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Mycobacterium Avium Subspecies Paratuberculosis - The Cause of Crohn's Disease

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Abstract

Objective: This systematic review assesses the causal relationship between Mycobacterium avium subspecies paratuberculosis (MAP) and Crohn's disease (CD).

Methods: A systematic review and meta-analysis of some impressive PCR based studies is provided aimed to answer among other questions the following question. Is there a cause effect relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease? The method of the conditio per quam relationship was used to proof the hypothesis whether the presence of Mycobacterium avium subspecies paratuberculosis guarantees the presence of Crohn's disease. In other words, if Crohn's disease is present, then Mycobacterium avium subspecies paratuberculosis is present too. The mathematical formula of the causal relationship k was used to proof the hypothesis, whether there is a cause effect relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease. Significance was indicated by a p-value of less than 0.05.

Results: The studies analyzed (number of cases and controls N=1076) were able to provide evidence that Mycobacterium avium subspecies paratuberculosis is a necessary condition (*conditio sine qua non*) and sufficient condition of Crohn's disease. Furthermore, the studies analyzed provide impressive evidence of a cause-effect relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease.

Conclusion: Mycobacterium avium subspecies paratuberculosis is the cause of Crohn's disease (k=+0.4435, p value < 0.0001).

Keywords: Mycobacterium avium subspecies paratuberculosis, Crohn's disease, cause effect relationship, causality

1. Introduction

Crohn's disease, described in 1904 by Polish surgeon Antoni Leśniowski (Leśniowski, 1904), later in 1913 by Dalziel (Dalziel, 1913) and ultimately by Crohn, Ginzburg and Oppenheimer in 1932 (Crohn et al., 1932), is a debilitating chronic inflammatory bowel disease (IBD) of unknown cause. Leśniowski-Crohn's disease affects about 6.3 per 100,000 people-years in Europe (Burisch, 2015). Many times, the initial signs and symptoms of Crohn's disease (CD) are non-specific and can overlap with symptoms of irritable bowel syndrome (IBS). A delay in the diagnosis of this inflammatory bowel disease (IBD) is associated with problems for both patients and physicians. Often Crohn's disease patients suffer from abdominal pain, malabsorption, steatorrhea, protein losing enteropathy, excessive diarrhea, rapid weight loss and other symptoms which may affect their quality of life (Danese et el., 2015). Several different extra-intestinal complications of Crohn's disease (events outside the gastrointestinal tract) may occur. Lesions of Crohn's disease begin as mucosal erosions and neutrophil infiltrates within crypts and crypt abscesses and may progress to transmural lymphogranulomatous enteritis while a cobblestoned appearance in the distal ileum and colon is observed in Crohn's disease patients (Greenstein, 2004). Medical treatment of Crohn's disease patients includes nutritional therapy, a medication having weak anti-MAP activity with anti-inflammatory drugs, immunosuppressants, and sometimes antibiotics too. A view monoclonal antibodies such as Adalimumab (Humira ®) and Infliximab (Remicade ®) are used too, to treat Crohn's disease patients. Most often, Crohn's disease patients require a dangerous, costly and time-consuming surgical intervention (laparoscopy, strictureplasty, anastomosis, bypass surgery et cetera). A number of theories regarding the etiology of Crohn's disease are discussed including diet, infections, other unidentified environmental factors, immune dysregulation and autoimmune theories. Still, the cause of Crohn's disease or some critical aspects in the pathogenesis of this disease are not known. Many authors are of the opinion that Crohn's disease is a syndrome caused by several etiologies. Mycobacterium avium subspecies paratuberculosis (MAP) is endemic in the bovine populations of many countries (Chaubey et el., 2017) and known to be a causative agent of Johne's disease, an inflammatory bowel disease in a variety of mammals including monkeys, chimpanzees, cattle, sheep, deer, bison and other animals. Johne's disease was discovered by Dr. H. A. Johne and Dr. L. Frothingham as visiting scientists from the Pathology Unit in Boston, Massachusetts at the Veterinary Pathology Unit in Dresden by investigating the tissues of a cow from the Oldenburg region of Germany. The first occurrence of Johne's disease (Pearson, 1908) in the U.S. was published by Leonard Pearson (1868-1909) in 1908. The first description of the similarities between Crohn's disease and Johne's disease in cattle was made in 1913 by the Scottish surgeon Thomas Kennedy Dalziel (Dalziel, 1913). The zoonotic capacities of MAP (Atreya et al., 2014) and the transmission routes to humans (Donaghy et al., 2004; Grant et al., 2002) have been discussed in literature widely. Humans are frequently exposed to MAP through different transmission routes. In this context, Mycobacterium avium subsp. paratuberculosis has been detected in retail cheese in about 31.7% of the samples (Ikonomopoulos et al., 2005). Due to the similarities between Johne's disease in cattle and Crohn's disease, it has been argued that Mycobacterium avium subspecies paratuberculosis (MAP), which causes Johne's disease, might also be a cause of Crohn's disease too. Historically, MAP became the leading infectious candidate as the causative agent of Crohn's disease. Meanwhile, the evidence to support a M. paratuberculosis infection as a cause of Crohn's disease is mounting rapidly. Studies were able to document that up to 83% of Crohn's patients showed evidence of serum antibodies (Elsaghier et al., 1992; Suenaga et al., 1999; El-Zaatari Fet al., 1999; Naser et al., 1999; Naser et al., 2000; Olsen et al., 2001) to M. avium ss paratuberculosis. In particular, critics of the mycobacterial theory argue that MAP is not a causal factor but a secondary invader (Chamberlin et al., 2006). The relationship between Mycobacterium avium subspecies paratuberculosis (M. paratuberculosis) and Crohn's disease is suspected but the evidence remains controversial.

2. Material and Methods

Chronic diarrhea is not the only but one of the most common presenting symptom of Crohn's disease. Severe disease-specific complications of this lifelong disease and a global health problem too are common and accompanied by disabling symptoms and impaired quality of life. The need for repeated courses of cost-expensive therapies, hospitalization and surgery determines a substantial healthcare burden which affects the patient, the healthcare systems and human society in general.

2.1 Search Strategy

For the questions addressed in this paper, PubMed was searched for case-control studies conducted in any country which investigated the relationship between Mycobacterium avium subspecies paratuberculosis (MAP) and Crohn’s disease at least by polymerase chain reaction (PCR). The search in PubMed was performed while using medical key words like “case control study” and “Mycobacterium avium subspecies paratuberculosis” and “Crohn’s disease” and “PCR DNA” et cetera. The articles found where saved as a *.txt file while using PubMed support (Menu: Send to, Choose Radio Button: File, Choose Format: Abstract (text). Click button “create file”). The created *.txt file was converted into a *.pdf file. The abstracts where studied within the *.pdf file. Those articles were considered for a review which provided access to data without any data access barrier; no data access restrictions were accepted. Additionally, references from relevant publications and review articles were checked. Case-control studies were included if they compared the prevalence of MAP in patients with Crohn’s disease with the prevalence in healthy controls. Studies were excluded if insufficient data were provided to calculate the measures of relationship or if there were data access barriers.

2.2 The Data of the Studies Analyzed

Mycobacterium avium subspecies paratuberculosis is an obligate intracellular pathogen (Naser et al., 2014) which is unable to synthesize mycobactin. MAP requires mycobactin to obtain iron from environmental sources. This is one of the reasons why MAP cannot replicate outside of a host. MAP can colonise the host for years without causing disease. In point of fact, is very difficult to detect Mycobacterium paratuberculosis using routine culture techniques. Many attempts to detect and isolate MAP by culture techniques from Crohn's tissues were unsuccessful. *M. paratuberculosis recovers very poorly by culture from Crohn's disease tissues while the incubation times (sometimes greater than one year) can be extremely long*. In this context, MAP DNA can be identified especially by PCR. Thus far, novel laboratory techniques (Chae et al., 2017) Southern Blot hybridization, Immunohistochemistry (IHC), introduced by Coons (Coons et al., 1941) in 1941, In-situ hybridization (ISH), described in the year 1969 by Joseph G. Gall (Gall et al., 1969), Fluorescent ISH (FISH), RNA in situ hybridization (RNA ISH), Polymerase chain reaction (PCR), Nested PCR, Quantitative polymerase chain reaction (QPCR) et cetera) can improve our understanding of the pathogenesis of Crohn’s diseases. The data of the studies (Sanderson et al., 1992; Fidleret al., 1994; Hulten et al., 2001; Ryan et al., 2002; Bull et al., 2003; Autschbach et al., 2005; Sechi et al., 2005; Romero et al., 2005; Szkaradkiewicz et al., 2007; Kirkwood et al., 2009; Mendoza et al., 2010; Tuci et al., 2011; Lee et al., 2011; Nazareth et al., 2015; Timms et al., 2016; Khan et al., 2016; Zamani et al., 2017) analyzed, are presented by the table 1 (Table 1). The meaning of the abbreviations a_t, b_t, c_t, d_t, N_t of table 1 (Table 1), table 2 (Table 2), table 3 (Table 3), table 4 (Table 4) are explained by a 2 by 2-table (Table 5).

Table 1. The data of the studies considered for a meta-analysis

	Mycobacterium avium subspecies paratuberculosis				Sample Size
	Crohn's Disease		Healthy control		
	MAP+	MAP-	MAP+	MAP-	
Study	$\mathbf{a_t}$	$\mathbf{c_t}$	$\mathbf{b_t}$	$\mathbf{d_t}$	$\mathbf{N_t}$
Sanderson et al., 1992	26	14	5	35	80
Fidler et al., 1994	4	27	0	30	61
Hulten et al., 2001	7	30	0	22	59
Ryan et al., 2002	6	9	0	12	27
Bull et al., 2003	34	3	9	25	71
Autschbach et al., 2005	22	14	4	73	113
Sechi et al., 2005	30	5	3	26	64
Romero et al., 2005	10	2	1	5	18
Szkaradkiewicz et al., 2007	10	6	1	11	28
Kirkwood et al., 2009	22	34	6	33	95
Mendoza et al., 2010	30	0	0	10	40
Tuci et al., 2011	21	10	11	21	63
Lee et al., 2011	5	14	0	19	38
Nazareth et al., 2015	27	13	11	18	69
Timms et al., 2016	6	15	0	21	42
Khan et al., 2016	16	53	3	46	118
Zamani et al., 2017	18	10	6	56	90
Total events	294	259	60	463	**1076**

2.3 Statistical Analysis

All statistical analyses were performed with Microsoft Excel version 14.0.7166.5000 (32-Bit) software (Microsoft GmbH, Munich, Germany). In order to simplify the understanding of this article, to increase the transparency for the reader and to correct some of the misprints of former publications, several of the following lines are *repeated word by word* and taken from former publications.

2.3.1 The 2x2 Table

The 2x2 table in this article is defined (Barukčić, 1989; Barukčić, 1997; Barukčić, 2005; Barukčić, 2006a; Barukčić, 2006b; Barukčić, 2011a; Barukčić, 2011b; Barukčić, 2012; Barukčić, 2016a; Barukčić, 2016b; Barukčić, 2016c; Barukčić, 2016d; Barukčić, 2016e; Barukčić, 2017a; Barukčić, 2017b; Barukčić, 2017c; Barukčić, 2017d; Barukčić, 2017e; Barukčić, 2017f; Barukčić, 2017g; Barukčić, 2018a; Barukčić, 2018b; Barukčić, 2018c) in general more precisely (Table 5) as follows.

Table 5. The sample space of a contingency table

		Crohn's disease (Conditioned B_t)		
		Yes = +1	No = +0	Total
MAP PCR DNA (Condition A_t)	Yes = +1	a_t	b_t	A_t
	No = +0	c_t	d_t	$\underline{A}_t$
	Total	B_t	$\underline{B}_t$	N_t

In general it is $(a+b) = A_t$, $(c+d) = \underline{A}_t$, $(a+c) = B_t$, $(b+d) = \underline{B}_t$ and $a_t+b_t+c_t+d_t=N_t$. Equally, it is $B_t+\underline{B}_t = A_t + \underline{A}_t = N_t$. In this context, it is $p(a_t)=p(A_t \cap B_t)$, $p(A_t) = p(a_t)+p(b_t)$ or in other words $p(A_t)= p(A_t \cap B_t)+p(A_t \cap \underline{B}_t)$ while $p(A_t)$ is not defined as $p(a_t)$. In the same context, it should be considered that $p(B_t) = p(a_t)+p(c_t) = p(A_t \cap B_t) +p(c_t)$ and equally that $p(\underline{B}_t) = 1- p(B_t) = p(b_t)+p(d_t)$. In point of fact, the joint probability of A_t and B_t is denoted by $p(A_t \cap B_t)$. It is $p(a_t)+p(c_t)+p(b_t)+p(d_t) = 1$. These relationships are viewed by the table (Table 6) as follows.

Table 6. The sample space of a contingency table

		Crohn's disease (Conditioned B_t)		
		Yes = +1	No = +0	Total
MAP PCR DNA (Condition A_t)	Yes = +1	$p(a_t)$	$p(b_t)$	$p(A_t)$
	No = +0	$p(c_t)$	$p(d_t)$	$p(\underline{A}_t)$
	Total	$p(B_t)$	$p(\underline{B}_t)$	$p(N_t)$

2.3.2 Independence

In the case of independence of A_t and B_t it is

$$p(A_t \cap B_t) \equiv p(A_t) \times p(B_t) \tag{1}$$

2.3.3 Sufficient Condition (Conditio Per Quam; Material Conditional)

The mathematical formula of the sufficient condition relationship (conditio per quam) (Barukčić, 1989; Barukčić, 1997; Barukčić, 2005; Barukčić, 2006a; Barukčić, 2006b; Barukčić, 2011a; Barukčić, 2011b; Barukčić, 2012; Barukčić, 2016a; Barukčić, 2016b; Barukčić, 2016c; Barukčić, 2016d; Barukčić, 2016e; Barukčić, 2017a; Barukčić, 2017b; Barukčić, 2017c; Barukčić, 2017d; Barukčić, 2017e; Barukčić, 2017f; Barukčić, 2017g; Barukčić, 2018a; Barukčić, 2018b; Barukčić, 2018c) of a population was defined as

$$p(A_t \to B_t) \equiv p(A_t \cap B_t) + p(\underline{A}_t) \equiv p(A_t \cap B_t) + (1 - p(A_t)) \equiv \frac{a_t + c_t + d_t}{N_t} \equiv +1 \tag{2}$$

and used to proof the hypothesis: *if* A_t *then* B_t .

2.2.3 Necessary Condition (Conditio Sine Qua Non)

The formula of the necessary condition (conditio sine qua non) relationship (Barukčić, 1989; Barukčić, 1997; Barukčić, 2005; Barukčić, 2006a; Barukčić, 2006b; Barukčić, 2011a; Barukčić, 2011b; Barukčić, 2012; Barukčić, 2016a; Barukčić, 2016b; Barukčić, 2016c; Barukčić, 2016d; Barukčić, 2016e; Barukčić, 2017a; Barukčić, 2017b; Barukčić, 2017c; Barukčić, 2017d; Barukčić, 2017e; Barukčić, 2017f; Barukčić, 2017g; Barukčić, 2018a; Barukčić, 2018b; Barukčić, 2018c) was derived as

$$p(A_t \leftarrow B_t) \equiv p(A_t \cap B_t) + p(\underline{B}_t) \equiv p(A_t \cap B_t) + (1 - p(B_t)) \equiv \frac{a_t + b_t + d_t}{N} \equiv +1 \tag{3}$$

and used to proof the hypothesis: *without* A_t *no* B_t.

2.2.4 Necessary and Sufficient Condition (Material Biconditional)

The *necessary and sufficient condition* relationship (Barukčić, 1989; Barukčić, 1997; Barukčić, 2005; Barukčić, 2006a; Barukčić, 2006b; Barukčić, 2011a; Barukčić, 2011b; Barukčić, 2012; Barukčić, 2016a; Barukčić, 2016b; Barukčić, 2016c; Barukčić, 2016d; Barukčić, 2016e; Barukčić, 2017a; Barukčić, 2017b; Barukčić, 2017c; Barukčić, 2017d; Barukčić, 2017e; Barukčić, 2017f; Barukčić, 2017g; Barukčić, 2018a; Barukčić, 2018b; Barukčić, 2018c) was defined as

$$p(A_t \leftrightarrow B_t) \equiv p(A_t \cap B_t) + p(\underline{A}_t \cap \underline{B}_t) \equiv \frac{a_t + d_t}{N} \equiv +1 \tag{4}$$

2.3.4 The X^2 Goodness of Fit Test of a Necessary Condition

Under conditions where the chi-square (Pearson K, 1900) goodness of fit test cannot be used it is possible to use an approximate and conservative (one sided) confidence interval as discussed by Rumke (Rumke, 1975), Louis (Louis, 1981), Hanley (Hanley et al., 1983) and Jovanovic (Jovanovic et al., 1997) known as *the rule of three*.

2.3.5 The Mathematical Formula of the Causal Relationship k

The mathematical formula of the causal relationship k (Barukčić, 1989; Barukčić, 1997; Barukčić, 2005; Barukčić, 2006a; Barukčić, 2006b; Barukčić, 2011a; Barukčić, 2011b; Barukčić, 2012; Barukčić, 2016a; Barukčić, 2016b; Barukčić, 2016c; Barukčić, 2016d; Barukčić, 2016e; Barukčić, 2017a; Barukčić, 2017b; Barukčić, 2017c; Barukčić, 2017d; Barukčić, 2017e; Barukčić, 2017f; Barukčić, 2017g; Barukčić, 2018a; Barukčić, 2018b; Barukčić, 2018c) is defined *at every single event, at every single Bernoulli trial t*, as

$$k({}_{R}U_{t}, {}_{0}W_{t}) \equiv \frac{\left(p({}_{R}U_{t} \times {}_{0}W_{t}) - \left(p({}_{R}U_{t}) \times p({}_{0}W_{t})\right)\right)}{\sqrt[2]{\left(p({}_{R}U_{t}) \times p({}_{R}\underline{U}_{t})\right) \times \left(p({}_{0}W) \times p({}_{0}\underline{W}_{t})\right)}} \tag{5}$$

where ${}_{R}U_{t}$ denotes the cause and ${}_{0}W_{t}$ denotes the effect while the chi-square distribution (Pearson K, 1900) can be applied to determine the significance of causal relationship k.

2.3.6 The Chi Square Distribution

The chi-squared distribution (Pearson K, 1900) is a widely known distribution and used in hypothesis testing, in inferential statistics or in construction of confidence intervals. The critical values of the chi square distribution are visualized by Table 8.

Table 8. The critical values of the chi square distribution (degrees of freedom: 1).

	p-Value	Critical one sided X^2	Critical two sided X^2
	0.1000000000	1.642374415	2.705543454
	0.0500000000	**2.705543454**	**3.841458821**
	0.0400000000	3.06490172	4.217884588
	0.0300000000	3.537384596	4.709292247
The critical values	0.0200000000	4.217884588	5.411894431
	0.0100000000	5.411894431	6.634896601
of the	0.0010000000	9.549535706	10.82756617
	0.0001000000	13.83108362	15.13670523
Chi square distribution	0.0000100000	18.18929348	19.51142096
	0.0000010000	22.59504266	23.92812698
	0.0000001000	27.03311129	28.37398736
	0.0000000100	31.49455797	32.84125335
	0.0000000010	35.97368894	37.32489311
	0.0000000001	40.46665791	41.8214562

3. Results

3.1 Without the Presence of Mycobacterium Avium Subspecies Paratuberculosis no Presence of Crohn's Disease

Claims.

Null hypothesis: The presence of Mycobacterium avium subspecies paratuberculosis is a necessary condition (a conditio sine qua non) of Crohn's disease. In other words, the sample distribution agrees with the hypothetical (theoretical) distribution of a necessary condition.

Alternative hypothesis: The presence of Mycobacterium avium subspecies paratuberculosis is not a necessary condition (a conditio sine qua non) of Crohn's disease. In other words, the sample distribution does not agree with the hypothetical (theoretical) distribution of a necessary condition. The significance level (Alpha) below which the null hypothesis will be rejected is alpha=0.05.

Proof.

The data reviewed by this article which investigated the relationship between the presence of Mycobacterium avium subspecies paratuberculosis and Crohn's disease are viewed by the table (Table 2). Altogether, 17 studies were meta-analyzed while the level of significance was alpha = 0,05. Altogether, 6 from 17 studies provided significant evidence of a conditio sine qua non relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease. The sample size of the study of Romero et al. (Romero et al., 2005) was small and has been analyzed and is significant according to the *Rule of three*. In the same respect, 17/17 studies analyzed provided evidence of a significant cause effect relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease. In other words, *without* a Mycobacterium avium subspecies paratuberculosis infection of human intestinal tract *no* Crohn's disease. Due to methodological inconsistencies, 11 of 17 studies reanalyzed failed to provide statistically significant evidence of this relationship. In point of fact, the presence of Mycobacterium avium subspecies paratuberculosis inside human intestinal tract is a necessary condition (a conditio sine qua non) of Crohn's disease. In other words, *without* the presence of Mycobacterium avium subspecies paratuberculosis inside human intestinal tract *no* Crohn's disease. **Q. e. d.**

Table 2. *Without* MAP infection of human intestinal tract *no* Crohn's disease

Study Id	Year	Country	N_t	a_t	b_t	c_t	d_t	p(SINE)	X^2 (SINE)	k	X^2(k)	p val (k)
Mendoza et al., 2010	2010	Spain	40	30	0	0	10	1	0.008333333	1	40	2.53963E-10
Bull et al., 2003	2003	UK	71	34	9	3	25	0.9577465	0.168918919	0.668723016	31.75052356	1.75302E-08
Romero et al., 2005	2005	USA	18	10	1	2	5	0.8888889	Rule of three	0.644658371	7.480519481	0.006237007
Sechi et al., 2005	2005	Italy	64	30	3	5	26	0.921875	0.578571429	0.7507418	36.071248	1.90233E-09
Tuci et al., 2011	2011	Italy	63	21	11	10	21	0.8412698	2.911290323	0.333669355	7.014120017	0.008086937
Zamani et al., 2017	2017	Iran	90	18	6	10	56	0.8888889	3.223214286	0.571683803	29.41401341	5.84534E-08
		Total	346	143	30	30	143	0.9132947	6.890328289		151.7304245	
								Alpha =	0.05		Alpha =	0.05
			Degrees of freedom (D. f.) =						5		D. f. =	6
			X^2 (Critical SINE) =						**11.07049769**	**X^2 (Critical k)=**		**12.59158724**
			X^2 (Calculated SINE)=						6.890328289	X^2 (Calculated k)=		151.7304245
											p value (k)=	3.33166E-30

3.2 If Presence of Mycobacterium Avium Subspecies Paratuberculosis then Presence of Crohn's Disease

Claims.

Null Hypothesis: The presence of Mycobacterium avium subspecies paratuberculosis is a sufficient condition (a conditio per quam) of Crohn's disease. In other words, the sample distribution agrees with the hypothetical (theoretical) distribution of a sufficient condition.

Alternative Hypothesis: The presence of Mycobacterium avium subspecies paratuberculosis is not a sufficient condition (a conditio per quam) of Crohn's disease. In other words, the sample distribution does not agree with the hypothetical (theoretical) distribution of a sufficient condition. The significance level (Alpha) below which the null hypothesis will be rejected is alpha=0.05.

Proof.

The data reviewed by this article which investigated the relationship between the presence of Mycobacterium avium subspecies paratuberculosis inside human intestinal tract and Crohn's disease are viewed by the table (Table 3). Altogether, 17 studies were meta-analyzed with n=1076 number of cases and controls while the level of significance was alpha = 0.05. Thus far, 17 out of 17 studies provided significant evidence of a conditio per quam relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease. The studies of Ryan et al. (Ryan et al., 2002), Romero et al. (Romero et al., 2005) and Szkaradkiewicz et al. (Szkaradkiewicz et al., 2007) were analyzed and are significant according to the *Rule of three*. In the same respect, 17/17 studies analyzed provided evidence of a significant cause effect relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease. In other words, *if* infection of human intestinal tract by Mycobacterium avium subspecies paratuberculosis *then* Crohn's disease. In point of fact, the presence of Mycobacterium avium subspecies paratuberculosis within human intestinal tract is a sufficient condition (a conditio per quam) of Crohn's disease. In other words, *if* presence of Mycobacterium avium subspecies paratuberculosis within human intestinal tract *then* presence of Crohn's disease. **Q. e. d.**

3.3 Mycobacterium Avium Subspecies Paratuberculosis is Necessary and Sufficient Condition of Crohn's Disease

Claims.

Null hypothesis: The presence of Mycobacterium avium subspecies paratuberculosis is a necessary and sufficient condition of Crohn's disease. In other words, the sample distribution agrees with the hypothetical (theoretical) distribution of a necessary and sufficient condition.

Alternative Hypothesis: The presence of Mycobacterium avium subspecies paratuberculosis is not a necessary and sufficient condition of Crohn's disease. In other words, the sample distribution does not agree with the hypothetical (theoretical) distribution of a necessary and sufficient condition. The significance level (Alpha) below which the null hypothesis will be rejected is alpha=0.05.

Proof.

The data reviewed by this article which investigated the relationship between the presence of Mycobacterium avium subspecies paratuberculosis and Crohn's disease are viewed by the table (Table 4). Altogether, in this proof, 17 studies were meta-analyzed while the level of significance was alpha = 0.05. In toto, 4 studies were able to provide significant evidence that Mycobacterium avium subspecies paratuberculosis is a necessary and sufficient condition of Crohn's disease. The study of Romero et al. (Romero et al., 2005) have been analyzed and was significant according to the *Rule of three*. In point of fact, more or less older studies failed to provide evidence of this relationship. In the same respect, 17/17 studies analyzed provided evidence of a significant cause effect relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease and thus far the absence of independence. In other words it is true that *without* the presence of Mycobacterium avium subspecies paratuberculosis within human intestinal tract *no* presence of Crohn's disease and at the same time it is equally true that *if* presence of Mycobacterium avium subspecies paratuberculosis in human intestinal tract *then* presence of Crohn's disease too. In particular, Mycobacterium avium subspecies paratuberculosis is a necessary and sufficient condition of Crohn's disease. **Q. e. d.**

Table 3. *If* MAP infection *then* CD

Study Id	Year	Country	N_t	a_t	b_t	c_t	d_t	p(IMP)	X^2(IMP)	k	X^2(k)	p val (k)
Mendoza et al., 2010	2010	Spain	40	30	0	0	10	1.0000	0.0083	1.0000	40.0000	0.0000000003
Romero et al, 2005	2005	USA	18	10	1	2	5	0.9444	Rule of three	0.6447	7.4805	0.0062370075
Szkaradkiewicz et al., 2007	2007	Poland	28	10	1	6	11	0.9643	Rule of three	0.5489	8.4349	0.0036807951
Hulten et al., 2001	2001	USA	59	7	0	30	22	1.0000	0.0357	0.2829	4.7225	0.0297712403
Ryan et al., 2002	2002	Geramny	27	6	0	9	12	1.0000	Rule of three	0.4781	6.1714	0.0129829729
Timms et al., 2016	2016	Australia	42	6	0	15	21	1.0000	0.0417	0.4082	7.0000	0.0081509716
Lee et al., 2011	2011	Canada	38	5	0	14	19	1.0000	0.0500	0.3892	5.7576	0.0164177087
Fidler et al., 1994	1994	UK	61	4	0	27	30	1.0000	0.0625	0.2606	4.1426	0.0418165336
Sechi et al., 2005	2005	Italy	64	30	3	5	26	0.9531	0.1894	0.7507	36.0712	0.0000000019
Khan et al., 2016	2016	India	118	16	3	53	46	0.9746	0.3289	0.2288	6.1773	0.0129398574
Autschbach et al., 2005	2005	Geramany	113	22	4	14	73	0.9646	0.4712	0.6190	43.2968	0.0000000000
Sanderson et al., 1992	1992	UK	80	26	5	14	35	0.9375	0.6532	0.5388	23.2258	0.0000014405
Kirkwood et al., 2009	2009	Australia	95	22	6	34	33	0.9368	1.0804	0.2579	6.3180	0.0119518827
Zamani et al., 2017	2017	Iran	90	18	6	10	56	0.9333	1.2604	0.5717	29.4140	0.0000000585
Bull et al., 2003	2003	UK	71	34	9	3	25	0.8732	1.6802	0.6687	31.7505	0.0000000175
Nazareth et al., 2015	2015	Portugal	69	27	11	13	18	0.8406	2.9013	0.2934	5.9407	0.0147955514
Tuci et al., 2011	2011	Italy	63	21	11	10	21	0.8254	3.4453	0.3337	7.0141	0.0080869370
		Total	**1076**	294	60	259	463	0.9442	12.20856	0.4435	272.91802	
						Alpha =0.05				Alpha =0.05		
						Degrees of freedom (D. f.) =14				D. f. =17		
						X^2 (Critical IMP) =23.6847913				**X^2 (Critical k)=27.587112**		
						X^2 (Calculated IMP)= 12.2085699				X^2 (Calculated k)=272.91802		
										p value (k)=4.229E-48		

3.4 Mycobacterium Avium Subspecies Paratuberculosis is the Cause of Crohn's Disease

Claims.

Null hypothesis (**no causal relationship**): There is no significant causal relationship between an infection by Mycobacterium avium subspecies paratuberculosis and Crohn's disease. (k=0).

Alternative hypothesis: (**causal relationship**): There is a significant causal relationship between an infection by Mycobacterium avium subspecies paratuberculosis and Crohn's disease. ($k \neq 0$). **Conditions.** Alpha level = 5%. The two tailed critical Chi square value (degrees of freedom = 1) for alpha level 5% is 3.841458821.

Proof.

The data for this hypothesis test were provided by different studies and are illustrated by a table (Table 3). The causal relationship k(Mycobacterium avium subspecies paratuberculosis, Crohn's disease) is calculated according to Barukčić (Barukčić, 1989; Barukčić, 1997; Barukčić, 2005; Barukčić, 2006a; Barukčić, 2006b; Barukčić, 2011a; Barukčić, 2011b; Barukčić, 2012; Barukčić, 2016a; Barukčić, 2016b; Barukčić, 2016c; Barukčić, 2016d; Barukčić, 2016e; Barukčić, 2017a; Barukčić, 2017b; Barukčić, 2017c; Barukčić, 2017d; Barukčić, 2017e; Barukčić, 2017f; Barukčić, 2017g; Barukčić, 2018a; Barukčić, 2018b; Barukčić, 2018c). Again, 17 studies were meta-analyzed with n=1076 number of cases and controls while the level of significance was alpha = 0.05. Thus far, all the 17 studies analyzed provided evidence of a significant cause effect relationship between Mycobacterium avium subspecies paratuberculosis and Crohn's disease. In other words, Mycobacterium avium subspecies paratuberculosis and

Crohn's disease are not only not independent of each other. Besides of the methodological difficulties associated with the studies analyzed the majority of the studies provided significant evidence of a necessary condition, of a sufficient condition and of a necessary and sufficient condition. The conclusion appears to be inescapable: Mycobacterium avium subspecies paratuberculosis *is the cause of* Crohn's disease (k~ +0.4435, p value < 0.0001). **Q. e. d.**

Table 4. MAP infection of human intestinal tract is a necessary and sufficient condition of CD

Study Id	Year	Country	N_t	a_t	b_t	c_t	d_t	p(IMP^SINE)	X^2(IMP^SINE)	k	X^2(k)	p val (k)
Mendoza et al., 2010	2010	Spain	40	30	0	0	10	1	0.0167	1	40.00	p<0.001
Romero et al., 2005	2005	USA	18	10	1	2	5	0.833333	Rule of three	0.644658371	7.48	0.0062
Sechi et al., 2005	2005	Italy	64	30	3	5	26	0.875	0.7680	0.7507418	36.07	p<0.001
Bull et al., 2003	2003	UK	71	34	9	3	25	0.830985	1.8492	0.668723016	31.75	p<0.001
		Total	193	104	13	10	66	0.880829016	2.6338		107.82	
								Alpha =	0.05	Alpha =		0.05
					Degrees of freedom (D. f.) =				3	D. f. =		3
					X^2 (Critical IMP^SINE) =				**7.8147**	**X^2 (Critical k)=**		**7.81**
					X^2 (Calculated IMP^SINE)=				2.6338	X^2 (Calculated k)=		107.82
										p value (k)<		0.0001

3.5 Antibiotic Therapy and Crohn's Disease

One of the most convincing contributions to the evidence of a role for MAP in Crohn's disease would be a proof that a treatment with appropriate antibiotics for sufficient duration able kill the organism leads to remission of Crohn's disease. In this context, different antibiotic drug combinations, called anti-MAP regimen, including clarithromycin (CLA), rifabutin (RIF) and Clofazimine (CLO) investigated in multiple randomized clinical trials have shown (Grant et al., 1996; Eltholth et al., 2009; Ricchi et al., 2014; Faria et al., 2014; Borody et al., 2002) promising results. A complete healing of ulcers in Crohn's disease patients after more than 6 months of treatment with a rifabutin (RIF) and clarithromycin (CLA) regimen, an inhibitor of CYP34A (Akiyoshi et al., 2013), has been reported by several studies. A 2007 case study reported that one Crohn's disease patient who was being treated with anti-MAP therapy drugs, attained complete clinical remission (Chamberlin et al., 2007). Selby et al. (Selby et al., 2007) conducted a prospective, parallel, placebo-controlled, double-blind, randomized treatment trial using the combination of clarithromycin, rifabutin, and clofazimine. Two hundred thirteen patients were randomized while the study design of the study of Selby et al. (Selby et al., 2007) included an initial 16-week phase in which all patients received prednisolone in addition to trial medications. Remission was defined as Crohn's Disease Activity Index < 150. Using combination antibiotic therapy with rifabutin, clarithromycin, and clofazimine Selby et al. (Selby et al., 2007) did not find evidence of a sustained benefit for Crohn's disease patient. At the end of week 16 period, there was only "a significantly greater percentage of subjects in remission in the antibiotic arm (67/102 [66%]) than in the placebo arm (55/111 [50%]) (P = .02)". Selby et al. (Selby et al., 2007) concluded that their finding does not support a significant contribution of Mycobacterium avium subspecies paratuberculosis in the pathogenesis of Crohn's disease. Thus, what unifies the conclusions of the abovementioned study group (Selby et al., 2007) is seemingly the disparate misuse of statistical methods and the data of their study made publicly available for further investigations to an extent which demands and justifies several critical remarks. Firstly. The inappropriate definition of remission of Crohn's disease (defined as *Crohn's Disease Activity Index* < 150) is highly subjective, unfair and has underestimated Crohn's Disease cases achieved remission. The use of Crohn's Disease Activity Index has overestimated the number of cases in the placebo group which achieved remission. Therapeutic monitoring can and should be grounded on objective parameters too like C-reactive protein, Erythrocyte sedimentation rate, Procalcitonin (PCT), antibody (Ab) against Mycobacterium avium ssp. Paratuberculosis, faecal calprotectin, colonoscopy and other. Secondly. The abovementioned study presented contradictory outcomes while at the same time it cannot be excluded that subjects were not exposed to the correct dose of drugs. Clofazimine capsules were reencapsulated and it was found that the clofazimine capsules did not rupture in vitro. "Because of this, there was a period of approximately 10 months during which it was possible that subjects were not exposed to the correct dose of clofazimine." (Selby et al., 2007). In contrast to Selby et al. (Selby et al., 2007), Gui et al. (Gui et al., 1997) treated 46 patients with severe Crohn's disease with rifabutin in combination with a macrolide antibiotic (clarithromycin or azithromycin). An improvement in inflammatory parameters was observed and the clinical remission based on the *Harvey–Bradshaw activity index* was induced in

43 (93.5%) patients. The studies concerning chemotherapy with antimycobacterial agents have given mixed or contradictory results in Crohn's disease patients. Thus far, a causal relationship between M. paratuberculosis and Crohn's disease derived from chemotherapy studies has not been demonstrated beyond any reasonable doubt.

4. Discussion

Mycobacterium avium subspecies paratuberculosis is a robust and phenotypically versatile pathogen which is widespread in domestic livestock, present in retail pasteurized cows' milk and potentially, elsewhere. A high-temperature short-time pasteurization of milk appears not effectively to kill M. paratuberculosis in milk (Grant et al., 1998). Even water supplies are also at risk. Mycobacterium avium subspecies paratuberculosis (MAP) has the potential to cause chronic inflammation of the intestine in many species, including primates. Crohn's disease has long been suspected of having a mycobacterial cause. Still, for many years the role of mycobacteria, specifically Mycobacterium paratuberculosis in Crohn's disease has aroused considerable controversy. The inconsistencies throughout the studies on the relationship between Mycobacterium paratuberculosis and Crohn's disease are very great and due to several factors. First of all, it is known that Mycobacterium paratuberculosis is very difficult to be detected by culture and due to the heterogeneous nature of CD the rate of detection of M. avium subsp. paratuberculosis in individuals with CD can vary extremely. The development of highly sensitive and M. paratuberculosis-specific polymerase chain reaction assays and an appropriate in situ hybridization method is highly welcomed. Further technical and methodological advances should allow the identification and/or isolation of M. paratuberculosis from a significantly higher proportion of Crohn's disease tissues compared too controls. Clinical differences in patients studied, including treatment regimens and the duration of disease are not considered in an appropriate manner. Patients studied were analyzed by different methods while the sensitivity of the methods used was not determined. In addition to these inconsistencies, the methodological uncertainty surrounding the role of Mycobacterium avium subsp paratuberculosis (Map) in Crohn's disease is great. The studies analyzed are full of contradictory findings due to small sample size, the lack of uniformity in the materials and methods used by many authors and other factors too. Under such circumstances, is it possible at all to provide new and convincing perspectives (Singh et al., 2011)? One might argue that in line with problems like these, the question is justified whether a decision about a relationship between M. avium subsp. paratuberculosis and Crohn's disease under such conditions is possible at all? Still, the extent to which several studies were able to determine a strong and significant relationship between Mycobacterium avium subsp. paratuberculosis and Crohn's disease reduces the penumbra of uncertainty surrounding the methodological problems of the studies dramatically. The need for such an explanation is especially pressing, since some studies provided data which are extraordinary and cannot be ignored.

Table 9. The data of Zamani et al. (Zamani et al., 2017)

		Crohn's disease (CD)		
		Yes	No	Total
Mycobacterium avium	Yes	18	6	24
subsp. paratuberculosis (MAP)	No	10	56	66
	Total	28	62	90
	Results:			
	$p(A_t \leftarrow B_t)=$	0.8889		$p(A_t \rightarrow B_t)=0.93333333$
$X^2(U \leftarrow W)$ =		**3.223214286**		**$X^2(A_t \rightarrow B_t) = 1.260416667$**
	$k(A_t, B_t)$ =	+0.5717		
	p val (k)=	**5.84534E-08**		

More particularly, according to Zamani et al. (Zamani et al., 2017) Mycobacterium avium subsp. paratuberculosis is necessary condition of Crohn's disease and equally a sufficient condition of Crohn's disease while the cause effect relationship between Mycobacterium avium subsp. paratuberculosis and Crohn's disease is highly significant. The data of are viewed by a 2x2 table (Table 9). In point of fact, i. e. how could Zamani et al. (Zamani et al., 2017) provide such an impressive evidence of the relationship between Mycobacterium avium subsp. paratuberculosis and Crohn's disease? In toto, 6 of 17 studies analysed using the ultra sensitive polymerase chain reaction (PCR) for the detection of M. avium subsp. paratuberculosis within Crohn's disease provided convincing evidence of a conditio sine qua non relationship between M. avium subsp. paratuberculosis and Crohn's disease patients. In other words, *without* M. avium subsp. paratuberculosis *no* Crohn's disease. The involvement of M. avium subsp. paratuberculosis in Crohn's disease (CD) in humans has been uncertain because of several substantial difficulties especially in detecting this pathogen. Still, even if 11 studies failed on the point aformentioned, the question is how could 6 studies document such an evidence. At the same time, M. avium subsp. paratuberculosis and Crohn's disease are causally related. *All studies* analyzed provide convincing evidence that the presence of M. avium subsp. paratuberculosis gurantees the presence of Crohn's disease. In other words**,** *if an infection with M. avium subsp. paratuberculosis is present***,** *then Crohn's disease is present too* ($X^2_{Calculated}$ = 12,2085699 and is less than $X^2_{Critical}$ = 23,6847913, degrees of freedom = 14). Three studies where analyzed according to the rule of three. At the same time, M. avium subsp. paratuberculosis and Crohn's disease were not independent of each other. Additionally, 4 out of 17 studies were able to provide evidence that *M. avium subsp. paratuberculosis is a necessary and sufficient condition of Crohn's disease*. In this context, 17 of 17 studies provided data which are consistent with the conclusion that there is a significant cause and effect relationship between M. avium subsp. paratuberculosis and Crohn's disease. Besides of the very limited number of cases and controls studied, the present study confirms previous reports of the association between M. avium subsp. paratuberculosis and Crohn's disease. Altogether, the results of this study support the hypothesis that *M. avium subsp. paratuberculosis is the cause of Crohn's disease* which implicates the necessity of an effective antibacterial treatment supported by a plant based lactose free diet of this mycobacterial intestinal infection. Once questions raised by methodological inconsistencies and their difficulty are acknowledged, a treatment of Crohn's disease patients with appropriate antibiotics for sufficient duration able kill Mycobacterium avium ssp. paratuberculosis (MAP) is worth being considered. Effective treatment of a mycobacterial infection can be difficult, due to *the structure of the mycobacterial cell wall*. In principle, the mycobacterial cell wall can hinder the entry of drugs and is able to make many antibiotics ineffective. Mycobacterium avium ssp. paratuberculosis (MAP) as the etiologic agent of cattle's paratuberculosis (Johne's disease) lacks its *cell wall* in humans (Naser et al., 2004) while more than 30 strains (Harris et al., 2001) of MAP have been identified. Cell wall deficient bacterial organisms may lack important cell wall antigens more than cell wall competent bacterial organisms, and so are less visible to host immune surveillance and theoretically may survive better than other organism. The human immune system shaped through evolution by the necessity of discriminating non-self pathogens from self tissues and extremely important for the survival of a multicellular organisms uses especially antigens to determine which cells are resident and which are foreign. In the following, antibodies can recognize an antigen and lock onto it without being capable of destroying it without help. Mycobacterium avium ssp. paratuberculosis appears to avoid recognition by the immune system and makes the triggering of immune responses against itself to a very great extent ineffective. Parameters like C-reactive protein, Erythrocyte sedimentation rate, Procalcitonin (PCT), IgG, IgM, IgA antibodies against Mycobacterium avium ssp. paratuberculosis may provide only a weak and approximate picture of the extent of inflammation. In particular, using antibiotic drugs which aim to target the cell wall of Mycobacterium avium ssp. paratuberculosis may lead to complications, the rise of multidrug resistance bacteria and inhibiting of normal flora. At the end, such a strategy could be inefficient for treatment of Crohn's disease patients. The evidence presented in this study based on a systematic review of other studies justify the assumption to treat Crohn's disease with antibiotic. The following or similar Phase I (Table 10) and Phase II (Table 11) drug regimen for the treatment of Crohn's disease patients is still not established. Whether does it makes sense to stop the therapy with Rifaximin 550 mg b.i.d. (i. e. or Metronidazol 400 mg b.i.d. and Ciprofloxacin 500 mg b.i.d.) which is used to control intestinal microbiome dysbiosis and Predinsolon 60 mg q.d. (in this context used to control clinical symptoms) after two months of therapy is another point which is not secured. Diagnostic (or therapeutic) colonoscopy if necessary with the support of a feeding / gastroduodenal tube is needed for therapeutic monitoring and to reduce the microflora of the gut mechanically. The therapy with *drugs free of lactose* (a disaccharide composed of galactose and glucose) like Clarithromycin 500 mg b. i. d., Rifabutin 450 mg q. d. and Colofazimine 50 mg q. d. should continue for at least further 4 months or longer until a complete remission is achieved. Although shortening the duration of therapy is a desirable target, the use of the standard 4-month rifabutin-containing regimen or longer is worth being considered.

Safety evaluation, risk control and therapeutic monitoring should consider at least prameters like C-reactive protein, Erythrocyte sedimentation rate, Procalcitonin (PCT), IgG, IgM, IgA antibodies against Mycobacterium avium ssp. paratuberculosis, faecal calprotectin and of course other too.

The novelty of this study is focused on the causal relationship between Crohn's disease and Mycobacterium avium ssp. paratuberculosis. The hypothesis that Mycobacterium paratuberculosis is the cause of Crohn's disease is older than 100 years (Dalziel, 1913). In particular, it necessary to emphasized that a lot of the controversy (Van Kruiningen, 1999) concerning MAP and CD stems from inconsistent methodology used to detect and isolate MAP, which have questioned the causal (Naser et al., 2014) relationship between Crohn's disease (CD) and cell wall-deficient (CWD) forms of Mycobacterium avium subspecies paratuberculosis (M. paratuberculosis). This study has been able to establish a causal link between Mycobacterium avium subspecies paratuberculosis (MAP) and Crohn's disease beyond any reasonable doubt. Due to small sample sizes and other factors, arguments to the contrary equally have to be considered. Besides of the difficulties mentioned growing evidence determined by all the studies presented suggests that Mycobacterium avium subspecies paratuberculosis is a sufficient condition of Crohn's disease. At the same time, all studies provided evidence of a significant cause effect relationship between and Mycobacterium avium subspecies paratuberculosis (MAP) and Crohn's disease (k~+0.4435, p value < 0.0001). This article provides a review of recent PCR DNA based works on the relationship between and Mycobacterium avium subspecies paratuberculosis (MAP) and Crohn's disease and invites us to consider the following inescapable conclusion.

Table 10. Phase I. The starting 2 months antibiotic regimen to treat MAP in Crohn's disease

Week	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Predinsolon 60 mg q.d.	(x)		(x)		(x)		(x)	
Rifaximin 550 mg b.i.d.	x		x		x		x	
Probiotics / 25 OH Vitamin D3 …		x		x		x		x
(diagnostic or therapeutic) colonoscopy		x		x		x		x
Furthermore:								
Colofazimine 50 mg q.d.	x	x	x	x	x	x	x	x
Clarithromycin 500 mg b.i.d.	x	x	x	x	x	x	x	x
Rifabutin 450 mg q.d.	x	x	x	x	x	x	x	x

Table 11. Phase II. The 4 months and longer antibiotic regimen to treat MAP in Crohn's disease

Month	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Colofazimine 50 mg q.d.	x	x	x	x	x	x	x	…
Clarithromycin 500 mg b.i.d.	x	x	x	x	x	x	x	…
Rifabutin 450 mg q.d.	x	x	x	x	x	x	x	…
(diagnostic or therapeutic) colonoscopy	x	(x)	(x)	x	(x)	(x)	x	…
Probiotics / 25 OH Vitamin D3 …	x			x			x	

5. Conclusion

In this review, data has been presented in the form of tables providing evidence for a cause effect relationship between MAP and CD by PCR. Based on this information, Mycobacterium avium subspecies paratuberculosis (MAP) is definitively the cause of Crohn's disease. Consequently, the need for more sophisticated and optimized antibiotic therapy is required so that CD patient can be cured accurately and definitely.

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