Digestive Diseases and Sciences
https://doi.org/10.1007/s10620-020-06653-0

INVITED REVIEW

Putting Crohn's on the MAP: Five Common Questions on the Contribution of *Mycobacterium avium* subspecies *paratuberculosis* to the Pathophysiology of Crohn's Disease

Gaurav Agrawal[1,2] · John Aitken[3] · Harrison Hamblin[1] · Michael Collins[4] · Thomas J. Borody[1]

Accepted: 2 October 2020

Abstract
For decades, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been linked to the pathogenesis of Crohn's disease. Despite many investigations and research efforts, there remains no clear unifying explanation of its pathogenicity to humans. Proponents argue Crohn's disease shares many identical features with a granulomatous infection in ruminants termed Johne's disease and similarities with ileo-cecal tuberculosis. Both are caused by species within the *Mycobacterium* genus. Sceptics assert that since MAP is found in individuals diagnosed with Crohn's disease as well as in healthy population controls, any association with CD is coincidental. This view is supported by the uncertain response of patients to antimicrobial therapy. This report aims to address the controversial aspects of this proposition with information and knowledge gathered from several disciplines, including microbiology and veterinary medicine. The authors hope that this discussion will stimulate further research aimed at confirming or refuting the contribution of MAP to the pathogenesis of Crohn's disease and ultimately lead to advanced targeted clinical therapies.

Keywords *Mycobacterium avium paratuberculosis* · Crohn's disease · Tuberculosis treatment · Mycobacteria PCR · Antibiotic treatment for Crohn's disease

Introduction

Crohn's disease is a chronic, inflammatory granulomatous disease that can affect the entire gastro-intestinal tract. Involving the full thickness of the bowel, it is characterized by granulomas in approximately 60% of cases. A recent marked increase in the prevalence of the disease, particularly in developing nations and in the pediatric population, has generated further interest and research into this incurable condition. Although no proven etiology exists as of yet, propositions such as autoimmune, T-cell-mediated immune responses to the resident gut flora, and numerous infective agents are among the most commonly debated and treated causes. Given the apparent similarities to Johne's disease, a chronic, granulomatous enteritis in ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the suspicion for the past 120 years is that MAP may be the causative agent of Crohn's disease according to the observations of the Scottish surgeon, TK Dalziel [1], and likely illustrated earlier by the Polish surgeon Antoni Leśniowski [2] in 1904. Although Dr Burrill Crohn commented initially upon its similarities to known mycobacterial infections of the gut, such as *Mycobacterium tuberculosis* complex *(*MTB), particularly in that it was of a "granulomatous enteritis" nature [3], the organism was never found or isolated; consequently, over time several etiologies came to the fore [4]. This conjecture is arguable due to the lack of a confirmatory diagnostic assay that implicates MAP in active disease. This review addresses common questions relating to the involvement of MAP in the pathogenesis of Crohn's disease.

✉ Gaurav Agrawal
drgagrawal@gmail.com

1 Gastroenterology and Infectious Diseases, Centre for Digestive Diseases, Sydney, Australia

2 Guy's and St. Thomas' NHS Foundation Trust & King's College, London, UK

3 Microbiology, Otakaro Pathways, Christchurch, New Zealand

4 Veterinary Microbiology, Department of Pathobiological Sciences, University of Wisconsin, Madison, USA

Published online: 22 October 2020

Mycobacteria

Members of the genus *Mycobacterium* are microaerophilic gram-positive, rod-shaped bacteria that can form a mycolic (myco) bacterial cell wall that is lipid and cholesterol rich, which often retains acidic stains (acid-fast). Members of this genus of Actinobacteria can exist in a myriad of forms with three distinct groups recognized: MTB, *M. leprae*, and non-tuberculosis mycobacteria (NTM), which includes MAP. These range from behaving as obligate parasites to environmental saprophytes with varying rates of growth. When infecting humans, mycobacteria have a preference for residing within the lysosomes of the macrophage cell cytoplasm, mirroring the preference of mycobacteria in the environment where they sequester within amoebae in order to survive [5]. MAP, like MTB, employs a variety of means to inhibit phago-lysosomal maturation that enables persistence within the macrophage [6]. Mycobacteria can exist in a number of adaptable forms such as extracellular/intracellular, cell wall deficient (CWD—also known as spheroplasts/L forms), acid-fast cell walled, and endospores. They have a relatively slow to the slowest growing rates of all bacterial species, which contributes to whether a particular mycobacterium is pathogenic or opportunistic (the host–microbe interaction is also an important factor [7]). Slower growing organisms are more difficult to eradicate due to the majority of treatments targeting active respiration or cell walled forms. There is evidence to suggest that zoönotic pathogens may cause Crohn's disease, from the emergence of Johne's disease in animals, where the pathogenic features are well described and understood.

A core principle is that an infection cannot be equated with pathogenicity. More recent discoveries, particularly in relation to the mycobacterial life cycle and of latency, strongly suggest the involvement of stealth CWD forms of mycobacteria. Further factors include the pathogenicity of the bacteria and strain varieties, host immune–microbe interaction, macrophage functioning, the influence of the gut microbiome, optimal testing and protocols, treatment trials, and appropriate antibiotic combination chemotherapy. Nevertheless, all of these observations require the gold standard of reliably and regularly culturing of MAP in human samples for confirmation and analysis. Recent discoveries continue to implicate MAP as the initial promoter of Crohn's disease through direct and indirect effects of inflammation. Possible indirect consequences of infection in a predisposed host are a re-modulation of the gut microbiome from a Gram-positive aerobic constituency to a Gram-negative, anaerobic environment [8]. The production of lipopolysaccharide and other signaling molecules from the latter groups of bacteria may also cause extraintestinal effects. The gut microbiome may be the common highway for a variety of inflammatory "autoimmune" diseases that cascade and overlap; it appears that MAP in Crohn's disease is more than a simple direct "cause and effect" infective pathology. Indeed, host–microbe interactions are essential to phenotypic expression of a disease. Whether this is genetic, flora-related (dysbiosis), acquired defects (autophagy dysfunction), it is clear that an opportunity is presented for MAP to reproduce rather than colonize in the host, with persistence dependent on factors such as L forms and growth rates.

It is estimated by the World Health Organization that one-third of the humanity has been exposed and infected with MTB [9], highlighting the widespread distribution and infectivity of mycobacteria in the environment. The observable characteristics of the genus *Mycobacterium* are diverse, reflecting the environmental and pathogenic functions of certain members. Nevertheless, there are some, such as the *M. avium* complex (MAC), which bridge the divide between environmental members and the mycobacterial human pathogens MTB and *M. leprae*. This gradual scale of pathogenic potential, from commensal organism to opportunistic pathogen to an obligate pathogen, highlights the difficulty of assigning pathogenic functions to MAP, which is a member of the MAC group. Organisms in this cluster are characteristically opportunistically pathogenic. As an example, *M. avium* is seen in both healthy human controls although the organism is pathogenic in the presence of a declining or absent immune response. Detection of MTB is considered to be proof of infection in the patient, whereas for MAC members it is not so, as they have been associated with transient asymptomatic human carriage. After 140 years, a consensus being considered is that MAP is not a human pathogen in its own right. Characteristics of MAP are summarized in Table 1.

In light of the foregoing discussion, the authors have formulated five questions aimed at further understanding the involvement pf MAP in Crohn's pathogenesis.

Five Questions That Require Answers Regarding the Involvement of MAP in Crohn's Pathogenesis

1. Why Is There Difficulty and Variability in Studies Which Aim to Detect MAP in Crohn's Patients?

Cell wall-deficient mycobacterial (CWDM) species were discovered in the tissue of Crohn's patients in 1986 [10], with subsequent identification of the isolates as a MAP after 18 months of incubation [11]. Attempts were made to prove, by the use of Koch's postulates (Table 2), the pathogenicity of the organism in humans in an attempt to link MAP with

Table 1 Specific characteristics of MAP

Slowest growing Mycobacteria known—takes 16 weeks to reproduce and > 24 h generation time
Multiple cellular forms, such as acid-fast bacilli with the ability to form spheroplasts or L forms
Strong tendency to form clumps. Important for antibiotic chemotherapy treatment
Resistance to first-line anti-tuberculous drugs, in part due to genes/biofilm. Low cure rates
MAP has been cultured and grown in human blood but took 18 months to do so
Can create dysbiosis of the local (gut) microbiome leading to inflammatory cascades
Disease is a result of host–microbe interaction and immune susceptibility of the host
Infection does not mean disease is expressed but may result in colonization/persistence/latency

Crohn's disease. This was by using the technical methods available at that time. Although further attempts to isolate MAP in Crohn's disease tissue have had less success, the cardinal steps to proof of causation were described. These included the detection and isolation in culture of the putative pathogen and the subsequent introduction of the isolate into an animal model with induction of a "Crohn's like" syndrome. To date, there have been few research attempts to assign causality that have duplicated that path likely due to the necessity of culturing the organisms until mycobacterial reversion of the CWDM occurs and the CWDM develop a cell wall. This necessity conflicted with the evidence that cell walled isolates of MAP in Crohn's were quite rare, making it unlikely that cell walled MAP was a pathogen. On the contrary, the cell walled and the CWDM variety were rarely ever cultured from tissue.

PCR Detection

This inability to culture the target organisms led to the adoption of the surrogate detection method (polymerase chain reaction; PCR) in order to detect MAP in tissue. These molecular methods are preferred for detecting pathogens where culture is difficult with certain caveats. In favor of PCR for proof of pathogenicity are its ease to use, timely results, lower costs, and relatively high sensitivity and specificity of results. There are, however, two problems with PCR—false positives and false negatives, which in the instance of MTB infection, is a major reason why culture accompanies PCR. Culture, the gold standard in MTB diagnosis, is also important for the detection of antimicrobial resistance, whereas PCR is confirmatory, providing a preliminary indication.

The specific difficulties with reliance on PCR in the diagnosis of Crohn's disease are numerous. It is an unreliable surrogate for culture. Despite this, probes to detect genes and insertion sequences have been designed in order to identify specific sequences within the genome of MAP. One sequence, thought to be specific for MAP, is the IS900 sequence though recent evidence suggests that other mycobacteria share this same gene sequence [12]. Although the F57 sequence appears to be more specific for MAP [13], in comparison with the 15–17 copies per organism observed for the IS900 sequence, the F57 gene only has 1 copy per organism. Importantly, the numbers of these repetitive sequences influence the PCR detection rate, often requiring the more sensitive nested PCR methodology [14]. Many research trials comparing the detection rates of MAP using PCR in patients with Crohn's disease versus healthy controls [15] observed transient carriage of MAP in healthy controls [16], predictable given that members of MAC are opportunistic pathogens. Although one investigation was thought to have successfully isolated MAP in the majority of Crohn's patients, subsequent investigations by other researchers demonstrated difficulties in reproducing the results using the same methods [17, 18], inferring that PCR for MAP is not reliable on its own due to a lack of disease correlation. On the basis of these observations, the utility of PCR for MTB diagnosis has been questioned. A study of 293 samples of real-time (RT)-PCR for MTB DNA as compared with MTB cultures showed that PCR was 100% sensitive and 38.9% specific having a positive predictive value of 57.2% and a negative predictive value of 100% [19]. A systemic review and meta-analysis showed that MTB RT-PCR "may be better utilized as a rule out, add-on diagnostic test." [20]. Some consider that there is no association between MAP and Crohn's disease, a conjecture supported by abundant

Table 2 Koch's Postulates

The bacteria must be present in every case of the disease
The bacteria must be isolated from the host with the disease and grown in pure culture
The specific disease must be reproduced when a pure culture of the bacteria is inoculated into a healthy susceptible host
The pathogen must be reisolated from the new host and shown to be the same as the originally inoculated pathogen

circumstantial evidence. Nevertheless, the American Academy of Microbiology concluded that people with Crohn's disease have a 7:1 odds of having MAP in their blood or gut tissues compared with those who do not have Crohn's disease [21]. Therefore, the current issue has progressed now to whether MAP causes CD or is an "innocent bystander" uninvolved in disease pathogenesis.

Culture

Growing and isolating an organism by culture of infected tissue is considered to be the gold standard in detecting bacteria [13]. Regarding MAP, the observed difficulties in the culture of CWDM forms has been a major obstacle for many researchers. The reasons for this are complex but can be distilled down to lack of experience with mycobacterial microbiological methods, and in developing media capable of sustaining growth of CWDM. The same culture methods used for Johne's disease may not guarantee success in the cultivation of CWDM in humans. The task of cultivation of CWDM, though difficult, has been successfully carried out by some researchers albeit by time-consuming and complicated methods. Lacking simple and straightforward methods and protocols for routine diagnoses, the proof of pathogenicity of MAP in Crohn's disease remains obscure and arcane.

2. Has MAP Fulfilled Koch's Postulates?

In 1876, the eminent German microbiologist Robert Koch formulated a set of principles termed "Koch's postulates" designed to assign causality of a disease to bacteria using an animal model (Table 2). The principles were first utilized for anthrax [22], a common disease of cattle caused by the bacterium *Bacillus anthracis* and subsequently launched the field of medical bacteriology [23]. Problems exist though, since *M. leprae* and *Helicobacter pylori* do not fulfill these criteria for causation, yet they are the recognized causes of leprosy and peptic ulcer disease, respectively. Even during their early use, Koch realized that his postulates could not explain the causative links between microorganisms and disease in all cases. An example was his acceptance of the causative bacteria of cholera (*Vibrio cholerae*) despite its isolation from both sick and healthy people [24]. Although researchers have since attempted to apply Koch's postulates to MAP and Crohn's disease [25], their efforts have largely been thwarted due to the protean nature of MAP, which exists as a CWD form in humans but is present in ruminants as a cell walled form [26]. Specific genetic factors with unique host—microbe interactions are also thought to be involved in the pathogenesis of CD. These observations are summarized in Table 3.

3. Why Does the Use of Immunosuppressive Therapy Not Worsen Crohn's Disease if It Is Caused by MAP, as Is Seen with MTB?

Crohn's disease patients do not deteriorate when treated with immunosuppressives, whereas in MTB infection, deterioration is common. The relative risk for MTB increases up to 1.6–25 times with anti-TNF therapy depending on certain factors [27]. Hence, if Crohn's disease is similarly the result of a mycobacterial infection, such as MAP, then there should be similar clinical deterioration instead of a positive response in a cohort of patients to anti-TNF therapy. Nevertheless, MTB and MAP do not share the same ability to establish disease. There are for example, no cavitary lesions in Crohn's disease that might suggest invariable pathogenicity, as is seen with pulmonary MTB. Practically, the difference is that MTB is invasive, whereas MAP appears proinflammatory (in Crohn's disease). Both however share the same ability to trigger formation of granulomas and T-cell responses and as such are the main drivers of inflammation in Crohn's disease and incidentally, are the main protective mechanism against intracellular pathogens in humans. Importantly, drugs displaying several different mechanisms of action have historically been utilized for multiple purposes, e.g., hydroxychloroquine for malaria and rheumatoid arthritis treatment. Similarly, the immunosuppressive drugs used in Crohn's disease, such as thiopurines and biological therapies demonstrate anti-mycobacterial properties [28]. Though, infliximab reduces MAP titers in vitro [29], there is a suspicion that this may also lead to resistance of the organism through environmental pressures [30].

Current immunotherapies are targeted against the overexpression of cytokines in Crohn's disease, such as Interleukin (IL)-1, IL-6, and TNF-α, with underexpression of other cytokines, such as IL-10. These mirror the known immune responses to mycobacteria. TNF-α is essential for the clearance of intracellular pathogens and the control of mycobacteria via the augmentation of T-cell responses. This

Table 3 Barriers to the proof that *Mycobacteria* are pathogenic

There is no animal model for Crohn's disease that supports an infectious trigger
There is no readily available human isolate of a CWDM from infected Crohn's tissue available to introduce into an animal
There are no reliable published methods to re-isolate the CWDM from an artificially infected animal

is through the promotion of macrophage activation and the differentiation and phagolysosome formation that optimizes CD4 + T-cell immunity; by promoting antigen presentation and apoptosis; and cross-priming CD8 + T-cells [31]. The inability to clear mycobacteria from macrophages persistently raises TNF-α levels. Furthermore, TNF-α is inhibited by induction of IL-10 production, a key virulence factor produced by mycobacteria [32] in order to create an intracellular sanctuary. This environment is created by down-regulating apoptosis and stimulating the release of soluble TNF receptor type 2 from macrophages, which also inactivates TNF-α [33]. IL-10 is well studied by MAP investigators, since this factor is essential for communication between host innate and adaptive immune responses, in particular by macrophages hoping to attract T-cells. Documentation of IL-10 mediated suppression of interferon gamma (IFN-γ) secretion in peripheral blood of cattle and goats exposed to MAP infection enables persistence of MAP within the macrophage [34]. The known excesses and deficiencies of cytokines and signaling molecules in Crohn's disease has been compared with those observed in MAP infection, revealing similar patterns between the two [35].

Newer small molecule inhibitors currently being introduced as Crohn's therapies such as pan and gut specific Janus kinase (JAK) inhibitors may also target the downstream effects of a pathological MAP infection, achieved by inhibiting JAK and signal transducer and activator of transcription (STAT)—the JAK-STAT pathway; a hallmark of IFN-γ induction, which is classically active in the clearance of intracellular pathogens. MAP subverts IFN-γ mediated activation of the infected macrophage with subsequent malfunctioning [36]. There is an inability of the infected host to respond to, rather than produce, IFN-γ, hence this factor is raised unresponsively [37]. This observation requires future confirmation in humans.

4. Why Is the Incidence of Crohn's Not Higher in At-Risk Subgroups, Such as Veterinarians or Farmers?

There are no observable increase in rates among farmers and veterinarians, who are regularly exposed to MAP-infected cattle. Therefore, environmental exposure appears to not be a risk factor for Crohn's disease due to MAP.

The prevalence of Crohn's is estimated to be about 0.3% in developed countries with an average of 320 people affected per 100,000 population [37]. Comparatively, subgroups who exhibit greater exposure to Johne's disease show only a mild increase of 0.47% in the prevalence of Crohn's disease, with studies reporting no association between the exposure to Johne's disease and the development of Crohn's disease [38]. Indeed, some studies have reported lower rates and a reduced mortality from inflammatory bowel diseases in farmers and in veterinarians when compared with the general population [39, 40]. Therefore, the hypothesis that MAP causes Crohn's disease appears to be erroneous [41].

From a microbiological viewpoint, the risk factor in CD for MAP involvement is a defective immune system. When coupled with an earlier exposure to the pathogen, the person is at a much higher risk of developing Crohn's disease. With human immunodeficiency virus (HIV), patients are at a far higher risk of opportunistic infection with *M. avium intracellulare* (MAC) due to their compromised immune system (reduced CD4 + count). It is also evident that Crohn's, like HIV, is an emerging prevalent human disease, unlike other "autoimmune" diseases, that demonstrate a constant disease rate.

This observation that the incidence of Crohn's disease is increasing indirectly suggests that a significant environmental risk factor is present. Observing that the rate of Johne's disease is increasing in parallel with Crohn's disease supports the hypothesis that Crohn's is driven by a pre-existing genetic susceptibility married to an emerging animal pathogen, a hypothesis that can be further explored by knowledge of how the organism behaves when causing an inflammatory enteritis in cattle herds. Indeed, in all animal species, MAP susceptibility is generally age-dependent—with exposure at an early age and during the pre-weaning phase essential for subsequent disease development [42]. At a point later in life of the organism, typically during sexual maturation, a phenotypic change occurs, resulting in Johne's disease [43]. Hence, according to this hypothesis, one's adult occupation should be irrelevant to the incidence of Crohn's disease although exposure to cattle early in life should be contributory. Furthermore, the majority of the aforementioned studies that compare the incidence of Crohn's disease with livestock exposure are cross sectional since they do not take into account exposure to MAP-infected animals only, rather including exposure to any and all animals.

Evidence suggests that the younger this exposure occurs the greater the chance of controlling the organism [44] with exposure to intracellular forms of MAP in early life incurring increased risk versus extracellular forms, which results in immune priming. Although with the recent increase in incidence in the pediatric demographic, this would conflict, but it could suggest other environmental factors are at play, with one possibility being an increasing virulence of the organism from evolutionary pressures. Another, is the host responses are influenced by the effect of dysbiosis, given the discoveries of the microbiome on mycobacterial susceptibility. Research suggests that particular microbial signatures within the lung microbiome [45] and even the gut microbiome [46] are associated with the development of pathogenic TB infection. Hence, one hypothesis is that the increasing prevalence of westernized diets and lifestyles reduces the diversity of resident microbiota [47, 48], including in

children [49] and thus contributes to immune susceptibility and the increasing incidence of Crohn's disease in this population. Since this hypothesis could explain the increasing incidence of Crohn's disease in the developing world, further studies are required in order to link this relationship with the epidemiology of Crohn's disease.

MAP is detectable in rural environments with live MAP present in retail pasteurized milk, cheese, raw meat, and in domestic water supplies [50–52]. Hence, the prevalence of MAP in agriculture and in food and water supplies [53] renders difficult the discrimination between neonatal exposure on the farm versus other modes of exposure. As a consequence, the identification of a suitable non-exposed group is challenging. Perhaps most interesting apropos of the observation that breast feeding appears to be protective against Crohn's disease, is the identification of MAP in retail powdered baby formula [54]. This likely represents the most interesting epidemiological comparison since breast-fed babies have a delayed exposure to MAP and most infant formula products are based on bovine milk. Further research is required in this area for these observations.

5. If MAP Causes Crohn's, Why Doesn't Atypical Mycobacterial Antibiotic Therapy (AMAT) Cure Crohn's: Have RCTs Shown that AMAT Is Ineffective?

Background

Effective therapies against pathogens are designed on the back of sound knowledge of the target organism, which is currently lacking due to challenges in isolating and observing MAP. A contemporary example of this are the recent advancements in the in vitro propagation of hepatitis viruses and PCR-based methods for detecting hepatitis C infection, which led to extremely effective antiviral therapies [55]. Importantly, the difficulties in developing efficacious treatments against *Mycobacteria* are not only present for MAP. Despite a wealth of research into the lifecycle of MTB, current gold standard treatment is effective in 81% of cases and drops significantly in resistant strains: 55% in multi-drug-resistant MTB (MDR-TB) and 35% in extensive-drug resistant TB (XDR-TB), respectively [56]. Therefore, applying this to MAP [57] explains, in part, the lower-than-expected cure rates with antibiotic chemotherapy. Although improved and evolved anti-mycobacterial therapy is required, the inability to culture the organism limits directly targeting organism-specific mechanisms, knowledge of which are needed to develop improved treatments. Alongside resistant strains, eradication of CWDM is especially problematic. Currently, AMAT is non-curative and the optimum length of treatment is unknown.

There are legitimate concerns of arrhythmias from QT interval changes/or prolongation, due to the involvement of a macrolide (clarithromycin) and a riminophenazine (clofazimine). Nevertheless, an underlying abnormal cardiac repolarization is a prerequisite for arrhythmia induction [58] and that QT prolongation is unique to the individual and is a poor predictor of ventricular arrhythmias [59]. Electrocardiographic monitoring has proven useful to optimize pharmacovigilance, identify those at risk from the interaction, and implement effective dosage adjustments. Guidance from *M. leprae*, *M. avium* and MDR-TB treatment suggest long-term regimens of > 18 months are required [60] and are safe [61, 62]. The WHO's International Drug Monitoring Programme reports that ventricular arrhythmia is not a common side effect of clofazimine [63] and even more safety data will be provided from the important STREAM phase 3 and Global TB Alliance Phase 2 trials [62]. As mentioned, guidance from *M. leprae* and *M. avium* treatment suggests that long-term regimens of > 18 months are required [58] for treatment of MAP. Yet, even the regimens themselves can vary substantially in their composition and efficacy, e.g., multi-drug therapy (MDT) and fixed duration (FD) therapy in leprosy treatment [64]. Despite this, several advancements in MAP treatment have occurred over time; rifabutin is now preferred to rifampicin due to its higher intracellular concentrations and tissue distribution that are essential for targeting intracellular MAP. Furthermore, rifabutin has a significantly longer half-life than rifampicin (35 h compared with 3.5 h). The higher lipophilicity of rifabutin (100-fold higher oil/water partition coefficient than rifampicin) could account for this difference alongside having a lower minimum inhibitory concentration (MIC) for most pathogens. Compared with rifampicin, rifabutin has a narrower induction spectrum and 30%–60% weaker CYP3A4 induction properties, thus altering its bioavailability and interactions with other medications. Furthermore, the previously utilized antibiotics isoniazid and pyrazinamide are now thought to be ineffective for CWD NTMs such as MAP, due to natural resistance [65]. Accumulating evidence suggests that a spheroplastic phase of MAP (a CWD form) is able to avoid antigenic immune recognition by the host, thereby helping explain the persistent relapsing nature of Crohn's disease, if the MAP hypothesis is indeed correct. Therefore, in order to target the known forms of MAP, it would be advisable to use at minimum a 4-drug combination, especially for the minimally metabolically active spheroplastic forms.

Data from Completed Clinical Trials

A significant argument against the contribution of MAP to Crohn's pathogenesis has been the lack of cure demonstrated with anti-mycobacterial therapy along with the lack of a significant p value in one large randomized control trial (RCT) of anti-mycobacterial therapy [66]. This study is often cited as a basis of evidence for ineffective treatment effects of

Table 4 Comparative analyses of intention-to-cure [66] versus intention-to-treat [72] clinical trials

Selby et al. [66]: PER PROTOCOL: 16 weeks RX: 67/102 (control 55/111) $p=0.02$
52: weeks $p=0.054$. 102: weeks $p=0.14$
Behr et al. [72]: INTENTION-TO-TREAT: RX: 16 weeks- RX: 67/102 (control 55/111) p value $=0.02$
52 weeks: RX: 41/102 (24/111) $p<0.003$. 104 weeks: RX: 31/102 (16/111) $p<0.005$

MAP-active antibiotics as the patients with Crohn's disease were not cured: a primary endpoint of the study. Nevertheless, care must be taken on the basis of a limited understanding of the target organism and unreliable detection methods. It may be more appropriate to conclude that rather than a lack of causation, there was a lack of efficacy in the treatment. In addition to the admissions made by the authors, there are several follow-up articles to the study that highlight flaws and inaccurate conclusions [67–71]. For a RCT, there was no placebo arm since it is considered to be unethical to have such a group for Crohn's patients, thus highlighting the flaws of RCTs in real-world practice. Prospective observational controlled studies are likely to be more clinically useful in this situation. Furthermore, the design of the study removed without replacement subjects who did not achieve clinical remission on prednisolone, rendering it underpowered. The "per protocol" analysis used fluctuating denominators and inverted criteria from "remission" to "relapse," confusing interpretation while rendering it statistically invalid. Importantly, an "intention-to-treat" reanalysis of the raw data demonstrated that AMAT was statistically of benefit for inducing and maintaining remission, as published in *The Lancet* [72]. These two studies are summarized in Table 4.

The RHB-104 Trial

A second large-phase III RCT trial of AMAP, awaiting peer review, appears to contain promising data. Although there are still limitations with this study, preliminary results have shown a statistically significant response in achieving remission using AMAT; ($p<0.013$) as the primary endpoint [73]. The MAP US RHB-104 study examined the safety and efficacy of AMAT in 331 moderate-to-severe Crohn's disease cases. Importantly, the AMAT doses used in this study were higher than those used previously [74]. The placebo arm was given standard immunosuppressive therapy due to the ethical considerations governing the control arm. At week 26, there were more subjects achieving the primary endpoint of remission (CDAI < 150) in the treatment arm than in the placebo arm (37% vs. 23%, $p=0.007$). Furthermore, the late induction of remission from week 16 to week 52 was also significantly greater than placebo (18% vs. 9%, $p=0.019$). The week 52 assessment does not represent preservation or durability of remission but rather captures the later induction of remission. The treatment arm showed a statistically significant reduction in biochemical markers of disease (CRP or fecal calprotectin) at week 16 (25.9% vs. 9.7%, $p=0.0002$), 24 (21.1% vs. 9.1%, $p=0.0003$), and 52 (16.9% vs. 7.9%, $p=0.02$) alongside an endoscopic response in a subgroup of patients. A follow-on RCT has a primary outcome measure of remission (CDAI < 150) at week 16. Importantly, key secondary outcomes include measurement of MAP by PCR of blood at baseline and at several timepoints during treatment, measurements not available in the first MAP US study [75]. This trial of AMAT was based on previous effectiveness in several case series and clinical trials reports [76–82] and would be an important contributor to the accumulating higher quality evidence required to determine the effectiveness of AMAT. A summary of recent published trials of AMAT in the therapy of Crohn's disease is presented in Table 5.

Summary

This review has attempted to acknowledge difficult questions regarding the contribution of MAP to Crohn's disease by involving other disciplines and knowledge. Zoönotic diseases are infections that originate in animals and jump species barriers to infect other species, including humans, and often occur unknowingly. Transmission and infectivity differ with the species reservoirs present. The bubonic plague is caused by *Yersinia pestis* from rats and more recently SARS-CoVID-19 is thought to have originated from bats or pangolins [86].

Current management of Crohn's disease relies on immunosuppression, with AMAT rarely, if ever, offered as a treatment option. Nevertheless, this could be regarded as a lost opportunity from a lack of specific mycobacterial knowledge and stems from core issues for the lack of progression in the treatment and cure of this condition. Specialization and reliance on current molecular technology entails a danger of neglecting and ignoring organisms for what they are and indeed, recognizing that assessment of bacterial behaviors could be beyond the current level of knowledge, awareness, and technology. The input of infectious diseases, microbiologists, and veterinarians are paramount to explain, present different viewpoints, and complement the knowledge base of the gastroenterologist. The future involves the essential and urgently required discovery of an accurate diagnostic test to truly assess the distribution, infectivity, and pathogenicity of MAP in Crohn's patients. Moreover, evolved forms of

Table 5 Recent trials using specifically targeted AMAT in Crohn's disease

Author	Title	No of cases	Outcome	*p* value
Graham et al. [73]	RHB-104, a fixed-dose, oral antibiotic combination against *Mycobacterium avium paratuberculosis* (MAP) infection, is effective in moderately to severely active Crohn's disease	Total 331. 165 Mod-severe CD 166 Placebo arm	Clinical remission (Crohn's disease Activity Index (CDAI) versus placebo Reduction in markers of disease activity (CRP or fecal calprotectin)	Week 16 (42.2% vs. 29.1%, $p=0.015$), Week 26 (37% vs. 23%, $p=0.007$). Week 16 (25.9% vs. 9.7%, $p=0.0002$) and Week 24 (21.1% vs. 9.1%, $p=0.0003$).
Selby et al. [66]	Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease Per Protocol; Intention to cure	Total 213. 102 Treatment arm 111 Control arm	Using combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for up to 2 years did not find evidence of a sustained benefit	16 weeks RX: 67/102 (control 55/111) $p=0.02$. 52 weeks $p=0.054$. 102: weeks $p=0.14$
Behr reanalysis [72]	Antimycobacterial therapy for Crohn's disease: a reanalysis Intention-To-Treat:	Total 213. 102 Treatment arm 111 Control arm	Atypical mycobacterial therapy in CD supports a role for antibiotics inducing remission versus "standard-of-care" therapy plus placebo	16 weeks RX: 67/102 (control 55/111) p value $=0.02$. 52 weeks—RX: 41/102 (24/111) $p<0.003$. 104 weeks—RX: 31/102 (16/111) $p<0.005$
Borody et al. [79]	Anti-mycobacterial therapy in Crohn's disease heals mucosa with longitudinal scars	52	Endoscopic ulcer healing in 56% of patients at 12 months follow-up colonoscopy (22/39)	
Gui et al. [76]	Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics	52 (6 dropped out and 46 analyzed)	A reduction in the Harvey–Bradshaw Crohn's disease activity index An improvement in inflammatory parameters observed at 18 months compared with pre-treatment levels An increase in serum albumin at 12 months	At 6 months ($p=0.004$) At 24 months ($p<0.001$). Reduction in ESR ($p=0.009$) and C-reactive protein ($p=0.03$) ($p=0.04$)
Honap et al. [83]	Anti-Mycobacterium paratuberculosis (MAP) therapy for Crohn's disease: an overview and update	41	Symptomatic benefit in 46% of patients. − 63% of these patients had improved biochemical markers, radiological or endoscopic indices	$p=0.04$
Agrawal et al. [30]	Targeted Combination Antibiotic Therapy Induces Remission in Treatment-Naïve Crohn's disease: A Case Series	8	Clinical remission (CDAI < 150) Improvement in inflammatory markers in all 8 patients receiving AMAT as sole therapy by 6 weeks Median CDAI score decreased from 289 prior to treatment to 62 at the 12-month follow-up	($p<0.001$).
Agrawal et al. [84]	Profound remission in Crohn's disease requiring no further treatment for 3–23 years: A Case series	10	Prolonged remission achieved for 3–23 years, with the majority using AMAT ± infliximab & FMT	
Agrawal et al. [85]	Anti-Mycobacterial Antibiotic Therapy Induces Remission in Active Paediatric Crohn's disease	16	− 47% clinical improvement − 60% mucosal healing	$p<0.001$ $p<0.0078$

ESR Erythrocyte Sedimentation Rate

therapy are needed such as optimal AMAT variations, vaccines, and phage therapy. Though great strides have been made, the true behavior of *Mycobacterium avium paratuberculosis* in humans cannot yet be fully understood or elucidated, though the resolution of this 120-year-old question could be close to being answered.

References

1. Dalziel T. Chronic interstitial enteritis. *BMJ*. 1913;2:1068–1070.
2. Leśniowski A, Towarzystwa P, Warszawskiego L. 1904; 100:630–631.
3. Janowitz HD, Lindner AE, Marshak RH. Granulomatous Colitis Crohn's disease of the Colon. *JAMA*. 1965;191:825–828. https://doi.org/10.1001/jama.1965.03080100043010.
4. Campbell J, Borody TJ, Leis S. The many faces of Crohn's disease: latest concepts in etiology. *Open J Int Med*. 2012;2:107.
5. Salah IB, Ghigo E, Drancourt M. Free-living amoebæ, a training field for macrophage resistance of mycobacteria. *Clin Micro Infect*. 2009;15:894–905.
6. Arsenault RJ, Maattanen P, Daigle J, et al. From mouth to macrophage: mechanisms of innate immune subversion by *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Res*. 2014;45:54. https://doi.org/10.1186/1297-9716-45-54.
7. Namasivayam S, Sher A, Glickman MS, Wipperman MF. The microbiome and tuberculosis: early evidence for cross talk. *mBio*. 2018;9:e01420–18. https://doi.org/10.1128/mbio.01420-18.
8. Loh G, Blaut M. Role of commensal gut bacteria in inflammatory bowel diseases. *Gut Microbes*. 2012;3:544–555. https://doi.org/10.4161/gmic.22156.
9. World Health Organisation. Latent TB Infection: Updated and consolidated guidelines for programmatic management. 2018. ISBN: ISBN 978-92-4-155023-9.
10. Chiodini RJ, Van Kruiningen HJ, Thayer WR, et al. Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. *J Clin Microbiol*. 1986;24:357–363.
11. Chiodini RJ, Van Kruningen HJ, Thayer WR, et al. Possible role of mycobacteria in inflammatory bowel disease. *Dig Dis Sci*. 1984;29:1080–1085. https://doi.org/10.1007/BF01317079.
12. Englund S, Bölske G, Johansson K-E. An IS900-like sequence found in a Mycobacterium sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Micro Lett*. 2002;209:267–271.
13. Donaghy JA, Johnston J, Rowe MT. Detection of *Mycobacterium avium* ssp. *paratuberculosis* in cheese, milk powder and milk using IS900 and f57-based qPCR assays. *J Appl Microbiol*. 2011;110:479–489. https://doi.org/10.1111/j.1365-2672.2010.04905.
14. Herthnek D, Bolske G. New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *Paratuberculosis*. *BMC Microbiol*. 2006;6:87.
15. Naser SA, Sagramsingh SR, Naser AS, Thanigachalam S. *Mycobacterium avium* subspecies *paratuberculosis* causes: Crohn's disease in some inflammatory bowel disease patients. *World J Gastroenterol*. 2014;20:7403–7415.
16. Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet*. 2004;364:1039–1044.
17. Sidoti F. Validation and standardization of IS900 and F57 real-time quantitative PCR assays for the specific detection and quantification of *Mycobacterium avium* subsp. *Paratuberculosis*. *Can J Microbiol*. 2011;57:347–354.
18. Naser SA, Collins MT, Crawford JT, Valentine JF. Culture of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from the Blood of Patients with Crohn's disease: a follow-up blind multi center investigation. *Open Inflam J*. 2009;2:22–23.
19. Nabil MG, Noomani AL, Mahmoud NA, et al. Clinical evaluation of real time PCR assay for detection of *Mycobacterium tuberculosis* DNA in respiratory specimens. *AAMJ*. 2010;8:3.
20. Babafemi EO, Cherian BP, Banting L, et al. Effectiveness of real-time polymerase chain reaction assay for the detection of *Mycobacterium tuberculosis* in pathological samples: a systematic review and meta-analysis. *Syst Rev*. 2017;6:215. https://doi.org/10.1186/s13643-017-0608-2.
21. Nacy C, Buckley M. *Mycobacterium avium paratuberculosis: Infrequent human pathogen or public health threat?*. Washington DC: American Academy of Microbiology; 2008:1–37.
22. Koch R. Die Ätiologie der Milzbrandkrankheit, begründet auf die Entwicklungsgeschichte des Bacillus Anthracis. *Beiträge zur Biologie der Pflanzen*. 1876;2:277–310.
23. Blevins SM, Bronze MS. Robert Koch and the 'golden age' of bacteriology. *Int J Inf Dis*. 2010;14:e744–e751.
24. Evans AS. Limitations of the Henle—Koch postulates. In: *Causation and Disease*. Springer, Boston, MA; 1993. https://doi.org/10.1007/978-1-4615-3024-4_7.
25. Greenstein RJ. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infect Dis Rev*. 2003;3:507–514.
26. Rosu V, Bandino E, Cossu A. Unraveling the transcriptional regulatory networks associated to mycobacterial cell wall defective form induction by glycine and lysozyme treatment. *Micro Res*. 2013;168:153–164.
27. Solovic I, Sester M, Gomez-Reino JJ, et al. The risk of tuberculosis related to tumour necrosis factor antagonist therapies: a TBNET consensus statement. *Eur Respir J*. 2010;36:1185–1206.
28. Chowdhry S, et al. Treatment of severe refractory erythema nodosum leprosum with tumor necrosis factor inhibitor Etanercept. *Int J Mycobacteriol*. 2016;5:223–225.
29. Bach H, Rosenfeld G, Bressler B. Treatment of Crohn's disease patients with infliximab is detrimental for the survival of *Mycobacterium avium* ssp. *paratuberculosis* within macrophages and shows a remarkable decrease in the immunogenicity of mycobacterial proteins. *J Crohn's Colitis*. 2012;6:628–629. https://doi.org/10.1016/j.crohns.2012.01.011.
30. Agrawal G, Clancy A, Sharma R, et al. Targeted combination antibiotic therapy induces remission in treatment-Naïve Crohn's disease: a case series. *Microorganisms*. 2020;8:371. https://doi.org/10.3390/microorganisms8030371.
31. Olsen A, Chen Y, Ji Q, et al. Targeting *Mycobacterium tuberculosis* tumor necrosis factor alpha-downregulating genes for the development of antituberculous vaccines. *mBio*. 2016;7:e01023–15. https://doi.org/10.1128/mbio.01023-15.
32. Balcewicz-Sablinska K, Gan H, Remold HG. Interleukin 10 produced by macrophages inoculated with *Mycobacterium avium* attenuates mycobacteria-induced apoptosis by reduction of TNF-α activity. *J Inf Dis*. 1999;180:1230–1237. https://doi.org/10.1086/315011.
33. Marlow GJ, van Gent D, Ferguson LR. Why interleukin-10 supplementation does not work in Crohn's disease patients. *World J Gastroenterol*. 2013;19:3931–3941. https://doi.org/10.3748/wjg.v19.i25.3931.
34. Hussain T, Shah SZ, Zhao D, Sreevatsan S, Zhou X. The role of IL-10 in *Mycobacterium avium* subsp *paratuberculosis* infection. *Cell Commun Signal*. 2016;14:29. https://doi.org/10.1186/s12964-016-0152-z.
35. Nakase H, Tamaki H, Matsuura M, et al. Involvement of *Mycobacterium avium* subspecies *paratuberculosis* in TNF-α production from macrophage: possible link between MAP and immune

response in Crohn's disease. *Inflam Bowel Dis*. 2011;17:E140–E142. https://doi.org/10.1002/ibd.21750.
36. Arsenault RJ, Li Y, Bell K, et al. *Mycobacterium avium* subsp. *paratuberculosis* inhibits gamma interferon-induced signaling in bovine monocytes: insights into the cellular mechanisms of Johne's disease. *Infect Immun*. 2012;80:3039–3048. https://doi.org/10.1128/iai.00406-12.
37. Ng SC, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet*. 2017;390:2769–2778.
38. Qual DA, Kaneene JB, Varty TJ, Miller R, Thoen CO. Lack of association between the occurrence of Crohn's disease and occupational exposure to dairy and beef cattle herds infected with *Mycobacterium avium* subspecies *paratuberculosis*. *J Dairy Sci*. 2010;93:2371–2376.
39. Hermon-Taylor J. *Mycobacterium avium* subspecies *paratuberculosis*, Crohn's disease and the Doomsday scenario. *Gut Pathog*. 2009;1:15. https://doi.org/10.1186/1757-4749-1-15.
40. Cucino C, Sonnenberg A. Occupational mortality from inflammatory bowel disease in the United States 1991–1996. *Am J Gastroenterol*. 2001;96:1101–1105.
41. Jones PH, Farver TB, Beaman B, Cetinkaya B, Morgan KL. Crohn's disease in people exposed to clinical cases of bovine *paratuberculosis*. *Epidemiol Infect*. 2006;134:49–56. https://doi.org/10.1017/S0950268805004681.
42. National Research Council (US) Committee on Diagnosis and Control of Johne's disease. Diagnosis and Control of Johne's disease. Washington (DC): National Academies Press (US); 2003. 2, Johne's disease in Domesticated and Wild Animals. Available: https://www.ncbi.nlm.nih.gov/books/NBK207655/.
43. Marquetoux N, Mitchell R, Ridler A, Heuer C, Wilson P. A synthesis of the patho-physiology of *Mycobacterium avium* subspecies paratuberculosis infection in sheep to inform mathematical modelling of ovine paratuberculosis. *Vet Res*. 2018;49:27. https://doi.org/10.1186/s13567-018-0522-1.
44. Radon K, Windstetter D, Poluda AL, Mueller B, von Mutius E, Koletzko S. Chronische Autoimmunerkrankungen und Kontakt zu Tieren (Chronic Autoimmune Disease and Animal Contact) Study Group: Contact with farm animals in early life and juvenile inflammatory bowel disease: a case-control study. *Pediatrics*. 2007;120:354–361. https://doi.org/10.1542/peds.2006-3624.
45. Hong B, Paulson J, Stine O, et al. Meta-analysis of the lung microbiota in pulmonary tuberculosis. *Tuberculosis*. 2018;. https://doi.org/10.1016/j.tube.2018.02.006.
46. Fu Y, Feng Y, Wu J, et al. The gut microbiome signatures discriminate healthy from pulmonary tuberculosis patients. *Front Cell Inf Micro*. 2019;9:90. https://doi.org/10.3389/fcimb.2019.00090.
47. Zinöcker MK, Lindseth IA. The western diet-microbiome-host interaction and its role in metabolic disease. *Nutrients*. 2018;10:365. https://doi.org/10.3390/nu10030365.
48. Statovci D, Aguilera M, MacSharry J, Melgar S. The impact of western diet and nutrients on the microbiota and immune response at mucosal interfaces. *Front Immunol*. 2017;8:838. https://doi.org/10.3389/fimmu.2017.00838.
49. Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. *Nutrients*. 2014;7:17–44. https://doi.org/10.3390/nu7010017.
50. Mullan WMA. Are we closer to understanding why viable cells of *Mycobacterium avium* subsp. *paratuberculosis* are still being reported in pasteurised milk? *Int J Dairy Technol*. 2019;72:332–344. https://doi.org/10.1111/1471-0307.12617.
51. Gerrard ZE, Swift BMC, Botsaris G, et al. Survival of *Mycobacterium avium* subspecies *paratuberculosis* in retail pasteurised milk. *Food Microbiol*. 2018;74:57–63.
52. Donaghy JA, Totton NL, Rowe MT. Persistence of *Mycobacterium paratuberculosis* during manufacture and ripening of cheddar cheese. *Appl Environ Microbiol*. 2004;70:4899–4905. https://doi.org/10.1128/AEM.70.8.4899-4905.2004.
53. Koutsoumanis KP, Lianou A, Sofos JN. Food safety: emerging pathogens. *Encycl Agric Food Syst*. *2014*; 250–272.
54. Botsaris George, Swift Ben, Slana Iva, et al. Detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in powdered infant formula by phage-PCR and confirmed by culture. *Int J Food Microbiol*. 2015;. https://doi.org/10.1016/j.ijfoodmicro.2015.09.011.
55. Feeney ER, Chung RT. The polymerase chain reaction: essential for the development of curative therapy for hepatitis C. *Dig Dis Sci*. 2015;60:2232–2235. https://doi.org/10.1007/s10620-015-3748-z.
56. World Health Organisation. Global Tuberculosis Report 2017.
57. Beckler DR, Elwasila S, Ghobrial G, Valentine JF, Naser SA. Correlation between rpoB gene mutation in *Mycobacterium avium* subspecies *paratuberculosis* and clinical rifabutin and rifampicin resistance for treatment of Crohn's disease. *World J Gastroenterol*. 2008;14:2723–2730. https://doi.org/10.3748/wjg.14.2723.
58. Abo-Salem E, Fowler JC, Attari M, et al. Antibiotic-induced Cardiac Arrhythmias. *Cardiovasc Ther*. 2014;32:19–25. https://doi.org/10.1111/1755-5922.12054.
59. Hondeghem LM. QT prolongation is an unreliable predictor of ventricular arrhythmia. *Heart Rhythm*. 2008;5:1210–1212. https://doi.org/10.1016/j.hrthm.2008.05.006.
60. Nunn AJ, Phillips PPJ, Meredith SK, et al. A trial of a shorter regimen for rifampin-resistant tuberculosis. *N Engl J Med*. 2019;380:1201–1213. https://doi.org/10.1056/NEJMoa1811867.
61. Haefner M, Funke-Kissling P, Pfyffer GE, et al. Clarithromycin, rifabutin and clofazimine for treatment of disseminated *Mycobacterium avium* complex disease in AIDS patients. *Clin Drug Investig*. 1999;17:171–178. https://doi.org/10.2165/00044011-199917030-00001.
62. Wang TJ, Dotsenko S, Jafarov A, et al. Safety and availability of clofazimine in the treatment of multidrug and extensively drug-resistant tuberculosis: analysis of published guidance and meta-analysis of cohort studies. *BMJ Open*. 2014;4:e004143. https://doi.org/10.1136/bmjopen-2013-004143.
63. WHO. A practical handbook on the pharmacovigilance of medicines used in the treatment of tuberculosis: enhancing the safety of the TB patient. Geneva, Switzerland: WHO, 2012.
64. Sotgiu G, Centis R, D'ambrosio L, Migliori GB. Tuberculosis treatment and drug regimens. *Cold Spring Harb Perspect Med*. 2015;5:a017822. https://doi.org/10.1101/cshperspect.a017822.
65. Malathi M, Thappa DM. Fixed-duration therapy in leprosy: limitations and opportunities. *Indian J Dermatol*. 2013;58:93–100. https://doi.org/10.4103/0019-5154.108029.
66. Zhang Y, Shi W, Zhang W, Mitchison D. Mechanisms of pyrazinamide action and resistance. *Microbiol Spectr*. 2013;2:1–12. https://doi.org/10.1128/microbiolspec.MGM2-0023-2013.
67. Selby W, et al. Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease. *Gastroenterology*. 2007;132:2313–2319.
68. Lipton JE, Barash DP. Flawed Australian CD study does not end MAP controversy. *Gastroenterology*. 2007;133:1742. **(author reply 1745-6)**.
69. Chamberlin W. Importance of the Australian Crohn's disease antibiotic study. *Gastroenterology*. 2007;133:1744–1745. **(author reply 1745-6)**.
70. Gitlin L, Biesecker J. Australian Crohn's antibiotic study opens new horizons. *Gastroenterology*. 2007;133:1743–1744. **(author reply 1745-6)**.
71. Kuenstner JT. The Australian antibiotic trial in Crohn's disease: alternative conclusions from the same study. *Gastroenterology*. 2007;133:1742–1743. **(author reply 1745-6)**.

72. Behr MA, Hanley J. Antimycobacterial therapy for Crohn's disease: a reanalysis. *Lancet Infect Dis*. 2008;8:344.
73. Graham D. Phase III Randomised, Double Blind, Placebo-controlled, Multicenter, Parallel Group Study to Assess the Efficacy and Safety of Add-on Fixed-Dose Anti-Mycobacterial Therapy in Moderately to Severely Active Crohn's disease (MAP US). United Eur Gastroenterol J 2018; 6.
74. Graham DY. Abstract 58: Oral Presentation. RHB-104, a fixed-dose, oral antibiotic combination against *Mycobacterium avium paratuberculosis* (MAP) infection, is effective in moderately to severely active Crohn's disease. American College of Gastroenterology 2019.
75. Open Label Efficacy and Safety of Anti-MAP (*Mycobacterium Avium* Ssp. *Paratuberculosis*) Therapy in Adult Crohn's disease: Full Text View: ClinicalTrials.gov [Internet]. [cited 2019 Sep 29]. https://clinicaltrials.gov/ct2/show/NCT03009396.
76. Gui GP, Thomas PR, Tizard ML, Lake J, Sanderson JD, Hermon-Taylor J. Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics. *J Antimicrob Chemother*. 1997;39:393–400.
77. Shafran I, Kugler L, El-Zaatari FA, Naser SA, Sandoval J. Open clinical trial of rifabutin and clarithromycin therapy in Crohn's disease. *Dig Liver Dis*. 2002;34:22–28.
78. Borody TJ, Leis S, Warren EF, Surace R. Treatment of severe Crohn's disease using antimycobacterial triple therapy–approaching a cure? *Dig Liver Dis*. 2002;34:29–38.
79. Borody TJ, Bilkey S, Wettstein AR, Leis S, Pang G, Tye S. Antimycobacterial therapy in Crohn's disease heals mucosa with longitudinal scars. *Dig Liver Dis*. 2007;39:438–444.
80. Chamberlin W, Borody TJ, Campbell J. Primary treatment of Crohn's disease: combined antibiotics taking center stage. *Expert Rev Clin Immunol*. 2011;7:751–760.
81. Borgoankar MR, MacIntosh DG, Fardy JM. A meta-analysis of anti-mycobacterial therapy for Crohn's disease. *Am J Gastroenterol*. 2000;95:75–79.
82. Feller M, Huwiler K, Schoepfer A, et al. Long- term antibiotic treatment for Crohn's disease: systematic review and meta-analysis of placebo-controlled trials. *Clin Infect Dis*. 2010;50:473–480.
83. Honap S, Johnston E, Agrawal G, et al. Anti-*Mycobacterium paratuberculosis* (MAP) therapy for Crohn's disease: an overview and update. *Frontline Gastroenterol*. 2020;. https://doi.org/10.1136/flgastro-2020-101471.
84. Agrawal G, Clancy A, Huynh R, et al. Profound remission in Crohn's disease requiring no further treatment for 3–23 years: a case series. *Gut Pathog*. 2020;12:16. https://doi.org/10.1186/s13099-020-00355-8.
85. Agrawal G, Hamblin H, Clancy A, Borody T. Anti-mycobacterial antibiotic therapy induces remission in active pædiatric Crohn's disease. Microorganisms.
86. Liu P, Jiang JZ, Wan XF, Hua Y, Li L, et al. Are pangolins the intermediate host of the 2019 novel coronavirus (SARS-CoV-2)? *PLOS Pathogens*. 2020;16:e1008421. https://doi.org/10.1371/journal.ppat.1008421.