in T2DM patients
Hashimoto's thyroiditis	P	Case report	PCR	100% positive	3/2/2	*	Two case reports, 1 family with 3 siblings. All had a history of Hashimoto's thyroiditis, bowel disease (undiagnosed) and Melkersson–Rosenthal syndrome (undiagnosed in 2/3)
Multiple sclerosis	P	Case-control	PCR	7.99 (3.41–18.64)	700/1/1	*	2/5 studies contributed to this summary. All conducted in Sardinia, Italy on the native Sardinian population. 3 excluded studies reported preliminary data to the results presented [101–103]. The global generalizability of these results is unknown. ELISA Heterogeneity $I^2 = 70\%$
	P	Case-control	ELISA	6.50 (2.86–14.75)†	1199/2/2	*	

OR, Odds ratio; CI, Confidence interval; P, Prevalence results (+/-); C, results on a continuous scale; † results of a random effect meta-analysis; PCR, polymerase chain reaction; MAP *Mycobacterium avium* ssp. *paratuberculosis*.

GRADE: GRADE Working Group grades of evidence (see explanations): **** high quality: very confident the true effect lies close the estimate of effect; *** moderate quality: moderate confidence the true effect is likely close to the estimate of effect, but is possibly substantially different; ** low quality: limited confidence in the estimate of effect, the true effect may be substantially different; * very low quality: very little confidence in the estimate of effect, the true effect is likely substantially different from the estimate of effect.

The potential association of *M. paratuberculosis* with diseases other than Crohn's disease was investigated in 20 studies (2 HIV, 2 sarcoidosis, 7 T1DM, 3 T2DM, 5 multiple sclerosis and 2 Hashimoto's thyroiditis). Methodological soundness of included studies was poor (case-control, case report and case series), and most were examining immune reactions, thus examining the association indirectly. T1DM and multiple sclerosis show consistent, statistically significant results across studies and outcomes. The hypothesis of *M. paratuberculosis* involvement in Hashimoto's thyroiditis requires investigation. No statistically significant association was reported for T2DM, sarcoidosis or HIV. Thus, additional studies on any of these diseases could change the conclusions and would likely alter the estimates from this summary-of-findings table.

Excluded: Reid & Chiodini (1993) [140] on sarcoidosis; excluded because the antigens were not specific to *M. paratuberculosis*.
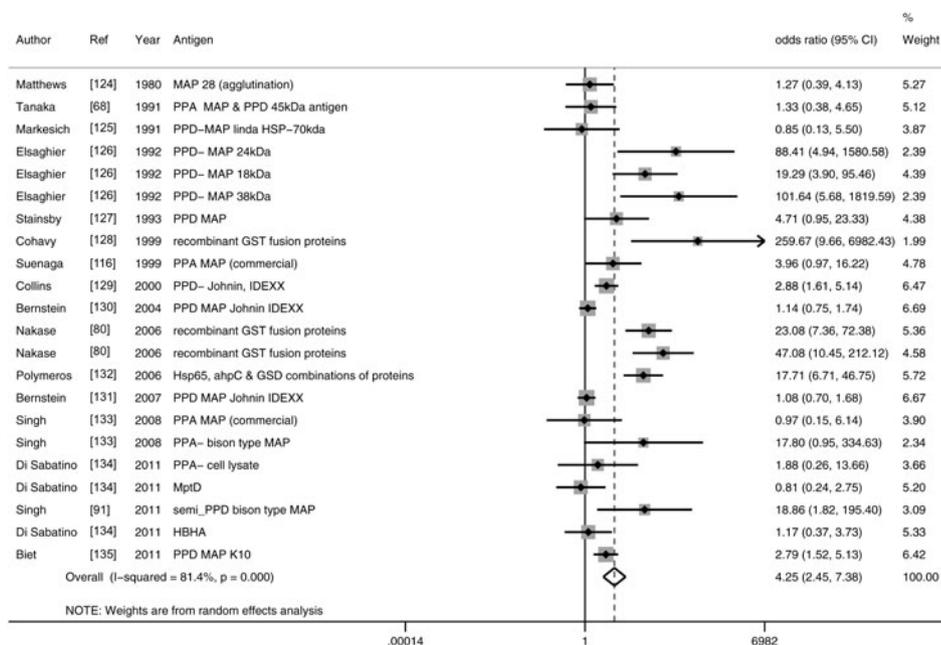
Fig. 2. Random-effects meta-analysis of 16 case-control studies ($I^2 = 81\%$) that examined the association of immune reactions measured by ELISA to *M. paratuberculosis* in Crohn's disease patients compared to controls. MAP, *M. paratuberculosis*; PPD, purified protein derivative; PPA, protoplasmic antigen, HSP, heat shock protein; AHP, alkyl hydroperoxide reductase; GSD, glycosyl transferase; HBHA, heparin-binding haemagglutinin.

patients compared to controls. Only IL-10, examined in four studies, had conflicting results in two studies [88, 89] and non-significant results in two studies [76, 90], Table 3. Overall studies examining immune reactions to *M. paratuberculosis* in CD patients and controls reported significant positive association in 22 studies and non-significant results in 15 studies (14 positive and one negative).

A small number of non-case control observational studies and experiments relevant to the evaluation of the association between *M. paratuberculosis* and CD were identified. As shown in Table 5, two cross-sectional surveys did not find an association between CD and high-risk occupations such as dairy farming and practising veterinary medicine on large animals [45, 46]. The third study was a case series of newly diagnosed CD patients that were followed and periodically tested for M. *paratuberculosis* over 5 years. In this study of 13 patients, three always tested positive, three were always negative and the other seven tested both positive and negative over time [47]. Two studies were challenge trials where *M. paratuberculosis* strains isolated from humans were used to experimentally challenge young goats; however, neither trial was of long enough duration to produce clinical Johne's disease in the goats and only one trial described Johne's disease-like lesions [48, 49].

Molecular epidemiology studies (*n*=7) make up the last type of relevant evidence by comparing isolates from different species and humans, some linked by geography and time, while others are from diverse sources around the world (Table 6). Globally, human isolates were considered most likely to be of bovine origin [51–55], although the human isolates from two studies were also closely related to ovine type *M. paratuberculosis* [52, 53]. In addition, two studies from India reported that a high proportion of human isolates were bison type, results so far unique to India [56, 91]. Wynne *et al.* [51] described human isolates that matched the bovine isolate from samples taken at the same time in the same region, lending evidence for potential sharing of *M. paratuberculosis* between species.

M. paratuberculosis association with human disease other than CD

The majority of research on *M. paratuberculosis*' association with human disease has focused on CD described in the previous section; however, there are 20 studies (16 case-controls, three case reports and one case series) that investigated the potential association of *M. paratuberculosis* with other human diseases (Table 4). The evidence within these studies was deemed very weak (GRADE *), meaning future

Table 5. *Summary of findings: association of* Mycobacterium paratuberculosis *and Crohn's disease in humans by non-case-control study design*

Population: Humans and some animal populations
Comparison: Humans with and without Crohn's disease
Outcome: Possible exposure to *M. paratuberculosis* by occupational exposure or confirmed by laboratory methods
Study design: Cross-sectional surveys, case series, case control

Author (year)	Study design	Country	Sample	Results	Comment	GRADE
Wagner (2011) [47]	Case series	Australia	CD patients at time 0 CD patients up to 5 years always positive	6/13 3/13	Over 5 years 7/13 CD patients were *M. paratuberculosis* at least once. 3/13 were always positive and 3/13 were always negative.	*
Jones (2006) [45]	Cross-sectional survey	UK	JD positive farms JD negative farms	2/1026 5/4173	No association between CD in farmers and a history of JD on the farm.	**
Qual (2010) [46]	Cross-sectional survey	USA	CD Control	4/7 89/1469	Veterinarians and producers (beef, dairy, swine) were surveyed across several states. No significant risk factors for CD and occupational *M. paratuberculosis* exposure.	**

JD, Johne's disease; CD, Crohn's disease.
GRADE: GRADE Working Group grades of evidence (see explanations): **** high quality: very confident the true effect lies close the estimate of effect; *** moderate quality: moderate confidence the true effect is likely close to the estimate of effect, but is possibly substantially different; ** low quality: limited confidence in the estimate of effect, the true effect may be substantially different; * very low quality: very little confidence in the estimate of effect, the true effect is likely substantially different from the estimate of effect.
There were a limited number of observational studies that looked at an association with JD, and results from prevalence and longitudinal prevalence surveys.

Table 6. *Summary of findings: molecular epidemiology evidence for the relatedness of* Mycobacterium paratuberculosis *from various animal species and humans*

Population: Ruminants, non-ruminants and humans
Outcome: M. paratuberculosis genotype
Study design: Molecular epidemiology

Author (year)	Country	Methods	No. of isolates	Human isolates	Comment	GRADE
Singh (2010) [50]	India	IS*1311* PCR-REA	32	9/10 (CD bison type) 1/10 (CD bovine type) 18/22 (nIBD bison type) 4/22 (nIBD bovine type)	Studies from India highlight prevalence of bison type *M. paratuberculosis* compared to bovine type *M. paratuberculosis*	*
Singh (2009) [56]	India	IS*1311* PCR-REA	26	20/26 (bison type) 6/26 (bison type)		*
Wynne (2011) [51]	Australia, Europe, USA	CGH: Read mapping using SHRiMP (K-10 genome)	41	7 (4 CD, 1 UC, 2 nIBD) 2 reference	All 7 human isolates were bovine type II, highly genetically similar and clustered with one bovine isolate	*
Ghadiali (2004) [52]	USA	Edit-Seq and MegAlign (DNASTAR, USA): identified alleles by G and GGT residues	94	11 CD	Two distinct alleles were identified among the 11 human isolates and each clustered with cattle, sheep, and goat isolates	*
Motiwala (2003) [53]	USA and 33 other countries	MPIL and ALFP genetic fingerprinting	247 (MPIL) 104 (ALFP)	7 human (MPIL) 2 human (ALFP)	MPIL: 4 human isolates clustered at A18, 2 were close to bovine A5 and ovine A14. 1 isolate had a distinct fingerprint (A13) AFLP: 2 human isolates appeared on independent branches	*
Whittington (2000) [54]	Australia	RFLP analysis: enzymes *Bst*EII. IS*900* RFLP type and IS*1311* PCR-REA type was determined	328	2 human	2 human samples were clustered with bovine type C1 and C5 that represented the most common bovine *M. paratuberculosis* types in Victoria	*
Francois (1997) [55]	USA and The Netherlands	Hybridization: enzymes *Bst*EII, *Pvu*II or *Pst*I with an IS*900* probe	9	4 CD	2 distinct profiles were observed in CD isolates, 1 clustered with the most common bovine type isolated	*

PCR-REA, Polymerase chain reaction-restriction enzyme analysis; CD, Crohn's disease; nIBD, non-inflammatory bowel disease; CGH, comparative genome hybridization; UC, ulcerative colitis; MPIL, multiplex PCR of IS*900* integration loci; AFLP, amplified fragment length polymorphism; RFLP, restriction fragment length polymorphism.
GRADE: GRADE Working Group grades of evidence (see explanations): **** high quality, very confident the true effect lies close the estimate of effect; *** moderate quality: moderate confidence the true effect is likely close to the estimate of effect, but is possibly substantially different; ** low quality: limited confidence in the estimate of effect, the true effect may be substantially different; * very low quality: very little confidence in the estimate of effect, the true effect is likely substantially different from the estimate of effect.
Only 7 studies have examined the relatedness of *M. paratuberculosis* isolated from humans and animals. Many studies used an isolate library for their sampling frame, thus, are representative of a geographic area or point in time. Most human isolates were reported to cluster with bovine isolates, however some from Ghadaili [52] and Motiwala [53] also clustered with ovine isolates and those from India were most likely to be bison type (Singh [50, 56]). Overall a close relationship between human and animal strains was reported, but it is still unclear if there is limit strain diversity in human *M. paratuberculosis*. Further research should focus on representative sampling and further evaluation of strain diversity from human isolates. Due to heterogeneity in the molecular methods and limited extrapolation of the samples evaluated future studies may completely change the conclusions of this summary-of-findings table.

research may completely change the conclusions of current research. The exception was T1DM that was recently investigated in seven studies (from the USA and Italy) all of which agreed an association may exist, providing weak but consistent evidence (GRADE * to **) for an association (Table 4).

Random-effects MA of the association between T1DM and direct detection of *M. paratuberculosis* by PCR yielded an OR of 2·91 (95% CI 1·33–6·33) [10, 92–94] and for the association between T1DM and detection of an immune reaction to *M. paratuberculosis* by ELISA yielded an OR of 9·95 (95% CI 5·75–17·25) [92–95] two additional ELISA studies not included in the MA reported significantly higher reactions to *M. paratuberbulosis* in T1DM patients compared to controls [96, 97]. Since our SR search, a study from India has reported finding no association with T1DM [98].

Recently, very weak evidence for a significant association with multiple sclerosis and *M. paratuberculosis* has been investigated in Sardinia, Italy, which is a unique isolated population that has very high levels of autoimmune disease [99, 100], three studies [101–103] were removed from this summary as they are preliminary data to Frau *et al.* [100] to avoid double-counting results (Dr Sechi, personal communication). The generalizability of this association needs to be investigated. A hypothesis was presented for an association between *M. paratuberculosis* infection and Hashimoto's thyroiditis based on two case reports involving one family [9, 104]. This has been further supported by a study published after our SR search, also from Italy, that provides evidence for an association with *M. paratuberculosis* [105]; however, future research on other populations is needed to provide further evidence for this hypothesis. By contrast, there was no observed association between *M. paratuberculosis* infection with T2DM [92, 94, 106] and nonsignificant results from HIV [107, 108] and sarcoidosis studies [109–111]. The latter two diseases were examined over a decade ago and no further studies examining this association have been published.

DISCUSSION

This SR is an in-depth analysis of the current global evidence underpinning the zoonotic potential of *M. paratuberculosis*. The 60 studies added to this review since the original search in 2005 [20], indicate that research on the potential association with human disease is still very active and not limited to CD, but now includes a possible association with T1DM, multiple sclerosis and Hashimoto's thyroiditis that will require further research to confirm or refute the existence of a true association. The MAs of case-control studies on CD conducted on different test categories demonstrate agreement in the direction and relative strength of the association. However, there is a general lack of evidence that explains the role of *M. paratuberculosis* in CD and the wide variation in control or disease group *M. paratuberculosis* prevalence across studies, indications that the evidence required to understand the role, if any, of *M. paratuberculosis* in the initiation or progression of CD has not been realized. Thus, we re-confirm our previous conclusions [20] that while the zoonotic potential of *M. paratuberculosis* cannot be ignored, due to important knowledge gaps in understanding its role and importance in the development or progression of human disease, its impact on public health cannot yet be quantified or described.

The strongest evidence for an association between M. *paratuberculosis* and human disease (mainly from studies on CD) arises from the studies that identify culturable organisms or DNA in appropriate samples; these studies have been conducted in increasing numbers since the original SR [20]. Moreover, these studies were based on CD populations around the world and included faecal and blood samples in addition to tissue samples (intestinal biopsy and resection). This group of 18 studies was homogenous, demonstrated culturable *M. paratuberculosis* in the samples and thus yielded the highest confidence in the MA conclusions. However, there were a number of methodological issues with this group of studies that ranked the risk of bias in eight of them (five published since 2005) as high or unclear due to inappropriate sampling frames and poor reporting of study design. There are many widely accepted guidelines for study design and reporting (e.g. STROBE statement for reporting observational studies) that if adhered to, should improve the utility and transparency of future studies [112–114].

Studies that used PCR and hybridization to detect *M. paratuberculosis* demonstrate the existence of *M. paratuberculosis* DNA (or RNA) in the sample. From this information we cannot know if the detected DNA is from a culturable organism, but we can compare the frequency of occurrence between disease and control groups. There were 57 PCR and hybridization studies, of which 22 were published since 2005. There was a lot of heterogeneity in the results of these studies from sample preparation to the vast numbers of

primers used across studies, including many that have been developed 'in house' and never validated. It should also be noted that some more widely used primers have produced only insignificant results for an association with CD, thus we encourage future researchers to consider using primers that have been validated to improve the interpretation and comparability across studies.

In the meta-regression significant heterogeneity was explained by accounting for the origin of the sampling frame (i.e. hospital vs. general population). The association between M. paratuberculosis and CD was only significant for case-control studies that used samples from hospitalized patients and tissue library samples, whereas samples taken from outpatient clinics and the general population showed no relationship. There are several possible reasons for this, including selection bias and different sampling strategies or the type of sample, which was not significant in the univariate meta-regression; however, outpatient studies were more likely to use faecal and blood samples and have less control over the medical history of the controls which could lead to misclassification. Alternatively, the outpatient findings may reflect a more realistic association than the secondary-based case-control studies. The potential discrepancy in results between primary- and secondary-based studies will require further appropriately designed research.

Across case-control studies there was considerable variability in the prevalence of M. paratuberculosis prevalence in both the control and diseased groups ranging from 0% to 100%. This inconsistency has not been explained in the literature, but may be due to true differences in prevalence, differences in test sensitivity and detection limits or even variation in sampling strategy. To complicate this further, a small cohort study by Wagner et al. [47] demonstrated that some CD patients intermittently test positive for M. paratuberculosis by PCR over time and infection may persist despite antimicrobial treatment. Research has not adequately addressed the effects of stage of disease and various treatments on the outcome of M. paratuberculosis testing and there is conflicting results on the relationship between M. paratuberculosis test results and CD activity status (measure of disease symptoms occurring at the time of sample collection) [86, 87, 115–117].

Studies that have shown an immune reaction to M. paratuberculosis provide evidence of prior exposure to the organism, and there is some evidence that there is a different immune response to M. paratuberculosis

in CD patients compared to controls. Overall, these studies provided evidence for an association at a similar magnitude to the direct detection studies, but individually, there were many studies that did not detect a significant association between M. paratuberculosis and human disease. Many of the lymphocyte and cytokine assay studies were working on the hypothesis that there is immune dysfunction in CD patients and they were attempting to demonstrate up-regulation or down-regulation of the inflammatory pathways. This type of work is also being done for Johne's disease with some recent successes in the identification of M. paratuberculosis activated immune pathways that lead to the clinical signs [118]. Across these studies there was little consistency in their approach, lymphocyte populations, antigens or cytokines, thus limiting the overall conclusions that can be drawn.

Evidence for the ecological association between Johne's disease and CD is limited and not strong. The two cross-sectional studies failed to find an association between high M. paratuberculosis exposure populations and CD [45, 46]. A recent molecular epidemiology study demonstrated evidence of M. paratuberculosis strain sharing between human and bovine isolates from the same geographical range [51]. This is in agreement with other studies that showed human isolates were more likely to be bovine type than ovine type M. paratuberculosis, including a recently published genome sequence from human breast milk [51, 53–55, 119]. Exceptions to these results are studies from India, where high proportion of an indigenous bison-type M. paratuberculosis, classified as newly evolved, have been reported [50, 56, 120–122]. The potential public health implications of high prevalence of M. paratuberculosis in humans from India remain unclear [50]. Future molecular research should seek to confirm the extent that different M. paratuberculosis strains may be shared between different species.

There are several limitations to the retrospective case-control studies, case series and case reports which make up 90% of the research captured in this SR. These include an inability to evaluate temporal relationships and M. paratuberculosis exposure [123]. Selection bias can easily occur in case and control selection and the studies lack generalizability to the whole population [123]. However, due to the long incubation period of mycobacterial infections and therefore long latency between likely exposure and disease, and the comparative low incidence of some of the studied diseases (particularly CD), case-control is often the only feasible study design [123]. While we

have gained more evidence over the past seven years, there is still a lack of longitudinal prospective cohort or case-controls studies in high exposure or low disease populations, respectively, that may help us understand the potential relationship between exposure to *M. paratuberculosis* and disease outcomes. Ultimately, we are still lacking an understanding of *M. paratuberculosis'* role in CD or other human diseases.

This updated SR summarizes the largely epidemiological research on the association between *M. paratuberculosis* and human disease (mainly CD). It highlights important knowledge gaps such as understanding *M. paratuberculosis's* role in disease and the relationship between exposure and disease development in humans. Although we presented some strong and consistent MA results of the association between *M. paratuberculosis* and human disease; the variability in *M. paratuberculosis* prevalence in controls and diseased groups and a lack of understanding whether some or all CD is caused or propagated by *M. paratuberculosis* are important knowledge gaps. These are the reasons for the lack of support for recommending further interventions against human exposure to *M. paratuberculosis* beyond what the dairy and ruminant industries have already developed for animal health and economic reasons. Thus, *M. paratuberculosis* may have a role in human disease; however the evidence is not strong enough to inform the potential public health impact of *M. paratuberculosis* exposure.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S095026881500076.

DECLARATION OF INTEREST

None.

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