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Human genetic defects and misinterpreted pharmacological data indicate that Crohn disease is consequent to a mycobacterial infection

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ABSTRACT

For more than a century, there has been controversy whether Crohn's disease is a cryptic zoonotic mycobacterial infection. In this manuscript we address two, usually ignored, critical elements that suggest that this concern is probably correct. First: human genetic defects identified in Crohn's disease are associated with increased susceptibility to mycobacterial infections. Second: multiple pharmaceutical agents that are used in Crohn's disease are misnamed. Many "anti-inflammatories" and "immune modulator" medications have dose dependent inhibition of mycobacteria in culture. Accordingly, their primary mechanism of action are as antimycobacterial antibiotics. Failure to incorporate these data renders all previous antibiotic studies in Crohn's disease irrevocably flawed. Finally, incorporating these genetic and antibiotic data, we suggest the necessary clinical studies to address the hypothesis that Crohn's disease is a zoonotic infection.

Introduction

The thesis that Crohn disease (CD) in due to a mycobacterial infection had been posited for more than a century[1]. The concept remains controversial and is conventionally met with denial[2,3], although a reanalysis of those data has been advocated[4]. It is important to consider that, to this day, Koch's postulates[5] have not been met for *M. Leprae* and leprosy. (*M. Leprae*, an obligate intracellular parasite, has never been grown in culture[6], consequent to massive gene decay[7]. Accordingly Koch's second and fourth postulates cannot be satisfied.) In contrast, in 2003 I posited that Koch's postulates had already been met for *M. avium* subspecies *paratuberculosis* (MAP) and Crohn disease, albeit in separate experiments[8].

This 22-minute video encapsulates my overall opinion about the Crohn's zoonotic conundrum. (https://vimeo.com/413736108/59ec7b1545)

The overarching opinion of those who contend that CD is infectious, is that it is a human form of Johne disease (JD), a mycobacterial disease, caused by MAP, that is found in in multiple vertebrates[9]. Johne disease is a ravage on the agricultural community[10]. Those concerned that CD is infectious contend that MAP is zoonotic[11] and that it is transmitted to humans in the food chain and potable chlorinated municipal water[12].

Map was first cultured from a human with CD in 1984[13]. MAP RNA (indicating viability) was first identified in the intestinal tissue of both CD and ulcerative colitis in1966[12]. This latter observation led to the hypothesis that all of inflammatory bowel disease was a spectrum of diseases caused by MAP[14].

Generally, the scientific community requires independent reproducible proof to accept a premise. In infectious diseases it is more than just documenting the presence of an organism. The suggestion that gastric "peptic' ulceration was consequent to a *H. pylori* infection[15,16] was initially met with almost universal derision and condemnation. It was only when "peptic" ulcers were cured with the eradication of *H. pylori* that the epiphenomenon (present but irrelevant) contention was discredited. 25 years later Warren and Marshall received their Nobel prizes for proving an infectious etiology for "peptic" ulcer disease.

If CD is indeed due to MAP, why is it not cured, or at least significantly ameliorated, by appropriate antiMAP therapies? The purpose of this manuscript is to address two aspects that are critical to understanding why all previous antibiotic studies of the CD/MAP conundrum are irretrievably flawed[17]. Finally, it will indicate what clinical studies are necessary to satisfactorily test the CD/MAP hypothesis.

First, it will focus on genetic defects that have been identified in inflammatory bowel disease (IBD.) Their function of the wild type genes will be addressed and why the presence of mutations indicate susceptibility to mycobacterial infections. Second, it will present an overview of unacknowledged and misnamed pharmacological agent that are commonly used in the therapy of IBD. Their primary function is as antiMAP antibiotics.

Genetics:

A detailed analysis of more than 200 loci associated with IBD (see[18-20] for reviews) is beyond the scope of this manuscript. In brief, IBD genetic analyses present a consistent, yet predominantly ignored, narrative on the potential susceptibility to mycobacterial infection as being associated with and possibly causative of IBD. Defects in these loci impair the host immune response to infectious organisms, particularly mycobacteria.

NOD2 was the first genetic defect identified in Crohn disease[21,22]. In 2005 Hugot, one of the initial NOD2 investigators[21], considered the NOD2 finding to be irrelevant in causation or therapeutic responses and was not worthy being identified in CD patients. (Opinion voiced at Inflammatory Bowel Disease Conference: Munster; Germany. Sept 2-3, 2005.)

Comparisons with acknowledged mycobacterial diseases, particularly tuberculosis, leprosy[23] and

Johne disease, provide critical insights into understanding IBD[8]. Wild-type NOD2 is associated with physiological responses to tuberculosis resulting in the production of protective pro-inflammatory cytokines, mediated in part through NF-kappa B pathways[24-26]. In contrast, human NOD2 defects are associated with susceptibility to tuberculosis[27]. With *M. leprae*, a NOD2 defect is associated with lepromatous (the more aggressive form of) leprosy[28,29]. An editorial accompanied the leprosy/NOD2 manuscript. It concluded "these common genetic signatures support, albeit indirectly, the proposal that a proportion of Crohn's disease cases may have a mycobacterial cause"[30]. It is also of considerable interest that NOD2 defects are associated with increased susceptibility[31] to Johne disease[9] (a bovine MAP infection evocative of Dalziel/Crohn disease[1,32])

Subsequently a study of 75,000 human genomes provided an unsurpassed insight[33] Half of the subjects had IBD, and half were healthy controls. The study was from 71 preeminent world-wide institutions, had 106 authors, 347 collaborators and the analyses were conducted at the Sanger Institution at the University of Cambridge UK. The manuscript concluded: "We also observe considerable overlap between susceptibility loci for IBD and mycobacterial infection"[33].

Yet another example of how an IBD genetic defects impairs responses to mycobacterial infections is LY75[18]. This encodes CD205 (aka DEC-205), which is highly expressed on gut dendritic cells (immobile macrophages which endocytose mycobacteria.) Wild-type CD205 regulates endocytosis, T-cell function and homeostasis[34], and protects against pulmonary Tb[35,36].

Genome wide association studies (GWAS) show common susceptibility loci in leprosy[23] and IBD. Of eight susceptibility loci identified in leprosy (NOD2[28], TNSNF15[37], LRRK2[38], Il23R & LCC1/CCDC122[38]) five are associated with IBD. Furthermore, by comparing known IBD susceptibility loci with leprosy, two more leprosy loci, IL18RAP/IL18R1 AND IL12B were identified[39].

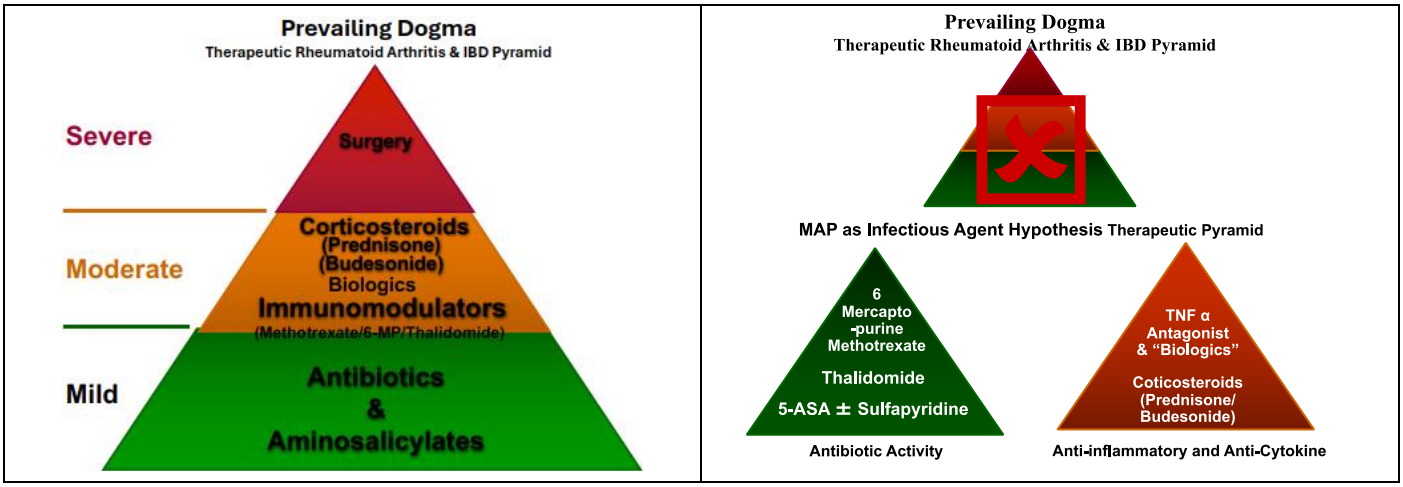
The authors accept prevailing dogma that "our study has further demonstrated the shared genetic susceptibility basis between inflammation and infectious diseases"[39]. We disagree with their conclusion. We consider that that both IBD and leprosy are mycobacterial infectious disease.

The question is: Why has an infectious etiology of UC and CD been missed?

Unacknowledged Pharmacological Effects.

There are two mainstream forms of therapies in IBD. The initial forms were either called "anti-inflammatories", because, when used, inflammation diminished, or "immune modulators", because pro-inflammatory cytokines diminished. These include 5ASA[40], methotrexate, 6MP and azathioprine. More recently there has been the introduction of multiple "biologics."

The central thesis of this manuscript is that the mechanism of action of "anti-inflammatories" and "immune modulators" has long been misinterpreted. We suggest that nomenclature simply describes a secondary physiological response. Their primary mechanism of action is, in my opinion, as antiMAP antibiotics.

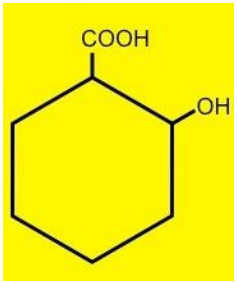


Legend to Figure 1: Shown on the left is prevailing therapeutic dogma for IBD. Shown on the right is our contention that the prevailing dogma is wrong. The green triangle identifies antiMAP antibiotics. The brown triangle identifies agents that are actual anti-inflammatories. These include steroids and a variety of cytokine inhibitors (conventionally referred to as "Biologics.")

In 1940 salicylic acid was shown to increase the growth of the tuberculosis bacillus[41]. This led to manipulation of the salicylic molecule to see whether it could decrease the rate of growth of mycobacteria in general. This was done by adding an amino group to the carbon ring of salicylic acid.

The Effect of Salicylate on the
Oxygen Uptake of the Tubercule Bacillus
Bernheim, F.
Science 1940, 92, 204

Salicylic acid



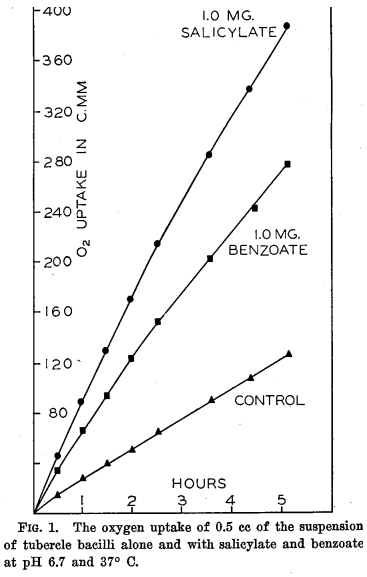


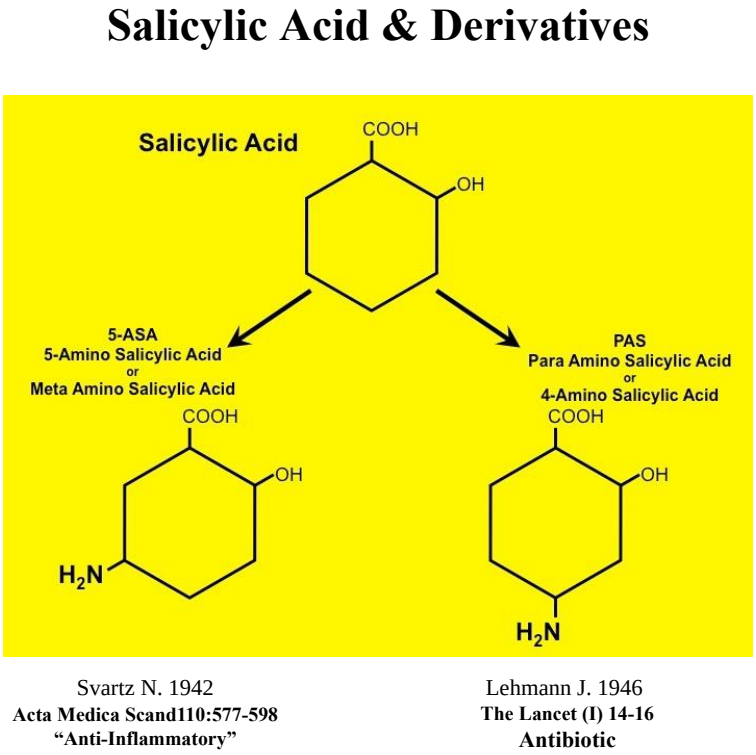
FIG. 1. The oxygen uptake of 0.5 cc of the suspension of tubercle bacilli alone and with salicylate and benzoate at pH 6.7 and 37° C.

Legend to Figure 2: Shown is the increase in O2 consumption when M. tb was supplemented by salicylic acid.

In 1942 Sulfanilamide was introduced in the therapy of rheumatoid arthritis as well as ulcerative colitis[42]. It was a combination of 5-ASA and sulphapyridine. In the therapy of IBD, sulphapyridine is no longer used. This due to the empirical observation of lack of clinical efficacy of the sulphapyridine moiety[43-45]. In contrast, 5ASA alone is now considered to be a "first line' therapy for several" inflammatory diseases including ulcerative colitis and some forms of CD"[46]. One formulation, Olsalazine, is simply two molecules of 5ASA.

The precise mechanism of action of 5-ASA remains unknown[47]. Amongst speculations are: "the hypothesis is that it modulates the inflammatory response derived from the cyclooxygenase and lipooxygenase pathways, decreasing the synthesis of prostaglandins and leukotrienes"[47], suppression of Il-1[48], blocking TNF-alpha suppression and NF kappaB activation[49] and decreased transcriptional activity due to Interleukin-1-stimulated NF-kB RelA/p65 Phosphorylation[50]. None of these postulated mechanisms are accepted[47]

We herein posit a data based, published[51], never refuted, hypothesis as to the primary mechanism of 5ASA in IBD. It is that 5-ASA is a bacteriostatic antiMAP antibiotic. Thirty years after clinical evidence showing no efficacy of sulphapyridine, we provide laboratory data explaining these observations. In radiometric culture 5-ASA, but not sulphapyridine, inhibit MAP. The fact that it is an antibiotic should not be surprising. 5-ASA was introduced in 1942[42]. Its author, Nanna Svarts, Chairperson of Medicine at the Karolinska Institute in Stockholm, hypothesized that its mechanism of action was as an antibiotic (for review see[46]) The structural difference between 5-ASA and the universally acknowledged antibiotic PAS (para-amino salicylic acid) is merely that the amino group, on the salicylic six carbon ring, is one carbon atom displaced.



Legend to Figure 2: The structural similarity between 5-ASA and PAS is immediately apparent. PAS could be called 4-ASA. 5-ASA could be called Meta Amino Salicylic Acid.

It is appropriate to inquire why it took 62 years from the introduction of 5-ASA in 1942[42] until our manuscript in 2005[51], to document dose dependent inhibition in culture of any mycobacterium, particularly MAP. It took an additional 13 years (2018) until 5-ASA dose dependent inhibition of *E. Coli* in culture was reported[52]. The explanations of this prolonged interval from introduction to documentation of antibiotic activity are technical. They have previously been addressed in detail[53]. In brief, reliable culture of MAP first had to be established. Initial isolation requires Herrold's egg yolk slopes and mycobactin J. The latter is obligatory, as MAP is unable to constitutively chelate iron. Using agar slopes, inhibition can only be detected with potent bactericidal antibiotics. Bacteriostatic dose dependent inhibition (such as we demonstrate with 5-ASA[51]) requires culture in liquid as well as an exquisitely sensitive detection

mechanism[54] Our published data were generated in $^{14}CO_2$ radiometric Bactec 460® (Becton Dickenson NJ.) Because of onerous radionucleotide regulation compliance[14],C disposal difficulties and the fact it was only semi-automatic, the Bactec 460 is no longer produced or supported. It has been replaced with the automated, fluorometric MGIT® Bactec 960® (Becton Dickenson NJ.) The MGIT system is less sensitive than the Bactec 460, particularly for non-tuberculous mycobacteria[55-57]. In contrast to our 5-ASA dose dependent inhibition data generated using Bactec 460[51], using MGIT we were not able to demonstrate MAP inhibition by 5-ASA (unpublished observations.)

In addition to 5-ASA, there are other medications that are routinely used in the therapy of IBD. Using Bactec 460 we show dose dependent MAP inhibition in many. We document antiMAP antibiotic activity of methotrexate[58], 6-MP[58], Cyclosporine A[59], Rapamycin[59], Tacrolimus[59], the thioureas methimazole[60] and thiourea[60] (used in the treatment of thyrotoxicosis) and the 1-hydroxypiperidine- 2,6-dione (but not the phthalimide) moiety of thalidomide[61]

Others show, using fluorometric MGIT culture, that 6-MP and azithromycin inhibit MAP in culture[62,63]. A non-culture study identified MAP DNA in IBD patients and controls[64] In two IBD cohorts MAP DNA was not found. These were IBD patients treated with methotrexate or 6-MP[64]. A possible rational, data based, explanation of this lack of MAP DNA in IBD is that the action of methotrexate and 6-MP is bactericidal, and that MAP had been eradicated, or diminished to undetectable levels.

Role of Vitamins A & D in inhibiting mycobacterial growth.

The utility of Vitamin A in combating infection was first published in 1929[65]. Subsequently the clinical use of Vitamin A in treating infectious diseases was extensively studied until the 1940's (see a 1999 comprehensive and informative review[66]). With the introduction of sulfa and subsequently penicillin antibiotics in the mid 1930's, investigation of the use of Vitamin A floundered. The precise mechanism of how Vitamin A acted in treating infectious disease was never conclusively defined or accepted but is generally thought to improve the immune system of the infected eukaryotic host.

In antiquity, the value of sunlight in the treatment of tuberculosis was recognized by Hippocrates, as well as by the Incas for multiple diseases (see[67] for review.) In 1903 Rollins began treating tuberculosis in Switzerland with "heliotherapy"[68]. It was assumed that elevation in vitamin D was the mechanism of efficacy. The precise role of Vitamin D as an anti-infective remains controversial[69]. Adequate doses or supplementation improves clinical outcomes in active tuberculosis[70]. Well studied is the host immune system, particularly the monocyte/macrophage response[71]. High dose vitamin D supplementation in Crohn's disease improves outcomes[72], but interestingly not in those with the NOD2 defect[73]. As with Vitamin A, benefits of supplementation are assumed to be predominantly on improving the immune response of a mycobacterial infected host.

We hypothesized that both Vitamins A and D could directly inhibit growth of prokaryotes, particularly mycobacteria. The effect of four fat soluble vitamins (A, D, E & K) on growth of three species of mycobacteria (*M. Tb*, *M. avium* subspecies *avium*, and MAP) was evaluated. Vitamins E, K and the Vitamin A precursor -Carotene were not inhibitory. Vitamin A, its metabolites Retinyl acetate, Retinoic acid, 13-cis Retinoic acid and Vitamin D inhibited all mycobacterial growth in all species studied[74].

We show that, in radiometric culture, Vitamins A, some of its metabolites and Vitamin D, inhibit several species of mycobacteria in a dose dependent manner. These data indicate that Vitamins A & D[75], at no risk, can be used as supplements and adequate levels should be maintained in a multitude of possible mycobacterial diseases.

Role of a veterinary "grown enhancer" in inhibiting mycobacterial growth.

Introduced in 1967, Monensin is a carboxylix polyether ionophore[76]. In the poultry industry it has been employed as a coccidiostat since 1971 (see[77] for review.) In the cattle industry it is extensively used as a "growth enhancer" because it decreases food intake, increases weight, prolongs lactation[78], and "lipogenic:gluco- genic volatile fatty acids and NH_3-N concentration were lower, and apparent digestibility of dry matter, organic matter, crude protein, and gross energy were higher with " Monensin[79]. Worldwide the veterinary cattle industry is ravaged by MAP[10]. Forty two years after Monensin's introduction, we show a heretofore previously undocumented dose dependent inhibition of MAP in culture[80]. Accordingly, we posit that the beneficial effect of Monensin in cattle may be due, at least in part, to antiMAP antibiotic activity.

We consider all prior antibiotic studies in CD are irrevocably flawed[17,81]. A pivotal antibiotic study in CD was considered negative[2,3], although others suggest it was positive[4]. In that study, at the discretion of the referring physician, patients were permitted "immunomodulator therapy with azathioprine/6-mercaptopurine (6/MP) at a stable dose for at least 6 months prior to enrolment; and 5-aminosalicylates at a stable dose for at least 4 weeks prior to entry"[3]. The accompanying editorial commented "Interestingly, in Selby *et al's* trial, concomitant use of immunomodulatory therapy was the only parameter that was associated with a significantly greater response in the antibiotic group"[2]. The editorial then cited our manuscript[58], as a possible explanation: "These data are compatible with the hypothesis that clinical improvement in patients with inflammatory bowel disease treated with immuno- modulators could be due to treatment of a MAP infection".

Effect of tobacco in Crohn & UC:

Empirical clinical data show a paradoxical effect of the use of tobacco in Crohn disease and UC. In CD clinical course is exacerbated[82-84], in UC it may be improved[85]. Genetic predisposing factors may account for these differences[86]. Multiple clinical studies on the role of tobacco in IBD has assumed that nicotine was the culpable agent (see Table 1 in[85] for review &[87]). In investigative studies, the role of nicotine has addressed the effect on the cell biology and immune response of the patient with IBD[88]. Investigators who consider MAP to be culpable in the etiology of CD have examined the role of nicotine on MAP in culture. At the doses tested, they found nicotine to be MAP-cidal[89].

All these observations and laboratory studies ignored the fact that tobacco has ≥ 4000 individual components[90,91]. We hypothesized that some of these ≥ 4000 molecules might affect the growth kinetics of MAP in radiometric culture. We studied eight mycobacterial strains. Four were MAP, two of which had been isolated from humans with CD and two were bovine isolates. Two were *M. avium* subspecies *avium* and two were BSL-2 *M. tb* strains[92].

Our previous studies had documented inhibition of MAP in radiometric culture[51,58-61,74,93]. Accordingly, we initially demonstrated that we could detect MAP growth enhancement. The salicylic acid increase shown by Bernheim in 1940[41], was first replicated[92]. Nicotine had no effect on MAP growth kinetics on any of the eight strains studied, at the doses we used (1-64µg/ml)[92].

In contrast, we found enhancement of both *M. avium* subspecies *avium* strain and BCG. This enhancement was only seen in one of four MAP strains. It was a bovine MAP, not human, isolate[92]. Interestingly, this salicylic acid enhancement was not found in MAP isolated from humans with CD.

At the 1-64µg/ml dose, Nicotinic Acid, and αt β Nicotinamide Adenine Dinucleotide enhanced MAP growth. Most remarkably Nicotinamide was 10 times more potent with enhancement detectable at 0.1 µg/ml. Of additional interest is that this remarkable sensitivity to Nicotinamide growth enhancement we detectable in MAP isolated from humans but not bovine MAP isolates

(see[92] Table 3 & Figure 4.) This sensitivity to Nicotinamide enhancement was also seen with *M. avium* subspecies *avium* but not *M. Tb*[92].

The role of tobacco in IBD is under intense scrutiny. Since our 2010 publication of growth enhancement of MAP isolated from humans with CD by several components of tobacco[92] has never been contradicted; it is simply ignored. A PubMed search cross-indexed IBD and tobacco. From 2010 to 2024, 126 citations were retrieved. Not a single manuscript addressed the protean implications of our tobacco related radiometric culture MAP enhancement studies[92].

Two additional vignettes.

We consider that the word "placebo" is inappropriate in antibiotic studies where the use of "anti-inflammatories" or "immunomodulators" is permitted in an uncontrolled and *ad libitum* manner. A more scientific, accurate and appropriate term is "Add On"[81]. Additionally, there is a simple rule-of-thumb to differentiate antiMAP antibiotic action from genuine anti-inflammatory action[81]. It is time to clinical response. As with leprosy and tuberculosis, antibiotic therapy requires weeks, months or years. In contrast, a genuine anti-inflammatory action is virtually immediate. Following an infusion, a patient feels clinical improvement immediately, colloquially referred to as "the parking lot effect."

What studies need to be performed?

Considering the forgoing, it is logical to ask how to refute or confirm that MAP is zoonotic. Appropriate clinical studies need to be re-analyzed[3] or performed in IBD in general[14] and in CD in particular[11]. The design must consider the unacknowledged antiMAP antibiotics that have been identified in this article as well as accepted antiMAP antibiotics[3]. More desirably naive IBD/Crohn patients could be divided into two groups. One, the "Add On"[81] group , will include any of the pharmaceuticals with antiMAP activity identified in this article as well as acknowledged antiMAP antibiotics[3]. The second will permit none of the agents used in group one, and have only true anti-inflammatories (such as steroids) and the biologics. Finally, because of the tardiness of the replication rate of MAP, as with prior studies[3] a minimum of two years will be required to get meaningful analyses.

MAP is ubiquitous in the environment, food and water. Accordingly, all treated individuals will be re-exposed to viable MAP. Predisposing genetic defects must be identified. This is because following cessation of antiMAP therapies, a recurrence of disease may occur. This may simply indicate re-exposure to MAP in a genetically compromised individual and indicate the need for lifelong therapy, rather than failure of the study.

Conflict of Interest:

None.

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Competing Interest statement:

RJG has submitted patents based on the hypotheses tested in prior publications.

Patents Issued

US Patent # 7,846,420: Issue Date Dec 7, 2010. *Mycobacterium Avium* Subspecies *Paratuberculosis* Vaccines and Methods for Using the Same. (Now lapsed)

US Patent # 7,902,350: Issue Date March 8, 2011. Method for Monitoring the Efficacy of a *Mycobacterium Avium*Subspecies *Paratuberculosis* Therapy. (Now lapsed).

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