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Is there evidence for a link between Crohn's disease and exposure to *Mycobacterium avium* ssp. *paratuberculosis*?

A review of current literature

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Abstract

Crohn's Disease is characterized by a severe, non-specific, chronic inflammation of the intestinal wall. The inflammation of CD most commonly affects the last part of the ileum, and often includes the colon and sigmoid. CD is a Th1 disease characterized by the production of pro-inflammatory cytokines like TNF-α, which is responsible for development of the lesions, and by the production of IFN-γ and Il-2. CD is a multi-factorial disease; based on epidemiological and geographical observations, several genetic (familial, racial) and environmental (geographic, hygienic) factors (especially microbial) have been associated with the disease. Mutations in the human CARD15 gene and differential expression of Toll-like receptors (TLRs) 2, 3 and 4 have been associated with CD. CARD15 and TLRs are part of the body's innate defence system against bacteria. They activate the immune system after recognizing specific bacterial components. Presence of intestinal bacteria seems to be a prerequisite for CD; the disease cannot develop or perpetuate without the presence of an intestinal flora. One of the bacteria that has been frequently associated with CD is *Mycobacterium avium* subspecies *paratuberculosis (Map). Map* causes a severe chronic intestinal disease in ruminants, Paratuberculosis. CD and Paratuberculosis share several clinical, immunological and histo-pathological characteristics. *Map* is present in many dairy herds and probably can be transmitted to humans via foodstuff. Many investigators have tried to prove, or controvert a common aetiology for CD and Paratuberculosis, and have applied several detection methods to accomplish this. Unfortunately, the quest for *Map* using PCR and culture methods, and the studies on the immune responses against *Map* in CD patients, have yet not resulted in conclusive data to support or discount the hypothesis that *Map* is the etiologic agent of CD. The fact that *Map* can be found in a high percentage of apparently healthy individuals, and that CD patients have significantly higher immune responses against several food antigens compared to healthy individuals, raises the question whether *Map* is a common passer-by of the human intestinal tract, or that a particular cofactor (a genetic aberration) is needed before *Map* can cause disease. The current hypothesis about the patho-physiology of CD is that in a genetic susceptible host, the intestinal flora triggers an aberrant immune response that results in a chronic intestinal inflammation and a damaged (leaky) intestinal mucosal barrier. Although a multi-factorial cause for CD is expected, the possibility, however, that an infectious agent like *Map* can play a key role in the causation of even a sub-set of CD patients remains, and clearly needs to be taken seriously.

Preface

Crohn's disease (CD) is a high burden of disease in humans, and has a dramatic negative impact on the patients' quality of life. Since more than 30 years, numerous researchers have indicated *Mycobacterium avium* ssp. *paratuberculosis* (*Map*) as the possible causative agent of CD in humans. This assumption is predominantly based on the clinical and pathological similarities between CD and the disease 'Paratuberculosis' in ruminants, which is unambiguously caused by *Map*, and the presence of *Map* in a subset of CD patients. In contrast, just as many investigators have discounted *Map* as the cause of CD based on the absence of *Map* in a subset of CD patients, and its presence in many healthy individuals. Nevertheless, since *Map* is an animal pathogen that may be transmitted from animals to humans via milk, meat, water and other foodstuff [37,38,84,145,146], concern has risen about the potential risk of *Map*-contaminated food for the development of CD. In this report we aim to answer the question: 'is there scientific evidence for a causal link between CD in humans and exposure to *Map*?' This report reviews the available information on the nature of CD and its likely causation, especially concerning a possible link with exposure to *Map*. Furthermore, the results of an extended cohort study in 59 CD patients and 79 control persons, conducted at the RIVM, are summarized. On the basis of what is known to date about CD and *Map* we will substantiate whether a link between *Map* and CD is conceivable.

Contents

Samenvatting

Doel van dit rapport

Mycobacterium avium ssp. *paratuberculosis* (*Map*) wordt door veel onderzoekers beschouwd als mogelijke verwekker van de ziekte van Crohn (morbus Crohn, MC) bij de mens. Dit is vooral gebaseerd op klinische en pathologische overeenkomsten tussen MC en de ziekte Paratuberculose bij runderen (herkauwers), die zonder twijfel veroorzaakt wordt door *Map*, en de aangetoonde aanwezigheid van *Map* bij een deel van de patiënten met MC. Echter, evenzoveel onderzoekers zijn van mening dat *Map* niet de verwekker is van MC omdat Paratuberculose en MC ook verschillen in een aantal kenmerken. *Map* kan bijvoorbeeld niet worden aangetoond bij alle MC patiënten. Verder kan *Map* vaak wel worden aangetoond bij een aanzienlijk deel van de onderzochte gezonde personen. Omdat *Map* niettemin een ziekteverwekker is die via melk, vlees, water en andere levensmiddelen de consument kan bereiken, bestaat er ernstige bezorgdheid over het mogelijke risico van dit *Map*-besmet voedsel voor het ontstaan van MC bij de consument. In dit rapport wordt een antwoord gegeven op de vraag ‘is het verband tussen de verwekker van Paratuberculose, *Mycobacterium avium* ssp. *paratuberculosis*, en de ziekte van Crohn overtuigend bewezen?’ De huidige wetenschappelijke kennis over MC wordt beschreven, waarbij het accent wordt gelegd op de informatie die direct of indirect betrekking heeft op, of een indicatie kan zijn voor een mogelijke relatie tussen MC en *Map*.

De ziekte van Crohn

Nederland telde in 2000 tussen de 27.000 en 56.000 mensen met de chronische darmontsteking ‘Inflammatory Bowel Disease’ (IBD) [97]. Hiervan stierven in 2000 in totaal 60 personen aan de gevolgen van deze ziekte [97]. De twee vormen van IBD zijn Colitis Ulcerosa en Morbus Crohn (MC, de ziekte van Crohn). Elk jaar worden in Nederland ongeveer 2.000 nieuwe patiënten gediagnosticeerd met IBD [97]. Vooral de incidentie van de MC lijkt toe te nemen. De meeste patiënten worden door MC getroffen in de leeftijdsfase tussen 15 en 35 jaar. MC is een niet-specifieke, chronische ontsteking van het maagdarmkanaal. De ziekte heeft een zeer dramatische invloed op het fysieke- en sociale leven van de patiënt, en is niet te genezen. Medicijnen die worden gebruikt voor de behandeling zijn er vooral op gericht om de ontstekingsreacties en de symptomen van de ziekte te onderdrukken.

Achtergrondinformatie over MC

MC kan op verschillende manieren tot uiting komen. Bij de meeste patiënten is het laatste deel van de dunne darm (terminale ileum) ontstoken, en vaak is ook de dikke darm (colon) aangetast. Minder vaak zijn andere delen van het maagdarmkanaal erbij betrokken, zoals mond, slokdarm (oesophagus) en twaalfvingerige darm (duodenum), en soms worden ook

ontstekingen buiten het darmgebied aangetroffen, zoals oogontstekingen en gewrichtsontstekingen. Het is evident dat MC niet een eenduidig en gelokaliseerd ziektebeeld geeft en daarom soms moeilijk is te diagnosticeren. Afhankelijk van de plaats van de ontstekingen en de aard van de afwijkingen wordt de ziekte in verschillende categorieën ingedeeld.
Bij ontstekingen speelt het afweersysteem een belangrijke rol. Het afweersysteem beschermt het lichaam tegen binnendringen van ziekteverwekkers en andere lichaamsvreemde stoffen. Er is een aangeboren en een verworven afweersysteem. Het aangeboren afweersysteem onderneemt actie tegen indringers voordat een specifieke afweerreactie (door het verworven afweersysteem) op gang komt, en maakt daarbij gebruik van speciale structuren (eiwit- en suikerstructuren) die aanwezig zijn bij micro-organismen (bacteriën, virussen) die proberen binnen te dringen. In tegenstelling tot het aangeboren deel is het verworven afweersysteem specifiek gericht op één bepaalde lichaamsvreemde stof, en is opgebouwd uit een cellulair en een humoraal deel. Om een goed geheugen tegen lichaamsvreemde stoffen te verwerven maken het cellulaire en humorale afweersysteem gebruik van T-helper (Th) cellen die deze afweerreactie besturen en coördineren. Er zijn twee belangrijke soorten T-helper (Th) cellen:

- Th1 cellen, die de afweerreactie sturen in de richting van het afdoden van door virussen of bacteriën geïnfecteerde cellen.
- Th2 cellen, die de afweerreactie sturen in de richting van antilichaamproductie. Th2 cellen spelen een belangrijke rol bij het afdoden van nog niet door macrofagen opgenomen virussen en bacteriën, onder andere bij acute infecties.

Bij gezonde personen is er een balans in de sturing door deze Th1/Th2 cellen. In de darm van gezonde personen ligt de balans naar de Th2 kant. Bij MC patiënten blijkt echter een verstoring te zijn opgetreden, waardoor er een te hoge activiteit van de Th1 cellen is ontstaan. Deze Th1 cellen sporen macrofagen aan om stoffen (cytokines) te produceren die ontstekingsreacties en weefselschade veroorzaken. Enkele van deze cytokines die een belangrijke rol spelen in MC zijn: Interferon gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-α) en Interleukine 6 (Il-6). Vooral TNF-α is verantwoordelijk voor de weefselschade bij MC.

Factoren betrokken bij MC

Sinds 1913, toen MC voor het eerst werd beschreven, zijn er allerlei hypotheses opgesteld over de mogelijke oorzaak van de ziekte. Bestudering van epidemiologische en geografische gegevens, die te maken hebben met het optreden en vóórkomen van MC, hebben geleid tot het identificeren van twee belangrijke elementen die betrokken zijn bij het ontstaan van MC: erfelijke factoren en omgevingsfactoren.
Dat erfelijke factoren betrokken zijn bij MC blijkt uit een relatie met:

- Bloedverwantschap: iemand die een eerstegraads bloedverwant heeft met MC heeft een grotere kans om de ziekte ook te krijgen dan iemand die geen bloedverwant met MC heeft. Dat blijkt vooral bij eeneiige tweelingen, waar de ziekte bij allebei vaker voorkomt dan bij twee-eiige tweelingen.

- Etnische achtergrond: MC komt vaker voor bij blanke mensen dan bij gekleurde mensen, en vaker bij joodse mensen dan bij niet-joodse mensen.

De betrokkenheid van omgevingsfactoren blijkt uit:

- De afgelopen 50 jaar is er een toename van MC te zien in het aantal nieuwe gevallen per jaar.
- Er is een geografische noord-zuid gradiënt te zien in het aantal MC gevallen per jaar. In noord-west Europa komt MC vaker voor dan in zuid-Europa, en vaker in Europa dan in Azië en Afrika.
- Mensen die vanuit een streek waar MC nauwelijks voorkomt emigreren naar een streek waar MC veel voorkomt, krijgen dezelfde kans om MC te ontwikkelen als de plaatselijke bevolking van die streek.
- Kinderen die opgroeien onder goede hygiënische omstandigheden lijken meer kans te lopen later MC te krijgen.
- Rokers hebben een verhoogde kans om MC te krijgen.

Door de grote verscheidenheid aan factoren die betrokken lijken te zijn bij MC en het feit dat tot nu toe geen individuele factor gevonden is die doorslaggevend is voor het ontstaan van de ziekte, wordt MC gezien als een multi-factoriële ziekte, waarbij het optreden van verschillende factoren samen bepalen of een persoon de ziekte ontwikkelt of niet.

Erfelijke factoren

De aanwijzingen voor betrokkenheid van erfelijke factoren bij het ontstaan van MC hebben onderzoekers ertoe gebracht op zoek te gaan naar afwijkingen in het erfelijk materiaal (het genoom, de genen op de chromosomen) van MC patiënten. Inmiddels zijn minstens zeven plaatsen in het genoom geïdentificeerd waar mogelijk afwijkingen (mutaties) optreden die leiden tot een verhoogd risico op het ontwikkelen van MC. De belangrijkste mutaties die tot nu toe ontdekt zijn liggen in het CARD15 gen en deze mutaties komen bij 15-20% van de MC patiënten voor. Het CARD15 gen codeert voor het NOD2 eiwit. Dit eiwit is een onderdeel van het aangeboren afweersysteem van het lichaam, en speelt een rol bij de afdoding van door de cel opgenomen bacteriën. Een ander onderdeel van het aangeboren afweersysteem waarbij afwijkingen zijn gevonden in MC patiënten zijn de Toll-like receptoren. Evenals CARD15 herkennen Toll-like receptoren speciale structuren bij microorganismen en zetten het specifieke afweersysteem in gang. Het feit dat bij MC patiënten afwijkingen zijn aangetroffen in twee verschillende onderdelen van het aangeboren afweersysteem tegen microorganismen (CARD15 en Toll-like receptoren) doet vermoeden dat erfelijke factoren die betrokken zijn bij de herkenning van microorganismen mede het ontstaan en/of het voortduren van MC bepalen.

Darmbacteriën

De theorie dat micro-organismen, en dan vooral de darmbacteriën (darmflora), een cruciale rol spelen bij MC wordt ondersteund door de waarneming dat proefdieren (muis en rat) geen

darmontsteking ontwikkelen als ze worden gekweekt in een steriele omgeving (waarin ze geen darmflora hebben). Verder bleek dat bij MC patiënten met een stoma waarbij een deel van de aangetaste darm afgesloten werd van voedseldoorstroming (en dus de darmflora ter plaatse verdween) na 6 maanden geen MC meer optrad, terwijl dat in controle MC patiënten, waar wel een normale darmflora aanwezig is, wel het geval was. Nadat het stoma was verwijderd en de darm weer een darmflora kreeg, ontwikkelde zich bij alle ex-stoma patiënten toch weer CD-achtige ontstekingen.

Mycobacterium avium ssp *paratuberculosis (Map)*

De bacterie die het meest met MC in verband wordt gebracht is *Mycobacterium avium* ssp. *paratuberculosis* (*Map*). *Map* hoort tot de familie van de *Mycobacteriacae,* waartoe ook *Mycobacterium tuberculosis* (de veroorzaker van tuberculose), *Mycobacterium leprae* (de verwekker van lepra) en *Mycobacterium avium* (deze bacterie veroorzaakt vaak infecties bij AIDS patiënten) behoren. Vooral de verwantschap tussen *Map* en *M. avium* is erg hoog. *Map* veroorzaakt in ieder geval Paratuberculose, een chronische darmontsteking bij herkauwers (rund, schaap, geit). De klinische, immunologische en pathologische verschijnselen van Paratuberculose lijken sterk op die van MC. Het is dan ook niet verwonderlijk dat onderzoekers op zoek zijn gegaan naar *Map* bij MC patiënten.

Detectie van Map

Verschillende methoden zijn toegepast om de betrokkenheid van *Map* bij MC aan te tonen:

- Isoleren en kweken van *Map* bacteriën uit weefsels van MC patiënten.
- Aantonen van *Map* in weefsels met behulp van moleculaire detectiemethoden zoals PCR en *in-situ* hybridisatie.
- Aantonen van een afweerreactie tegen *Map* in MC patiënten.
- Gebruik van antibiotica die werkzaam zijn tegen mycobacteriële infecties.

In de afgelopen 20 jaar zijn meer dan 40 publicaties verschenen die betrekking hebben op de aanwezigheid van *Map* in MC patiënten. De resultaten van die verschillende onderzoeken zijn in drie categorieën in te delen:

1. *Map* werd significant vaker aangetroffen bij MC patiënten dan bij personen zonder MC (controle personen).
2. *Map* werd even vaak aangetroffen in MC patiënten als in controle personen.
3. *Map* werd niet aangetroffen in MC patiënten en niet in controle personen.

In de meeste onderzoeken werd *Map* zowel bij een deel (tot 90%) van de MC patiënten als bij een deel (tot 40%) van de controle personen aangetoond. Het blijft hierbij vreemd dat als *Map* de enige oorzaak zou zijn van MC, deze bacteriën niet kunnen worden aangetoond bij alle CD patiënten, en dat *Map* ook bij veel gezonde personen wordt aangetroffen.
Mogelijke reden hiervoor zijn:

- *Map* is altijd betrokken bij MC, maar alleen bij het ontstaan van de ziekte. Na binnendringen van *Map* komt het afweersysteem op gang en wordt *Map* snel opgeruimd. Echter door een (genetisch) defect in het afweersysteem is er als het ware een defect aan de rem die het systeem weer tot rust moet brengen; het systeem slaat op hol met MC als gevolg.
- *Map* is niet altijd betrokken bij MC. Ook andere factoren (microorganismen) spelen een belangrijke rol.
- *Map* is betrokken bij MC maar kan niet altijd worden aangetoond. Soms zijn de aantallen bacteriën in een geïnfecteerde patiënt zo laag dat er niet altijd voldoende van aanwezig zijn in het weefselmonster dat voor analyse wordt gebruikt. In dat geval geldt: hoe meer weefselmonsters er geanalyseerd worden, hoe groter de kans is dat de bacterie wordt aangetroffen.
- *Map* is helemaal niet betrokken bij MC. De reden dat *Map* aangetroffen wordt in het darmkanaal van MC patiënten en controle personen komt doordat *Map* een toevallige passant van de darm is. Mycobacteriën komen vrij algemeen in de natuur voor en kunnen dus gemakkelijk via het voedsel (al dan niet in levende vorm) worden aangevoerd. Dat *Map* in sommige onderzoeken vaker in MC patiënten wordt aangetroffen dan in controle personen kan komen doordat de darm van MC patiënten zodanig is aangetast dat *Map* zich er gemakkelijker in kan nestelen. In dit geval is dus de aanwezigheid van *Map* niet de oorzaak van MC, maar het gevolg.

Afweerreacties tegen *Map*

In alle onderzoeken die tot nu toe zijn uitgevoerd naar antilichamen tegen *Map* worden eiwitten gebruikt die niet specifiek zijn voor *Map,* maar ook voorkomen bij infecties met andere mycobacteriën. Als er dus in MC patiënten een afweerreactie gemeten wordt tegen '*Map'*, dan betekent dit dat de patiënt ook geïnfecteerd zou kunnen zijn (geweest) met andere, nauw verwante mycobacteriën zoals *M. avium*. In de meeste onderzoeken worden antilichamen aangetoond tegen mycobacteriën in zowel een deel van de MC patiënten als een deel van de controle personen. Antilichamen tegen *Map* worden dus niet in alle MC patiënten aangetroffen. Zoals eerder vermeld is in deze ziekte vooral het cellulaire afweersysteem van belang en minder het humorale (antilichamen). Het is daarom de vraag in hoeverre de aanwezigheid van specifieke antilichamen tegen *Map* in het serum een belangrijke parameter is in de rol die *Map* wellicht speelt in deze ziekte. Bovendien werd in alle onderzoeken alleen gekeken naar afweerreacties in het bloed van patiënten. Bekend is dat er in de darm lokale afweerreacties optreden die niet terug te vinden zijn in het bloed. Het zou dus beter zijn om specifiek de ontstoken darm te onderzoeken op afweer tegen *Map* (of elke andere mogelijke veroorzaker van MC) en de aanwezigheid van specifieke IgA antilichamen te bepalen en niet het perifere bloed als bron te gebruiken.

Intolerantie en een lekkende darm

De meest recente hypothese over het ontstaan en voortduren van MC gaat uit van het idee dat er drie factoren bij MC betrokken zijn:

- Erfelijke aanleg.
- Prikkeling van de darm door de darmflora.
- Afwijkende afweerreacties in de darm.

Dus in een persoon die daar genetisch gevoelige voor is wekken één of meerdere componenten van de darmflora een abnormale afweerreactie op die leidt tot de chronische darmontsteking die karakteristiek is voor CD. Dit proces gaat gepaard met een lekkend darmslijmvlies (mucosa) waardoor allerlei voedselcomponenten en microorganismen gemakkelijker kunnen binnendringen, en op hun beurt ook weer voor een afweerreactie kunnen zorgen. Het afweersysteem in de darm wordt voortdurend geprikkeld door alle mogelijke antigenen (dit zijn stoffen die een afweerreactie opwekken, zoals micro-organismen) die door het voedsel worden aangevoerd. Afweercellen staan permanent paraat om snel schadelijke antigenen te neutraliseren. Aan de andere kant moeten gunstige of niet schadelijk antigenen, zoals die van de darmflora wel worden getolereerd. In gezonde personen is daarom in de darm permanente paraatheid van het afweersysteem noodzakelijk, zonder dat het evenwicht omslaat naar de verkeerde kant. Als er een ontregeling plaatsvindt in het evenwicht tussen tolerantie van, en afweer tegen antigenen kan in genetisch gevoelige personen een ongecontroleerde reactie ontstaan die leidt tot een chronische ontsteking van de darm. Ontregeling van het evenwicht in de darm zou kunnen ontstaan als gevolg van een veranderde samenstelling van de darmflora, bijvoorbeeld door verhoogde koolhydraatinname, roken en andere milieufactoren. Opvallend is bijvoorbeeld dat zeer veel MC patiënten antilichamen hebben tegen bakkersgist (*Saccharomyces cerevisiae*). Dit duidt erop dat er bij MC patiënten een verminderde tolerantie is tegen normale voedselantigenen zoals bakkersgist, doordat deze als gevolg van een lekkend darmslijmvlies gemakkelijker tot de diepere lagen van de darm kunnen doordringen.

Conclusie

In dit review laten we zien dat er veel verschillende factoren betrokken zijn bij MC. Tot nu toe is echter geen enkele factor gevonden waarvan onomstotelijk bewezen is dat deze individueel verantwoordelijk is voor het ontstaan van MC, of die in samenwerking met andere factoren als een causatief agens kan worden aangemerkt; dit geldt ook voor *Map*. Wel bestaat de reële mogelijkheid dat *Map*, in samenwerking met andere factoren, als trigger betrokken is bij een deel van de MC patiënten. Bijvoorbeeld door:

- de darmwand te beschadigen en daarmee een afwijkende afweerreactie op te wekken.
- van de beschadigde (lekke) darmmucosa gebruik te maken om binnen te dringen en in een daarvoor gevoelige persoon een chronische infectie te veroorzaken.

- als normale, niet pathogene darmpassant gebruik te maken van een ontstoken darm om zich in te nestelen en te vermeerderen.

Hoewel MC gezien wordt als een ziekte waarbij meerdere factoren samen een rol spelen bij het ontstaan en voortduren van de ziekte, blijft de kans aanwezig dat in een deel van de MC patiënten een infectieus organisme zoals *Map* wel een hoofdrol speelt. Ondanks het feit dat een duidelijk verband tussen MC en *Map* (nog) niet is bewezen blijft het dus noodzaak om hun mogelijke relatie serieus te nemen.

1. Crohn's disease

Inflammatory bowel disease (IBD) is recognized as an important gastrointestinal disease in human adolescents and adults. IBD exist can manifest in two major forms, Crohn's disease (CD) and ulcerative colitis (UC). Both diseases are characterized by a chronic inflammation of the intestinal wall. CD is a non-specific chronic inflammation, which extends through all the layers of the intestinal wall, and may involve the mesentery as well as the regional lymph nodes. The inflammation of CD most commonly affects the last part of the ileum, and often includes the colon and sigmoid. In more rare cases other segments of the alimentary tract (mouth, oesophagus, stomach and duodenum) are affected, but this usually occurs in association with involvement of the distal ileum. CD may be diagnosed at any age, although most diagnoses are made between the age of 15 to 35. Men and women have an equal chance to develop the disease. Figures from the US indicate that 20% of cases are diagnosed before the age of 20, and 40% between 20-29 [134]. The prevalence (existing cases) of CD in Europe varies between 10 to 200 cases per 100,000 persons (Appendix 1, Table 2). The incidence (new cases) of CD in southern Europe ranges from 1-4 cases per 100,000 persons per year, and in northern Europe from 4-10 cases per 100.000 persons per year (Appendix 1, Table 1) [132]. In the Netherlands, the prevalence of IBD in 2000 was estimated between 10.000 and 21.000 cases for CD and between 17.000 and 35.000 for UC; mortality in 2000 was determined at 34 cases for CD and 26 for UC [97]. There are somewhat indications that the incidence of CD is increasing, especially in low incidence areas [11,97].

Early pathological features of CD include hyperaemia and oedema of the intestinal mucosa and of the mesentery, associated with enlarged inflammatory lymph nodes. Another typical, pathological lesion of CD is a mucosal ulceration, commonly located in areas with Peyer's patches in the small intestine and over the lymphoid aggregates in the colon. Later during the course of the disease the ulcerations tend to enlarge and to coalesce. Histologically, CD is characterized by lymphoid infiltration of the submucosa and to a lesser degree of the muscularis mucosa. Moreover, non-caseating granulomas are often present in the intestinal mucosa and submucosa, as well as in the mesentery, peritoneum and lymph nodes. The predominant clinical symptoms observed in CD patients are diarrhoea, abdominal pain and weight loss. The clinical course of CD in most patients is characterized by periods of relapses and remissions. Although spontaneous improvement and disappearance of clinical symptoms may be seen in some patients without medical or surgical treatment, well over 60% of all patients with CD will require surgery at one or another phase during the course of their disease, and about half of these patients will require more than one operation over time. About 5-10% of all CD patients will die of their disease, primarily due to massive infection [63].

The pathology and epidemiology of CD share many features with those of UC, though they can be distinguished on histological and immunological characteristics:

- The inflammation of CD may be discontinuous, meaning that areas of involvement in the intestine may be separated by normal, unaffected segments of intestine.

- The inflammation of CD affects all layers of the intestinal wall, while ulcerative colitis affects only the inside layer of the intestine. Also, ulcerative colitis is usually limited to the rectum without extension to the other colon segments or the ileum.
- Immunologically, CD is a Th1 disease, whereas UC is a Th2 disease (this will be discussed later).

CD lesions occur predominantly where there is a high density of lymphoid follicles: in the small bowel where the follicles are grouped in Peyer's patches, and in the colon where they are isolated. In the small intestine, lesions are more frequent in the distal ileum where Peyer's patches are most present. In addition to this spatial relationship, a temporal relationship has also been suggested [228]. Peyer's patches are more numerous in younger individuals and become less prominent with age [4]. Interestingly, the age-dependent incidence curve for CD is roughly parallel to the number of Peyer's patches with a peak in the third decade of life [100]. A delay of 5–10 years is seen between the development of Peyer's patches and the occurrence of symptoms. Such a delay is compatible with chronic infections. Conversely, the ileal location of CD is less frequent in elderly people after the involution curve of Peyer's patches [174]. In the colon, in contrast with the small intestine, the number of lymphoid follicles is less subject to variation. This observation can account for the fact that colonic CD can occur at any age [100]. At present there is no cure for the disease, which tends to pursue a variable course, characterized by periods of activity interspersed with remissions, when the disease is either absent or relatively quiescent. A range of factors is thought to increase the risk of relapse of quiescent disease, including stress and dietary factors. Many therapies have been tried to induce or maintain remissions. Typically, treatment is by a combination of medical therapies (anti-inflammatory drugs to reduce the inflammation, drugs to treat the symptoms, and antibiotics to reduce infections associated with the disease), attention to diet to control weight loss, and surgery to deal with the structural effects of the inflamed intestines. Although only carried out as a last resort, a significant proportion of patients needs a colostomy or an ileostomy. It is clear that CD has a dramatic negative impact on the patients' quality of life and frequently also has serious consequences for the rest of their family.

- *Summary: CD is an incurable chronic inflammation of the human intestinal wall. CD has a dramatic negative impact on the patient's quality of life. CD occurs particularly in the Western community.*

2. Features of CD

2.1 Classification of CD

It is becoming more and more recognized that CD is not a homogeneous pattern of disease but a variety of different patterns of inflammatory disease of the intestinal tract [87]. Previous attempts of classification have been based primarily on anatomic location and behaviour of disease. During the World Congresses of Gastroenterology in Vienna in 1998, an international Working Party has developed a simple classification of CD based on objective variables [71]. Eight outcome-related variables relevant to CD were identified and evaluated in detail. Three variables were finally chosen: 'Age at Diagnosis' (below 40 years, A1 ; equal to or above 40 years, A2), 'Location' (terminal ileum, L1 ; colon, L2; ileocolon, L3; upper gastrointestinal, L4), and 'Behaviour' (non-stricturing non-penetrating, B1; structuring, B2; penetrating, B3). This Vienna classification of CD provides distinct definitions to categorize CD patients into 24 subgroups (2 x 4 x 3) or disease clusters. A cross-table analysis reveals associations between Age at Diagnosis and Location, and between Behaviour and Location (all $p < 0.001$) [71]. However, the initial 'behavioural' classification of B1 (non-stricturing non-penetrating) at the onset of CD hardly ever remains stable over the lifetimes of the patient but almost invariably progresses in time to either B2 (stricturing) or B3 (penetrating) disease.

- *Summary: in terms of pathological criteria, CD is not a homogeneous pattern of disease and can be categorized into 24 subgroups.*

2.2 Immune responses in CD patients

The present knowledge about the immunological mechanisms involved in perpetuation of the chronic inflammation in CD is that the mucosa of patients with established CD is dominated by CD4+ lymphocytes with a Th1 phenotype, characterized by the production of cytokines like Interferon gamma (IFN-γ) and Interleukin-2 (Il-2) [173]. In contrast, the mucosa in ulcerative colitis is dominated by CD4+ lymphocytes with a Th2 phenotype, characterized by production of Transforming Growth Factor beta (TGF-β) and Il-5 [173]. In Crohn disease the Th1 cytokines activate macrophages, which in turn produce Il-12, Il-18, and macrophage migration inhibitor factor and thus further stimulate Th1 in a self-sustaining cycle [137,139,183]. Macrophages produce a potent mix of broadly active inflammatory cytokines, including Tumor Necrosis Factor (TNF), Il-1 and Il-6; in CD, Il-6 levels correlate with the severity of the disease [136]. Recruitment of additional leukocytes from the vascular space to sites of disease activity is especially important in maintaining inflammation. In CD, TNF-α is the key pro-inflammatory cytokine in the development of the lesions and in the persistence of IFN-γ production by Th1-cells [4]. On the other hand, production of anti-inflammatory cytokines such

as Il-10, Il-4 and Il-13 seems deficient or at least insufficient to counteract the pro-inflammatory loop of the immune response [4,137,159].

- *Summary: CD is a Th1 disease characterized by the production of pro-inflammatory cytokines like TNF-α, which is responsible for development of the lesions, and by the production of IFN-γ and Il-2.*

2.3 Aetiology of CD

Since Thomas Dalziel's initial description of CD in 1913, many ideas have been postulated about the cause of the disease [45]. Based on epidemiological and geographical observations, two major factors were identified that contribute to the development of IBD: genetic factors and environmental factors.

Genetic factors
At present, substantial evidence is available that suggest genetic factors are important determinants of susceptibility and disease behaviour in IBD. Reports of familial clustering of IBD date back to the 1930's. Concordance rates in siblings and monozygotic twins suggest that the genetic contribution to disease pathogenesis is at least equivalent to that in other common immune mediated diseases (Multiple Sclerosis, insulin-dependent Diabetes Mellitus). First-degree relatives of an affected patient have a risk of IBD that is 4 to 20 times as high as that among the background population; the absolute risk of IBD is approximately 7% among first-degree family members of IBD patients [173]. A substantial higher rate of disease concordance has been observed in monozygotic twins than in dizygotic twins, especially in those with CD [173]. A positive family history is more common with CD than UC, and relatives of patients with CD have a higher risk of developing IBD than do relatives of patients with UC [173]. Furthermore, Caucasian people are more frequently affected than other racial groups, and the incidence of IBD among Jewish people compared with non-Jewish are 16.8% versus 7.0%, respectively, for CD and 4.6% versus 0.9%, respectively, for UC. Within Jewish populations, Ashkenazi (Eastern European) Jews have a higher incidence of IBD than Sephardic (Middle Eastern, African, or Spanish) Jews. These data strongly suggest a genetic background for IBD; so persons can have a predisposition to CD. However, the absence of a simple mendelian inherited pattern of disease suggest that IBD is a polygenic disease, and that multiple gene products, whether or not in combination with environmental factors, contribute to a person's risk of IBD [173].

Environmental factors
The incidence of CD has increased in developed countries over the past 50 years [205]. There is a slight north to south gradient of IBD incidences worldwide as well as in Europe; for instance, the incidence rates in Scandinavia, UK and the Netherlands are 2-3 fold higher compared to southern Europe (Appendix 1, Table 1)[132,206]. In developing countries infectious

intestinal diseases are common, whereas IBD's, especially CD, are rare. However, the incidence and prevalence of IBD are beginning to rise in low-incidence areas such as southern Europe, Asia, and much of the developing world [132]. Since people who have immigrated from regions with a low IBD prevalence to regions with a high prevalence acquired the same disease prevalence as the local population [94,132] there is little doubt that environmental factors are involved: Western lifestyle and hygiene. This is supported by the finding that good domestic hygiene in infancy has been shown to be a risk factor for CD, but not for UC [63,72]. The risk of developing CD was shown to be increased 3-fold if a separate toilet is available and 5-fold if there is hot tap water in the household [63,72]. Furthermore, CD and UC patients in childhood were less likely to have been breast-fed [112] and less likely to have ingested unpasteurised milk. CD seems to occur more frequently in members of small families. Since intra-familial transmission of common pathogens is frequent, the single child is particularly prone to be raised under more hygienic conditions with lower risk of acquiring gut infections [74,171]. Most likely these various factors associated with the incidence of CD serve as indicators of a rather clean environment, leading to a diminished confrontation with (non)-pathogenic micro-organisms. As a result, the intestinal innate immune system is probably not trained to confront minor infections without recruiting the full array of specific immune functions that act only at the expense of a relevant inflammation [63]. Another interesting new aspect of the hygiene hypothesis is the decline in infections from helmintic parasites in the developed world, which is in line with more hygienic conditions. Helmintic infections are mechanistically associated with the rise in prevalence of CD [60,205]. Helminths are associated with a Th2 response that would counterbalance the Th1 response of CD. There are however also publications that indicate that infections in childhood increase the chance of IBD. Babies with a recorded 'prenatal health event' (i.e., infection or serious illness in mother or child) had a four-fold increased risk for IBD, and infants from families of low socio-economic status (often associated with low hygiene) were three times more likely to develop IBD later in life. Furthermore, several studies have noted a higher frequency of gastroenteritis or diarrhoeal illness during infancy among future IBD patients. Unfortunately, in many retrospective population studies, recall bias is of great concern. Nevertheless, despite these somewhat controversial data, micro-organisms seem to play an important role in development of IBD, and perhaps also in preventing from IBD. CD has also been associated with increased intake of carbohydrates (refined sugar) [179] and oral contraceptives. The strongest environmental factors identified so far are cigarette smoking and appendectomy [19,55,132]. Whereas smokers have a significantly decreased risk of UC, they have an increased risk of CD, and appendectomy appears to be protective for the development of UC, whereas it is associated with future risk of CD [132,189].

- *Summary: CD is a multi-factorial disease. Several genetic (familial, racial) and environmental (geographic, hygienic) factors -especially microbial- have been associated with the disease.*

3. The role of genetic factors in CD

3.1 CARD15/NOD2 mutations and CD

At present, at least seven genomic regions located on chromosomes 1, 5, 6, 12, 14, 16 and 19 are thought to be involved in the predisposition to IBD [18,34,54,90,91,101,180,181]. Thus far, most emphasis is put on mutations found in the CARD15 gene, located on chromosome 16q12. CARD15 encodes NOD2, a cytoplasmic protein that is mainly expressed in monocytes/macrophages and granulocytes, but has also been identified in epithelial cells [77,173]. Three main mutations in the CARD15 gene (R702W, G908R and 1007fsinsC/3020insC) have been identified that are carried by 20-25% of Caucasian CD patients and only 7-15% of healthy controls [101,102,163]. Interestingly, mutated homozygotes and compound heterozygotes (double dose mutation carriers) are significantly more frequent than expected by chance in CD patients (15-20%), which demonstrates a mutation dose effect [102]. However, so far no association between CD and CARD15 has been found for Japanese patients, suggesting that a subgroup of CD is not related to a dysfunction of CARD15 [104,247], and little is known about presence of CARD15 mutations in other ethnic groups. The frameshift mutation (1007fsinsC) of the CARD15 gene is associated with ileal involvement and the fistulizing or fibrostenotic phenotype, which are characterized by an increased expression of Th1 cytokines, such as TNFα. CARD15 is a component of the innate immune system. Innate immunity is the most ancient system of defence against pathogens in humans and animals. This system takes action against an invading organism before a specific, acquired response has been established towards that particular organism. The innate immune system relies on the expression of a restricted set of receptors that enable the recognition of highly conserved microbial motifs, often also named ‘pathogen-associated molecular patterns’ (PAMPs); these PAMPs are not present on the host cell. PAMPs are generally structural bacterial components that display little or no variation because of selection pressure. In bacteria, examples of such PAMPs include lipopolysaccharides (LPS), lipoproteins, flagellin, CpG DNA and peptidoglycan [77,130]. Peptidoglycan is a major constituent of the cell wall of Gram-positive bacteria. In Gram-negative bacteria a thin layer of peptidoglycan is located in the periplasmic space [77]. The 1007fsinsC frameshift mutation, the most frequent CARD15 variant associated with CD, fully abrogates NOD2-dependent detection of peptidoglycan [76].

NOD2 plays a role in the intracellular killing of bacteria with its function as an intracellular receptor for PAMPs and activates the ‘nuclear factor-kappa B’ (NF-κB) pathway following inflammatory stimuli, which results in increased transcription of proinflammatory cytokines [211]. NF-κB plays a central role in the cellular immune system. The C-terminal domain of CARD15 is required for bacterial PAMP dependent activation of NF-κB activity [16,163]. The three main CD associated mutations are located within this C-terminal domain, suggesting that an important causative factor in CD may be an initial defect in the response to bacterial

PAMPs. This loss of function model, also suggested by the dosage effect observed for CARD15 mutations, has been confirmed in experiments with cells transfected with the 1007fsinsC mutated CARD15 gene, where mutated NOD2 protein had a lower ability to respond to PAMPs and to induce NF-κB activation [16].
In CD lesions, an excessive stimulation of the NF-κB pathway is observed. This seems in contradiction to the functional model of reduced NF-κB activation due to a mutated CARD15 gene. However, this paradox between the *in vivo* situation in CD and reduced NF-κB activity *in vitro* is counterbalanced by Toll-like receptors. This will be discussed in the next paragraph.

- *Summary: mutations in the human CARD15 gene have been convincingly associated with CD. CARD15/NOD2 is part of the body's innate defence system against bacteria. It activates the immune system after recognizing bacterial components.*

3.2 Toll-like receptors and CD

Recently, an additional component of the innate immune system was discovered in mammals, the Toll-like receptors (TLRs). Toll-like receptors are related to the Toll protein, a molecule that is implicated in defence against fungal infection in the fly [126,158]. Toll-like receptors are anchored in the cell membrane. Out of the ten TLRs present in the human genome, at least eight detect PAMPs at the cell surface. TLRs are thought to play a significant role in IBD.
In patients with active IBD, TLR4, TLR3 and TLR2 are differentially modulated compared to control patients [25]. In biopsies from patients with CD or UC, an abundant expression of TLR4 by epithelial cells and lamina propria cells has been reported. In contrast, TLR4 is only minimally detectable in intestinal epithelial cells (IECs) of non-IBD intestines [25]. TLR4 recognizes LPS from Gram-negative bacteria [217]. Luminal LPS is usually well tolerated in large quantities within the healthy intestine. It has been shown that mice with a single point mutation in their TLR4 gene are highly susceptible to developing a more severe form of induced colitis.
In active CD biopsy specimens, the epithelial expression of TLR3 was significantly decreased in the IECs of the colon, compared with specimens from UC patients and normal controls [25]. TLR3 is constitutively expressed in the IECs of UC patients and controls. Reduced expression of TLR3 on IECs seems to be consistent in active CD, independent of location or inflammatory activity. So reduced TLR3 does not simply reflect the local effect of some inflammatory mediator.
TLR2 is involved in responses to Gram-positive bacteria, yeast, and LPS from Gram-negative bacteria [109,217,224,237], and is suggested to be essential for the induction of a protective immune response to Mycobacteria [224]. TLR2 is present in monocytes, polymorphonuclear phagocytes, dendritic cells and epithelial cells [158]. Stimulation of cells expressing TLR2 with LPS strongly activates the NF-κB pathway [20,109]. Point mutations in TLR2 abrogate inflammatory responses to yeast and Gram-positive bacteria, but not to Gram-negative bacteria, whereas

TLR4 mutations do abrogate LPS signalling [223]. Except for changes in TLR3 and TLR4 expression in CD patients, there is a significant up-regulation of TLR2 protein expression observed in active CD and UC. This up-regulation is restricted to inflammatory cells of the lamina propria [25].
Because TLRs are located on the outside of the cell membrane, they are considered as membrane pattern recognition receptors of the innate immune system [102]. TLRs would thus define a membrane-associated counterpart of the NOD2 protein, which is located intracytoplasmatic. Interestingly, both TLRs and NOD2 are involved in bacteria pattern recognition and both receptors may be abnormal in CD. Very recently, Watanabe *et al*. used NOD2-deficient mice to show that NOD2 signalling normally inhibits the TLR2-driven Th1 response by regulating NF-κB signalling. In addition, in the absence of NOD2-signalling, such that occur in the presence of a CARD15 mutation, NOD2-mediated inhibition is abrogated, resulting in increased TLR2-mediated NF-κB activation and more Il-12 production. Thus the CARD15 mutations lead to increased Th1 cytokine production [238]. This suggests that, at the molecular level, CD results from an inappropriate activation of the mucosal immune system driven by bacteria like the intestinal flora. The different disease patterns found in CD are likely to be based on the type of receptor and presence of specific commensal (not necessarily pathogenic) luminal bacteria. These data give additional indications that the innate response to the bacterial flora in the intestinal lumen is ineffective.

- *Summary: differential expression of Toll-like receptors 2, 3 and 4 has been associated with CD. TLRs are part of the body's innate defence system against bacteria. They activate the immune system after recognizing specific bacterial components. Enhanced expression of TLR2 followed with increased production of Th1-related cytokines is related with CARD15 mutations.*

4. Intestinal flora and CD

For many years, micro-organisms are thought to play a pivotal role in the development of CD. Microbial agents such as *Yersinia enterocolitica*, pseudomonas-like organisms, *Escherichia coli*, *Streptococcus faecalis*, *Chlamidiae*, Mycoplasma, Measles virus, *Saccharomyces cerevisiae*, *Helicobacter pylori*, and Mycobacteria have been postulated to play a role in the aetiology of the disease [46,50,79,98,100,118,129,182,196,234,243]. Accumulating evidence suggest that especially the intestinal flora is a prerequisite, and perhaps a central factor in the development of IBD. The discovery of mutations in genes involved in the reaction to bacteria in the gut, CARD15 and Toll-like receptors, has strengthened this theory. This assumption is supported by studies in mouse and rat models of colitis established through genetic manipulation. The development of colitis in rats and mice under experimental conditions appears to require the presence of a luminal flora; colitis does not occur in animals when they are maintained in a germ-free environment, but it develops rapidly when they are colonized by commensal bacteria. Knockout mice lacking several immunological relevant genes, including Il-2 or IL-10 develop experimental colitis only when raised under contaminated but not under sterile conditions [201]. Furthermore, in CD patients after curative resection, a diverting terminal ileostomy was constructed thereby excluding the neoterminal ileum and the colon from intestinal transit. After six months of exclusion, transit was restored. None of the patients had endoscopic lesions in the neoterminal ileum after that six months of exclusion and biopsies did not show inflammatory changes characteristic of CD. In contrast, 71% of patients with one-step surgery had recurrence in the neoterminal ileum within six months of surgery. All five patients had an important recurrence of disease, both endoscopically and histologically, six months after transit was restored [43,92,190,194]. These data show that the intestinal flora plays an important role in the development of CD.

- *Summary: CD cannot develop or perpetuate without the presence of an intestinal flora. Presence of intestinal bacteria seem to be a prerequisite for CD*

5. *M. avium* ssp. *paratuberculosis* and CD

One of the bacteria that has been frequently associated with CD is *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). In 1972, Patterson and Allen put emphasis on the strong similarities between CD in humans and Paratuberculosis (Johne's disease) in cattle [166]. The characteristics of CD and Paratuberculosis are compared in Appendix 1 Table 3 and 4. Paratuberculosis is a severe chronic intestinal disease caused by *Map*. Besides Cattle, *Map* can also cause Paratuberculosis in other ruminants like sheep and goats. *Map* is widespread in nature, and has been isolated from many wildlife species including rabbit, fox, stoat, weasel, crow, rook, jackdaw, rat, wood mouse, hare, badger and deer [7,8,167]. Indications of a possible etiological link between CD and Paratuberculosis came when Chiodini *et al*. isolated a *Mycobacterium* species from surgical gut specimens of four patients with CD [32,33]. This *Mycobacterium* was biochemically and genetically similar to *Map*. The investigators hypothesized that this slow growing *mycobacterium* played an etiological role in at least some cases of CD. *Map* belongs to the genus *Mycobacteriacea*. Well know members of this family are *M. tuberculosis*, the cause of tuberculosis, and *M. leprae*, the cause of Leprosy. *Map* is very closely related to *M. avium* ssp. *avium* (*Maa*) and together with *M avium* ssp. *silvaticum* and *M. intracellulare* belong to the *Mycobacterium avium*-Complex (MAC). On the nucleotide sequence level *Map* and *Maa* are 98% identical, but can be differentiated by the presence of a chromosomal insertion sequence, IS*900*, which is (thus far) unique to *Map*, and is present in 17-18 copies in its genome [85]. Phenotypically, *Map* can be distinguished by its *in vitro* dependence on Mycobactin-J, an iron-chelating compound, and its slow growth. In contrast to *Map, Maa* is not a major pathogen of cattle, but it in HIV-infected patients it can cause severe complications [6,27,177]. *Map* has a complex cell wall, relatively impermeable and rich in lipids, which confers acid-fast properties. Due to its firm cell wall *Map* can survive for a long time in the environment (for more than a year in a dry fully shaded environment) [244]. Neonatal and juvenile animals are at the highest risk for acquiring an infection of *Map* [95]. Young animals are most commonly infected through the fecal-oral route. This occurs either by ingesting the organism through contaminated milk or food products or by accidental ingestion of the micro-organism from contaminated surfaces [95].
Preferentially *Map* targets the mucosa-associated lymphoid tissues of the upper gastrointestinal tract, where it is endocytosed by the M-cells of the ileal Peyer's patches and subsequently phagocytosed by subepithelial and intraepithelial macrophages [4,70,95,138]. Data suggest that *Map* can arrest the development of the phagosomal compartment into a mature phagosome [99,115,220]. The Peyer's patches reach their maximum development about the time of birth and progressively disappear afterwards, though patches in the jejunum and ileocaecal valve can persist in adults [148,176]. This could explanation why the highest susceptibility for a *Map*-infection occurs in young animals. *Map* probably remains in the phagosome, where it multiplies intracellularly [115]. A recent study showed that *Map*-infected macrophages were able to stimulate production of IL-2 and IFN-γ in CD4+ cells [250]. Cytokine production and the initiation of a cellular immune response by the host causes the appearance of an intestinal

granuloma, and a cellular response is initiated in the nearby lymph nodes in an attempt to clear the infection [95,138]. This inflammatory process leads to the clinical manifestations of a corrugated intestinal epithelium and the corresponding characteristic malnutrition syndrome associated with Paratuberculosis [95]. Cattle become infected with *Map* as calves but often do not develop clinical signs until 3-5 years of age. After this subclinical stage, extensive granulomatous inflammation developes in the terminal ileum, which leads to chronic diarrhea, shedding high numbers of *Map*, diffuse edema, malabsorption of nutrients and decreased milk production. Though, not all infected cattle will develop clinical disease signs [110,122].

Paratuberculosis shows a wide immunological and histopathological spectrum: at one end the tuberculoid form, which is located where the host offers a strong cellular immune response with a very low humoral response; at the opposite end, the lepromatous form, associated with a weak cellular response but a strong humoral response. Between these forms is the borderline form, which shows the most severe clinical signs [4]. Animals from an infected flock can present varying immunological and pathological pictures and can be located in different positions in the spectrum.

Gross changes in *Map*-infected sheep and goat are often lacking or difficult to detect, and may not resemble those of Paratuberculosis in cattle [4]. In cattle, sheep and goats, two distinct types of pathology are present, based on the abundance of Mycobacteria and cellular infiltrate [22,170]. The more common form (predominantly found in cattle), the multibacillary (also named 'pluribacillary or lepromatous') form, is characterized by numerous acid-fast *Map* in the cytoplasm of the many large macrophages that infiltrate the mucosa. Lymphocytes and granulocytes are present in much lower numbers. These changes cause marked thickening of the intestine [4]. The other form of Paratuberculosis, the lymphocytic (also 'paucibacillary or tuberculoid') form (which is predominantly found in sheep and goat), is characterized by a more marked lymphocytic infiltrate with scattered, small focal granulomata and giant cells. Lesions may exhibit caseation, calcification or fibrosis. Acid-fast *Map* are sparse or undetectable in lymphocytic lesions and are usually absent from caseous or calcified foci [4]. The two types of pathology in Paratuberculosis correlate with different host responses to the bacterium [4], and perhaps also to the type of *Map* strain [1,190]. Sheep with multibacillary disease have a strong antibody response but a weak or absent cell-mediated immunity, predominant Th2-like cytokines Il-4 and Il-10, and *Map* appears to multiply in epitheloid cells in these lesions [4,24,75]. Animals with the lymphocytic form show a strong CMI response and poor or absent antibody response and predominant Th1-like cytokines Il-2 and IFNg. In these lesions the bacteria appear to degenerate in epitheloid macrophages [4]. This form of Paratuberculosis appears to be able to reduce the bacterial load in the intestine [4]. This lymphocytic form of Paratuberculosis accords best with the pathological and immunological features of CD.

Although CD and Paratuberculosis share numerous clinical, histo-pathological and immunological features, there are however also significant differences between these two diseases, as shown in Appendix 1 Table 3 and 4 [4,227]. This is one of the reasons why several investigators believe that CD and Paratuberculosis do not share a common etiologic agent [175,227].

- *Summary: Map causes a chronic intestinal inflammatory disease in animals, Paratuberculosis. Especially the paucibacillary variant of Paratuberculosis shares clinical, histo-pathological and immunological characteristics with CD. Map is present in many dairy herds and probably can be transmitted to humans via foodstuff like milk, meat, water, and via direct contact with contaminated soil.*

6. Is CD caused by *Map*?

For many investigators the resemblance between CD and Paratuberculosis was sufficient to assume *Map* to be the mutual causative agent. However, gross pathology and clinical data alone are not sufficient to confirm this hypothesis. Therefore the question remains whether *Map* is indeed the etiologic agent of CD. An important dogma concerning the aetiology of infectious diseases is Koch's postulates (Robert Koch, 1843-1910). According to Koch, to prove that any organism is the cause of an infectious disease, four postulates should be fulfilled:

1. The specific organism should be present in all individuals suffering from a specific disease, but should not be found in healthy individuals.
2. The specific organism should be isolated from the diseased individual and propagated in the laboratory.
3. This freshly propagated organism, when inoculated into a healthy individual, should cause the same disease seen in the original individual.
4. The organism should be re-isolated from the experimental infection.

Although Koch's postulates are slightly out of date, many investigators have tried to prove, or discount Koch's first postulate concerning CD and *Map.* They have applied different methods to achieve this [2,5,21,23,26,30,33,35,47,48,56,58,65,68,83,103,107,114,144,146,151,156,160,164,165,178,185,191-193,197,198,200,213,215,218,229,236,239].

- Isolation and culturing of *Map* bacteria from affected CD tissue.
- Demonstration of the presence of a cellular and/or humoral immune response against *Map* antigens in CD patients.
- Detection of *Map* DNA in CD tissue using PCR and/or *in situ* hybridisation (ISH).
- Investigation of the efficacy of anti-mycobacterial drugs.

In the next section we will evaluate the different attempts made to detect *Map* in human tissue. Furthermore, we will put forward arguments to support, or to discount the hypothesis about an etiologic link between CD and *Map*.

6.1 Detection of *Map* in CD tissue

The two techniques that are mostly used for the detection of *Map* in tissue samples from CD patients are culturing and PCR. Since it is rather difficult to culture *Map* bacteria from affected tissue, most studies have been done using the PCR technique, which is able to detect as little as a few copies of the *Map* genome. PCR has resulted in a much higher detection rate for *Map* in tissues. However, great care needs to be taken to avoid contamination of samples, and negative control samples should be included in the assays, because the high sensitivity

means inadvertent contamination can easily give rise to false-positive results. Furthermore, the assay will not discriminate between viable and dead cells. The fact that PCR also detects dead *Map* bacteria may account for its higher detection rate compared to culturing techniques. In Appendix 1 Table 5 and 6 the results of several recent studies on the presence of *Map* in tissue are depicted. An important issue to keep in mind to interpret these results is that there is neither consensus in patient selection nor in sample selection. Several investigators took tissue samples from patients with only a history of CD, irrespective of the patients' clinical state (whether or not an inflamed intestine) at the time of sampling. Other investigators only took samples from heavily inflamed intestines. In some studies either resections or biopsy samples were taken, while other studies used both. Resections cover the whole intestinal wall, but biopsies only the most outside layers (mucosa and lamina propria). Unfortunately, most if not all patients already had a history of IBD, and probably were (or had been) treated with medicines. Medicines change the course of the disease, and this of course could have influence on the endurance of a possible disease mediator, in particular micro-organisms.
The results of *Map* detection in CD tissue by PCR and culturing can be categorized into three groups:

1. *Map* was detected significantly more often in the CD group of patients than in the UC and control groups [47,48,65,103,156,192,193,197,198].
2. *Map* was detected in a number of CD, UC and control patients, but in neither the groups the detection rate was significantly higher [35,83,146](RIVM, Appendix 2).
3. *Map* could not be detected in CD, UC or control patients [2,5,26,30,56,68,107,178,185].

If CD was an infectious disease solely caused by *Map,* than according to Koch, *Map* should be present in all cases of individuals suffering from CD, but should not be found in healthy individuals. It is clear from the data in Appendix 1 Table 5 and 6 that *Map* cannot be detected in all individuals suffering from CD. But if *Map* were the etiologic agent of CD, why is it not possible to detect *Map* in all CD patients, and why does *Map* not cause disease in so many *Map*-positive healthy individuals? Four hypotheses can be postulated to account for the absence of *Map* (nucleic acid or antigens) in CD patients:
1. *Map* is always involved in CD, but solely at the onset of the disease. *Map* only triggers the disease and is subsequently cleared from the gut. Th1 cell-mediated immune responses are considered as efficient against Mycobacteria. The continuous Th1 stimulation seen in CD may therefore be appropriate to eradicate offending *Map* bacteria. However, this relative success in killing the bacteria may be counteracted by an insufficiency feedback in the immune system associated with a genetically induced failure to produce those anti-inflammatory cytokines that can stop the immune response. This run-away immune response leads to disease perpetuation and mucosal damage. If this first hypothesis is right, Koch's first postulate cannot be applied to CD.
2. *Map* is not always involved in CD. Apart from *Map*, other factors (micro-organisms, chemical substances, genetic defects, impaired intestinal mucosa) are essential (etiological) agent(s) for development of CD. This will be discussed later.

3. *Map* is involved in CD but cannot be detected. The landmark of most mycobacterial infections is the presence of acid-fast bacilli: in Paratuberculosis one can see swarms of acid-fast bacilli, in CD there are none. Failure to see acid fast bacilli in *Map* infections is not uncommon. In the lymphocytic or paucibacillary form of Paratuberculosis, often seen in sheep and goat, acid-fast *Map* cells are sparse or undetectable in lymphocytic lesions, and are usually absent from caseous or calcified foci [4,41]. Furthermore, *Map* cannot be cultured from all animals known to be infected with *Map* [41]. From studies on Leprosy it is evident that sensitisation with Mycobacteria can cause different host immune responses. Depending on the type of immune response, the clinical spectra in mycobacteriosis can vary from no symptoms; diffuse inflammatory mycobacteriosis with bacillary load such as lepromatous leprosy; self healing with no demonstrable bacilli (paucibacillary) such as the lymphocytic (tuberculoid) form of leprosy; intermediate type often with the presence of bacilli such as borderline leprosy. Hence, CD may resemble the tuberculoid form of leprosy in which hardly if any *Map* can be detected.
Some investigators believe that *Map* subsists in a 'cell-wall deficient' form in CD [31,59,83,142,151,198,236]. This cell-wall deficient form is supposed to be responsible for triggering the abnormal immune response that leads to CD [86]. Cell-wall deficient forms are very difficult to isolate. Due to the lack of a protective cell wall they do not (or hardly) survive the harsh decontamination procedure applied for culturing. Although cell-wall deficient *Map* cells have been produced artificially *in vitro*, there is however no conclusive evidence that they do exist in natural *Map* infections.
Another possible reason why *Map* cannot be detected in most CD patients is the sampling error. Even a very sensitive detection assay like PCR can only yield a positive result if the target (in this case *Map* DNA) is present in the reaction mixture. If *Map* is not uniformly distributed in CD tissue, and/or only present in minimal amounts, the place of sampling becomes very critical. It is a matter of chance to pick the right spot.
4. *Map* is not involved in CD. *Map* is just an opportunistic passenger that was coincidentally passing through the intestinal tract of the individual from whom it was isolated. Leading to the chicken and egg scenario of which came first, CD or *Map*. This scenario may explain why in about half of the published studies *Map* is uniformly distributed between Crohn's patients and controls. Other mycobacterial species are also known to be uniformly distributed between CD patients and controls [56,213]. This is consistent with the known environmental distribution of Mycobacteria, which are present in up to 30-50% of all environmental samplings, including water, soil and even air. For instance, *M avium* ssp. *silvaticum* is equally distributed among people with or without CD. In contrast, in the other half of the reported studies *Map* is detected in a significant higher percentage of CD patients compared to control patients. This may suggest that *Map* has an affinity for inflamed tissue, which is in accordance with the observations that chronic inflamed tissues are readily colonized by other microbial agents. However, in that case *Map* would also be more frequently found in biopsies of similar inflammatory diseases like UC, but this is not the case.

6.2 *Map* does not always cause disease

Recently, the RIVM has performed a large survey on the presence of *Map* in CD, UC and control patients. Detection of *Map* was performed using different techniques: PCR, two different culture methods and immunohistochemistry (immunoperoxidase staining, IP). The results, which are presented in the Appendix 2 section of this report, clearly show that *Map* is widely distributed between both IBD and control persons. When culturing and IP-staining techniques were applied, RIVM found even a higher prevalence of *Map* among control persons compared to CD patients; 29% versus 20% for IP-staining, 28% versus 27% for MGIT and 25% versus 22% for BACTEC culturing, respectively. Hence, *Map* is frequently present in healthy individuals. This is not unexpected, because *Map* (and most other pathogenic micro-organisms) does not always cause disease after infection [95]. The (in)ability of *Map* to cause disease may be attributed to the genetic background of the host, the virulence of *Map* in the host species and/or the nutritional state of the host [110,111]. In experimentally infected animals, *Map* infections do not consistently reproduce disease symptoms. For example, oral inoculation of rabbits with approximately 10^8 CFU of *Map* gave clinical and histopathological lesions in only 62 to 75% of the animals [147]. In contrast, 100% of calves orally inoculated with 10^6 CFU of *Map* developed disease [123]. Certain inbred and outbred strains of mice, such as the C57 black and Swiss white strains, are more susceptible to *Map* infections than the CBA strain [28,29,95]. Resistance to mycobacterial infections in mice, including *Map*, is associated with the *Bcg* locus on mouse chromosome 1 [69,95,208,209]. Two allelic forms of the *Bcg* gene, Bcg^s and Bcg^r, confer susceptibility or resistance to infection, respectively. The nutritional and hormonal status of an animal may also influence its susceptibility to *Map* infections. Reduced dietary calcium protects beige mice from *Map* infections, but a corresponding increase in endogenous vitamin-D levels reverses the beneficial effects of low Calcium levels [95,211,212]. Additionally, transient exposure of monocytes to growth hormone or prolactin enhances intracellular multiplication of *Map* in primary bovine monocytes [64]. From these examples it is clear that individuals can be contaminated with *Map* without *Map* being infectious or causing disease symptoms. Therefore the second part of Koch's first postulate 'and should not be found in healthy individuals' may not be applicable to *Map* infections (actually, this holds true for many other infectious agents). Nevertheless, if *Map* were the single etiologic agent of CD which can be found in up to 30% of healthy individuals (RIVM, Appendix 2)[242], it is clear that the presence of *Map* in human intestines is very common but hardly leads to CD (as shown in Appendix 1 Table 2, the highest incidence of CD is only 15.6 cases per 100,000 persons per year). If it were possible to investigate a large number of intestinal samples per person, *Map* may be found in many more individuals with or without CD.

- *Summary: so far, the quest for Map using PCR and culture methods, have not resulted in conclusive data to support or discount the hypothesis that Map is the etiologic agent of CD. The fact that Map can be found in a high percentage of apparently healthy individuals raises the question whether Map is a common passer-by of the*

human intestinal tract, or that a particular cofactor (i.e. a genetic dysfunction) is needed before Map can cause disease.

6.3 Immune responses against *Map* in CD

Detection of *Map* using PCR, ISH, (immuno) staining and culturing has the disadvantage that it requires the current presence of nucleic acids, proteins or life *Map* cells. If the first *Map*-CD hypothesis (paragraph F2) is right, and an individual has encountered a *Map* infection in the past and has cleared the infection, no *Map* nucleic acids or proteins may subsist at present. Detection of immune responses therefore has the advantage that these responses can maintain for a long period after the initial stimulus has been cleared. Several investigators have attempted to demonstrate immune responses against *Map* in CD (see Appendix 1 Table 7 for results).
As did the quest for *Map* in CD patients using PCR and culturing, the results of antibody detection in CD patients does not lead to conclusive data to support a mycobacterial aetiology of CD. It can be concluded that CD patients in general fail to elicit a consistent humoral or cellular immune response to *Map* antigens, while on the opposite many healthy individuals demonstrate a significant immune response. Unfortunately, most effort to study immune responses against *Map* is put into detection of antibodies. However, the pathologic lesions in CD in general can be considered a delayed type hypersensitivity reaction resembling a tuberculoid lesion. Such a lesion is mediated by cellular elements of immunity, which may not be accompanied by a humoral response. Hence, the absence of a humoral response to mycobacterial antigens in CD is not unexpected, and therefore measuring cellular immune responses against *Map* would be more beneficial. On the other hand, no peripheral cellular response to mycobacterial antigens in CD has been consistently demonstrated so far. CD is a mucosal disease and the sequestered nature of the lesions suggests that immune cells may be compartmentalized within the intestine. Therefore, examination of peripheral reactivity may not be appropriate, and only measuring a cellular immune response in localized areas of the gut may lead to a consistent answer about the possible *Map*-aetiology of CD lesions.
It is not surprising to find immune responses against *Map* in several IBD as well as control patients. Most investigations have focussed on antibody responses against whole *Map* cells. The use of these crude antigens will also detect immune responses to the other members of the *M. avium*-complex (MAC), and to many other Mycobacteria. After all, a substantial proportion of the population has been exposed and has immunity to Mycobacteria. To circumvent cross-reactions with other Mycobacteria, resent publications use selected recombinant *Map* proteins for antibody recognition [6,58,160]. The authors claim that these recombinant antigens are specific for *Map*. However, now the complete genome sequence of *Maa* and *Map* has become available for comparison, it is clear that in contrast to what is claimed, no *Map*-specific recombinant antigens have been used for antibody detection in literature to data. Consequently, all studies done so far to detect a *Map*-specific immune response suffer from cross-reaction with other micro-organisms, especially Mycobacteria.

The presence of antibodies to micro-organisms in IBD has been acknowledged for a long time: antibodies to colon extract were reported in the late 50s of the previous century. One of the strongest antibody responses seen in CD patients is against mannose epitopes of the yeast *Saccharomyces cerevisiae* (bakery/brewery yeast) [169,230]. A considerable number of studies have examined the prevalence and nature of yeast antibodies in CD [3,93,119,128,154,169,196,203,226,230,235]. Anti-*Saccharomyces cerevisiae* antibodies (ASCA) were strongly associated with CD, and less with UC [225,226,235]. The prevalence of ASCA in CD ranges from 49%–73% [3,93,119,169,203,219,235]. The high prevalence of ASCA in CD, and their lower prevalence in UC suggest that they are not raised solely due to antigenic exposure through a breached epithelium [225]. Furthermore, in a placebo-controlled study, the mean CD-activity index of CD patients taking baker's yeast was significantly greater than during yeast exclusion [225]. Besides *S. cerevisiae*, the antibody response to a number of other food antigens has also been examined in CD. In a monozygotic twin study of patients with CD, serum antibodies against ovalbumin, betalactoglobulin, gliadin, whole *S. cerevisiae* and yeast cell wall mannan were measured [128]. Twins, who both had CD, displayed higher antibody titres to yeast cell wall mannan and whole yeast than control persons. In contrast the response to the other antigens was equal to, or lower than controls [128]. These results again argue against an increased systemic antigen presentation caused by an impaired mucosal barrier in IBD. Rather they suggest that yeast antigens, or cross-reacting with yeast antigens, may play an important (etiological?) role in CD [225].
Another significant antibody response in IBD patients is seen against an unidentified 50-kilodalton nuclear lamina protein present in human neutrophils (atypical pANCA) [36,119,169,195,230]. The prevalence of this autoantibody in CD is 23-63% and in UC between 40%–87% [119,169,195,230]. More recently, antibodies to the outer-membrane porin C of *Escherichia coli* (OmpC), and against a *Pseudomonas fluorescens* associated sequence (I2) were reported in CD patients [119,154,243]. A recent study by Landers *et al*. showed that 85% of CD patients elicit antibody responses to at least one of the four antigens ASCA, ANCA, OmpC and I2; only a minority (4%) of patients respond to two or more of these antigens [119]. Lately, human histone H1 was identified as a target for pANCA. H1 homologous sequences were identified in the *M. tuberculosis* genome, encoding a protein named HupB. Furthermore, recombinant HupB was shown to be a pANCA antigen and to react with serum IgA, but not serum IgG, from CD patients [36]. Although this study suggests that a specific mycobacterial antigen, HupB, might be responsible for the high percentage of pANCA responses in IBD patients, it is very well possible that this is merely based on cross-reaction between HupB and other, unknown antigens involved in IBD. Furthermore, anti-HupB IgA in CD patients is only indicative of mycobacterial presence rather than pathogenesis. As already said, Mycobacteria are common inhabitants of the healthy gut, and an anti-mycobacterial antibody response may simple be secondary to IBD-associated mucosal disruption and local immune activity [36].

- *Summary: up till now, studies on the immune responses against Map in CD patients did not result in conclusive data to support or discount the hypothesis that Map is the etiologic agent of CD. Moreover, CD patients have significantly higher immune*

responses against several food antigens, especially Saccharomyces cerevisiae, compared to healthy individuals.

6.4 Antibiotics against *Map* in CD

It is conceivable that if CD is primarily caused by *Map*, it should be possible to cure the disease by treating the patient with antibiotics. Mycobacterial infections are however difficult to eradicate; prolonged treatment and relapses are common. *M. tuberculosis* takes months and *M. lepra* takes years to treat. In all cases it is conceivable that viable bacteria remain even after curative treatment. From Paratuberculosis we know that is very hard to treat *Map* infections with antibiotics. Treatment of cattle suffering from Paratuberculosis with antibiotics against *Map* has very little effect, and no animal with a *Map* infection has ever been cured [86]. Yet, researchers started trying antibiotics active against *M. tuberculosis* and thought they may be effective against *Map* in CD. Most pulmonary Tuberculosis can indeed be cured with bacteriostatics, however, when *M. tuberculosis* moves from the lung to the intestine and cause intestinal Tuberculosis, antibiotics alone cannot cure it. In addition, members of the *Mycobactrium avium*-complex, including *Map*, are in general resistant to standard anti-tuberculosis drugs. MAC can prevent these drugs from penetrating the cell and rapidly develop mutations, which confer drug resistance. For instance, MAC infections in HIV patients are difficult to eradicate; prolonged treatment is required and relapse is common [4]. For that reason, early results of treating CD patients with antibiotics were disappointing. The breakthrough came with the use of rifabutin and macrolides like clarithromicin and azithromycin. Clarithromycin was found to be most effective against *Map*. Macrolides block microbial protein synthesis and are, in contrast to other antibiotics, able to penetrate and accumulate inside human cells [78]. The latter is of extra advantage because *Map* is an intracellular pathogen. Especially simultaneous administration of Rifabutin and Macrolide (Rifabutin and Macrolide Antibiotic Therapy, RMAT) has been shown to be moderately successful in treating Crohn's. Recent studies with patients treated with RMAT showed a significant improvement, together with the absence of the need of all other Crohn's medications, such as immuno-suppressants and corticosteroids in up to 50% of the patients [17,89]. However, the same number of patients noticed no marked improvement at all while on rifabutin and macrolide antibiotic therapy or noticed significant improvements, but required other Crohn's medications concurrently with rifabutin and macrolide antibiotic therapy, to achieve and sustain improvement. Hence, although treatment with antibiotics active against *Map* may result in a substantial clinical improvement in CD [17,89,202], antibiotic treatment has no, or just a temporary improvement in just as many CD patients [80,125]. Furthermore, Rifabutin and Macrolides are broad-spectrum antibiotics, and their effect is not restricted to *Map* or Mycobacteria. The fact that these antibiotics have an advantageous effect in a subset of CD patients does not automatically confirm that *Map* is involved in CD.

- *Summary: broad-spectrum antibiotic therapy can be moderately successful in treating CD patients, suggesting that micro-organisms play a significant role in CD. However, studies with antibiotic therapies have yet not established that Map is involved in CD.*

6.5 Prevalence of CD versus prevalence of *Map*

One would expect that if *Map* were the major cause of CD, the regions in which there is a high prevalence of CD should overlap with the regions with a high prevalence of Paratuberculosis. The prevalence of CD is shown in Appendix 1 Table 2. Data about the prevalence of *Map* or Paratuberculosis are sparse: In the US up to 18% of cattle is infected. The disease is of low prevalence or almost absent in Austria, Norway, and Sweden; herd prevalence exceeds 15% in the USA, Denmark, Belgium and Costa Rica. In Australia the incidence is 11%. In the EC no national surveys have been carried out in the UK, France and Germany. Data from Belgium found 17% of herds infected based on serology. In Denmark bulk milk tested for antibodies against *Map* showed 70% of herds had evidence of infection [186]. In the Netherlands 55% of dairy herds had serological evidence of infection [157]. While sufficient data is lacking, a review of the epidemiology of Paratuberculosis compared with the epidemiology of CD found that CD is primarily seen in areas where they drink milk (Australia, southern Africa, Europe, the United States, Canada, New Zealand). Interestingly, CD is not seen in India, where they do drink milk, and in Sweden there is a high prevalence of CD while their cattle are reportedly Paratuberculosis free. For the present, all that can be said with certainty is that there are not enough data available on the incidence and prevalence of the two diseases, both in time and geographical spread to enable any conclusions on correlations or causality to be made.
Interestingly, Collins *et al*. [39] found that BCG vaccination (tuberculosis vaccination) was associated with a lower rate of *Map*-positive PCR findings for IBD patients between the US and Denmark (30% versus 13%) and within the Danish IBD patient population (33% for non-BCG vaccinates versus 9% among BCG vaccinates). BCG vaccination induces PBMCs that responded to mycobacterial antigen exposure by release of very high levels of the proinflammatory cytokine IFN-γ, offering a plausible explanation for the apparent protective effect of BCG vaccination for *Map* infection. In correspondence with this is the fact that in developing countries the incidence of *M. tuberculosis* infections is very high, while the incidence of CD is very low. However, BCG is a potent non-specific immune stimulator, so the observed association of BCG vaccination status and *Map*-positive status could as well be related to factors dissimilar to Mycobacteria. More important, the incidence of CD and UC in the United States and Denmark is very similar [131,155], so even if BCG vaccination could prevent *Map* infection, it cannot prevent from developing CD.

Summary: there is not enough epidemiological data available on the incidence and prevalence of CD and Paratuberculosis, both in time and geographical spread, to allow any conclusions on correlation or causality to be made.

7. Interpretation of the data in literature

7.1 Summary

Investigations to confirm the (etiological) link between CD and *Map* based on the presence of this organism in CD patients have given only controversial data, and have yet not solved the enduring chicken-and-egg-dilemma of which came first: *Map* could be either the cause or the result of CD. On the other hand, during the past decades we have learnt a lot about other significant factors in CD:

1. CD is an incurable chronic inflammation of the human intestinal wall. CD has a dramatic negative impact on the patient's quality of life. CD occurs particularly in the Western community.
2. In terms of pathological criteria, CD is not a homogeneous pattern of disease and can be categorized into 24 pathological subgroups.
3. CD is a Th1 disease characterized by the production of pro-inflammatory cytokines like TNF-α, which is responsible for the development of the lesions, and by the production of IFN-γ and Il-2.
4. CD is a multifactorial disease. Several genetic (familial, racial) and environmental (geographic, hygienic) factors –especially microbial- have been associated with the disease.
5. Mutations in the human CARD15 gene have been convincingly associated with CD. CARD15 is part of the body's innate defence system against bacteria. CARD15 activates the immune system after recognizing bacterial components.
6. Differential expression of Toll-like receptors 2, 3 and 4 has been associated with CD. TLRs are part of the body's innate defence system against bacteria. They activate the immune system after recognizing specific bacterial components. Enhanced expression of TLR2 followed with increased production of Th1-related cytokines is related with CARD15 mutations.
7. CD cannot develop or perpetuate without the presence of an intestinal flora. Presence of intestinal bacteria seems to be a prerequisite for CD.
8. *Map* causes a chronic intestinal inflammatory disease in animals, Paratuberculosis. Especially the paucibacillary variant of Paratuberculosis shares clinical, histo-pathological and immunological characteristics with CD. Map is present in many dairy herds and probably can be transmitted to humans via foodstuff like milk, meat, water, and via direct contact with contaminated soil.
9. So far, the quest for Map using PCR and culture methods have not resulted in conclusive data to support or discount the hypothesis that *Map* is the etiologic agent of CD. The fact that *Map* can be found in a high percentage of apparently healthy individuals raises the question whether Map is a common passer-by of the human

intestinal tract, or that a particular cofactor (i.e. a genetic dysfunction) is needed before Map can cause disease.

10. Up to now, studies on the immune responses against *Map* in CD patients did not result in conclusive data to support or discount the hypothesis that *Map* is the etiologic agent of CD. Moreover, CD patients have significantly higher immune responses against several other food antigens, especially *Saccharomyces cerevisiae*, compared to healthy individuals.
11. Broad-spectrum antibiotic therapy can be moderately successful in treating CD patients, suggesting that micro-organisms play a significant role in CD. However, studies with antibiotic therapies have yet not established that Map is involved in CD.
12. There is not enough epidemiological data available on the incidence and prevalence of CD and Paratuberculosis, both in time and geographical spread, to enable any conclusions on correlations or causality.

Emphasizing points 2, 4, 5, and 6, it is clear that CD is a multi-factorial disease. Several (genetic and/or environmental) factors together are responsible for -or at least have influence on- initiation, course and perpetuation of the disease, but none of them is strictly required. Consequently, CD is not a homogeneous pattern of disease. The diverse facets of genetic predisposition, modified by environmental factors may lead to very different forms of disease with respect to localization, natural course and therapeutic responses. It is clear that micro-organisms play a significant role in CD. However, if CD were solely an infectious disease caused by *Map* (or any other micro-organism), it would be very remarkable that epidemiological factors that promote *Map*'s transmission, like poor hygiene and sanitation and drinking unpasteurised milk, seem to protect against the disease [72,205]. Further, a conspicuously higher incidence of CD in the spouses of CD patients and in occupational groups having regular contact with *Map*-infected animals (farmers, veterinarians, abattoir workers) has never been reported.

Most recent hypotheses about CD start from the idea that CD consists of three interacting elements [205]:

1. Genetic susceptibility factors.
2. Priming by the intestinal microflora.
3. Immune-mediated tissue injury.

Intestinal inflammation arises from abnormal immune reactivity to bacterial flora in the intestinal tract of individuals who are genetically susceptible. Infectious agents such as *Map* could be cofactors that condition mucosal immune responsiveness and may trigger the disease [205]. Considering the involvement of the intestinal flora, there are three theories of disease mechanisms in CD [4]:

1) Reaction to a persistent intestinal infection.
2) Existence of a defective mucosal barrier to luminal antigens.
3) A dysregulated host immune response to ubiquitous antigens: breakdown of the tolerance against the intestinal microflora.

7.2 Loss of tolerance and disturbed mucosal barrier

The current hypothesis about the pathophysiology of IBD is that in a genetic susceptible host, the intestinal flora triggers and drives an aberrant immune response resulting in chronic inflammation of the gut. This so-called loss of tolerance to commensal bacteria can result in an impaired intestinal mucosal barrier [49-53,216,231,249]. This loss of tolerance is supported by the occurrence of specific serum antibody responses to various microbial antigens in CD, including anti-*Saccharomyces cerevisiae* antibodies which can be found in up to 73% of CD patients [3,93,119,169,203,219,235]. In healthy individuals the intestinal mucosa is in a constant state of controlled physiological inflammation. Maintenance of mucosal homeostasis needs controlled responsiveness to dietary antigens and the resident microflora, while retaining the capacity for effective immune responsiveness against episodic threats from pathogens [205]. Errors in interpretation or regulation of immune alertness and responsiveness disrupt mucosal homoeostasis, and predispose the individual to uncontrolled or pathological inflammation. In this setting, depending on the genetic susceptibility of the host, the usual intestinal flora, which is in generally protective, could become a threat, and the distinction between a pathogen and a harmless commensal becomes less clear [205]. In this scenario, chronicity of inflammation results from an interaction of the persistent stimuli of microbial antigens with genetically determined host susceptibility factors that determine the individual's immune response, and/or mucosal barrier function. The crucial step in CD might not be an excessive immunological activity, but may be compatible with an impaired ability to resolve the microbiological confront at the mucosa [67]. Evidence for a break in mucosal tolerance and the importance of bacterial flora in intestinal inflammation came with the observation that knockout mice, lacking several relevant cytokine genes, develop experimental colitis only when raised in contaminated but not in sterile conditions [201].

Alterations in the intestinal flora may account for CD as a twentieth-century phenomenon in industrialized countries. A possible relevant change in the second half of the 20th-century may be a shift in intestinal flora. Once established in infancy, the bacterial flora undergoes some changes after weaning but remains remarkably constant through life [113]. The protective role of breast-feeding against development of CD [112] may be through promoting Bifidobacteria and limiting *Bacteroides* [9]. The demonstrated association of increased intake of refined carbohydrates with CD may be explained through its influence on gut flora as well [113,179]. Indeed, alterations have been observed in the intestinal flora in patients with active and patients with inactive CD compared to healthy controls. CD patients harbour significantly more *Bacteroides* and *Enterobacteriacea* while *Bifidobacteriacea* and *Lactobacillus*, which are considered to be protective, are diminished [62,73,108,199]. Of course, whether these observed differences in intestinal flora between CD patients and healthy controls are a cause of, or a result from CD remains to be elucidated.

A summary of published results linking an impaired mucosal barrier and enteric bacterial flora with pathogenesis of CD is presented below [205]:

- The bacterial flora affects development, structure, and function of the mucosal immune system, and have a conditioning effect on mucosal integrity [10,81].

- Adherent and sometimes invading bacteria entering from the lumen heavily contaminate the mucosa in IBD. Lesions of CD arise mainly in regions of the bowel with highest bacterial counts [57]
- Diversion of the faecal stream is associated with distal improvement in patients with CD and the disease consistently relapses upon restoration of the faecal stream [106,204].
- Lesions can be induced experimentally in susceptible individuals by direct instillation of faecal material into non-inflamed loops of bowel [43,92,194].
- Immune reactivity against enteric bacteria can be seen in patients with CD, suggesting loss of tolerance to the indigenous flora [49].
- Colonisation with normal enteric bacterial flora is required for expression of disease in animals with CD irrespective of the underlying defect [15,106,245].
- CD-like disease can be transferred with T-cells reactive against enteric bacteria from animals with colitis to healthy animals [40]. This indicates that T-cells reactive with conventional antigens of the enteric bacterial flora can mediate chronic inflammatory bowel disease.
- Alteration of the flora with probiotics and antibiotic strategies have putative beneficial effects in human beings and other animals [204].
- Increased intestinal permeability to several specific molecular probes has been observed in patients with CD and their first-degree relatives [105,168].

In line with the concept of a disturbed intestinal mucosal is the observation of an impaired expression of peptides with antibacterial activity, so called defensins, in CD patients. Defensins (HBD) are an important peptide family of endogenous antibiotics**,** which are up-regulated following bacterial or other inflammatory stimuli under normal conditions [42,63,240-242]. A lack of this innate defence system of anti-microbial peptides may lead to a permanent but slow bacterial invasion triggering the inflammatory process. [63]. The decrease of HBD-1 (Defensin-1) in both IBD and the lack of induction of both inducible beta-defensins HBD-2 and HBD-3 in CD suggest a deficient mucosal barrier function [63,240,241]. A deficiency of these endogenous antibiotics may explain why exogenous antibiotics are a reasonable treatment option in CD: the exogenous antibiotics may compensate for the deficient endogenous antibiotic response to infections or commensal bacterial invasion.

Summary: the current hypothesis about the pathophysiology of CD is that in a genetic susceptible host, the intestinal flora triggers an aberrant immune response that results in a chronic intestinal inflammation and a damaged (leaky) intestinal mucosal barrier.

8. Conclusion

It is clear that agents that trigger a disease (initiators) may be quite different from agents that cause relapses or that are found during the chronic phase of that disease. Although it is clear that *Map* cannot be considered the sole causative agent of CD, there is certainly a possible significant role for it left. *Map* can be hypothesized to play a part in:

- Damaging the bowel in a 'normal' person and so permitting an abnormal response to develop.
- Taking advantage of abnormal permeability in a susceptible sub-set of patients and so causing a disease in that susceptible subset.
- Being normally an 'innocent bystander' that takes advantage of a diseased bowel caused by inflammation or caused by other factors, and then multiply and exist in the damaged bowel.

In the latter case *Map* could either aggravate symptoms from the basic pathological process, or could merely grow in the favourable environment without causing any symptoms. Maybe the immune system can keep *Map* under control, but is not capable to completely clear the infection. Consequently, the immune stimulus maintains. It is even quite conceivable that also death *Map* cells can induce an immune response and exacerbate the basic pathological process, or even initiate a basic (perpetuating) immune response in CD patients. In that case it becomes much more difficult to prevent consumers from exposure to *Map*, because only making every effort to kill *Map* in foodstuff is not enough.

Support for the hypothesis that there is a link between CD and *Map* varies widely amongst researchers. The persuasiveness of the evidence at present depends on one's scientific background: clinicians and immunologists tend to be sceptical about the possible link, microbiologists and public health specialists are more cautious about discounting the hypothesis (occupational disability?). Probably no one will entirely discount the possibility of a link for some cases of CD, neither will anyone suggest the link is now proven [186].

Nevertheless, at present there is only controversial data and circumstantial evidence to prove that *Map* is the etiologic agent of CD; hence, no conclusive statement can be made. Although the cause of CD remains unknown, it is expected to be due to a combination of:

- A genetic predisposition.
- An abnormal immune response.
- Environmental factors probably relating to a response to micro-organisms in the bowel but also possibly related to other dietary factors (which are certainly important in the management of the disease once present).

Although a multi-factorial cause for CD is expected, the possibility, however, that an infectious agent like *Map* may play a key role in the causation of even a sub-set of CD patients remains, and clearly needs to be taken seriously. This may offer a promising way of preventing at least a subset of the disease, and could also offer possible ways of treating the disease, and even of curing some of those suffering from the disease [186].

Yet, although the *Map*-CD hypothesis is an intriguing one, the burden of proof remains on the proponents of the hypothesis to demonstrate unequivocally the link between *Map* and CD [132]. A good start to establish the truth about the role of *Map* in CD would be a properly funded, carefully planned, blinded, international multi-laboratory study with good defined controls and free exchange of agreed optimised methods to detect *Map* in CD patients [175].

Appendix 1 Tables

Table 1. Incidence Rates of UC and CD from selected registries. Adapted from [132]

Author(s) (reference)	Setting	Case ascertainment	Incidence dates	Incidence of UC[a]	Incidence of CD[a]
North America					
Pinchbeck *et al*. [172]	Northern Alberta	Population	1981	6	10
Hiatt *et al*. [96]	Northern California	HMO	1980–1981	10.9	7.0
Stowe *et al*. [214]	Monroe County, NY	Hospital	1980–1989	2.3	3.9
Kurata *et al*. [116]	Southern California	HMO, outpatient	1987–1988	NA	3.6
		HMO, hospital	1988	NA	5.4
Loftus *et al*. [133,135]	Olmsted County, MN	Population	1984–1993	8.3	6.9
Bernstein *et al*. [12]	Manitoba	Population	1989–1994	14.3	14.6
Blanchard *et al*. [14]	Manitoba	Population	1987–1996	15.6	15.6
Europe					
Shivananda *et al*. [207]	8 N. European cities	Population	1991–1993	11.8	7.0
Shivananda *et al*. [207]	12 S. European cities	Population	1991–1993	8.7	3.9
Scandinavia					
Bjornsson *et al*. [13]	Iceland	Population	1990–1994	16.5	5.5
Munkholm *et al*. [155]	Copenhagen County	Population	1980–1987	9.2	4.1
Langholz *et al*. [120]					
Moum *et al*. [152,153]	S.E. Norway	Population	1990–1993	13.6	5.8
Roin *et al*. [184]	Faroe Isles, Denmark	Population	1981–1999	20.3	3.6
Lapidus *et al*. [121]	Stockholm County	Population	1985–1989	NA	4.9
United Kingdom					
Rubin *et al*. [187]	North Tees	Population	1985–1994	13.9	8.3
Yapp *et al*. [869	Cardiff, Wales	Population	1991–1995	NA	5.6
Kyle [117]	N.E. Scotland	Population	1985–1987	NA	9.8
Northern Europe					
Russel *et al*. [188]	S. Limburg, The Netherlands	Population	1991–1994	10.0	6.9
Gower-Rousseau *et al*. [82]	N.W. France	Population	1988–1990	3.2	4.9
Southern/Central Europe					
Mate´-Jiminez *et al*. [143]	2 Spanish regions	Hospital	1981–1988	3.2	1.6
Manousos *et al*. [140,141]	Heraklion, Crete	Population	1990–1994	9.4	3.3
Vucelic *et al*. [232,233]	Zagreb, Croatia	Population	1980–1989	1.5	0.7
Trallori *et al*. [222]	Florence, Italy	Population	1990–1992	9.6	3.4
Tragnone *et al*. [221]	8 Italian cities	Population	1989–1992	5.2	2.3
Asia					
Odes *et al*. [162]	Southern Israel	Population	1987–1992	NA	4.2
Sood *et al*. [210]	Punjab, India	Survey	1999–2000	6.0	NA
Yang *et al*. [248]	Seoul, Korea	Population	1992–1994	1.2	NA
Morita *et al*. [150]	Japan	Survey	1991	1.9	0.5
Africa					
Wright *et al*. [246]	Cape Town, South Africa	Population, white	1980–1984	5.0	2.6
		Population, colored	1980–1984	1.9	1.8
		Population, black	1980–1984	0.6	0.3
Latin America					
Linares de la Cal *et al*. [127]	Colon, Panama	Hospital	1987–1993	1.2	0
Linares de la Cal *et al*. [127]	Partido General de Pueyrredon, Argentina	Hospital	1987–1993	2.2	0.03

[a] Cases per 100,000 person per years.
NA, not available.

Table 2. Prevalence of UC and CD from selected registries. Adapted from [132]

Author(s) (reference)	Setting	Case ascertainment	Prevalence Date	Prevalence Of UC[a]	Prevalence of CD[a]
North America					
Pinchbeck *et al*. [172]	Northern Alberta	Population	12/31/1981	37.5	44.4
Kurata *et al*. [116]	Southern California	HMO	1988	NA	26.0
Loftus *et al*. [133,135]	Olmsted County, MN	Population	1/1/1991	229	144.1
Loftus *et al*. [131]	Olmsted County, MN	Population	1/1/2001	246	162
Bernstein *et al*. [12]	Manitoba	Population	12/31/1994	169.7	198.5
Europe					
Langholz *et al*. [120] Munkholm *et al*. [155]	Copenhagen	Population	12/31/1987	161.2	54
Kyle *et al*. [117]	N.E. Scotland, United Kingdom	Population	12/31/1988	NA	147
Mate´-Jiminez *et al*. [143]	2 Spanish regions	Hospital	12/31/1988	43.4	19.8
Vucelic *et al*. [232,233]	Zagreb, Croatia	Population	12/31/1989	21.4	8.3
Trallori *et al*. [222]	Florence, Italy	Population	12/31/1992	121	40
Rubin *et al*. [187]	North Tees, United Kingdom	Population	1/1/1995	243	144
Daiss *et al*. [44]	Tubingen, Germany	Population	12/31/1984	24.8	54.6
Montgomery *et al*. [149]	United Kingdom	Survey	1996	122	214
Asia					
Fireman *et al*. [66] Grossman *et al*. [88]	Central Israel	Population	1980	55.2	19.5
Odes *et al*. [161]	Southern Israel	Population	12/31/1985	70.6	NA
Odes *et al*. [162]	Southern Israel	Population	12/31/1992	NA	50.6
Sood *et al*. [210]	Punjab, India	Survey	1999	44.3	NA
Morita *et al*. [150]	Japan	Survey	1991	18.1	5.8
Lee *et al*. [124]	Singapore	Hospital	1985–1996	6.0	3.6
Yang *et al*. [248]	Seoul, Korea	Population	12/31/1997	7.6	NA

[a] Cases per 100,000 person per years.
NA, not available.

Table 3. Pathological features in CD and Paratuberculosis. Adapted from [4]

	Crohn's Disease	**Paratuberculosis**
Lesion Location		
-oesophagus and oral cavity	Yes	No
-ileum and colon	Yes	Yes[1]
-mesenteric lymph nodes	Yes	Yes
-rectum, anus	Yes	Advanced cases[2]
-segmental	Yes	Yes
Macroscopic Features		
-macroscopic appearance	Oedema of affected bowel wall, 'garden hose' like appearance	Thickened bowel wall[2]
-parietal oedema	Yes	Yes
-stenosis	Yes	Rare
-perforation	Yes	Rare
-fistula	Yes	No
-pseudopolyps	Yes	No
-mucosal aspect	Cobble stone appearance	Corrugated[2,3]
Microscopic appearance		
-transmural involvement	Yes	Yes
-fibrosis	Yes	No
-lymphoid aggregates	Yes	Yes[3]
-granuloma	Yes (50% -70% of cases)	Yes
-caseation	No	Usually not[4]
-fissures	Yes	No
-visible acid fast bacilli	No	Yes[5]

1 Ileum and jejunum are the initial and most frequent locations
2 not always in sheep
3 predominant feature in lymphocytic/paucimicrobial form
4 Varies with species
5 Scarce or absent in lymphocytic/paucimicrobial form

Table 4. Clinical features of CD and Paratuberculosis. Adapted from [4]

	Crohn's Disease	**Paratuberculosis**
Preclinical Stage		
-symptoms and signs	Not known	Decreased milk yield
-incubation period	Not known	Minimum 6 months
Clinical Stage		
-presenting symptoms and signs	Chronic diarrhoea Abdominal pain Weight loss	Chronic diarrhoea[1] Dull hair Weight loss Decrease in lactation
Gastro-intestinal symptoms and signs		
-diarrhoea	Chronic (3 weeks +)	Chronic[6]
-blood in stools	Rare	Rare
-vomiting	Rare	No
-abdominal pain	Yes	No evidence
-obstruction	Yes	No
Extra-intestinal manifestations		
-polyarthritis	Yes	No
-uveitis	Yes	No
-skin lesions	Yes	No
-amyloidosis	Yes	No
-hepatic granulomatosis	Yes	Yes
-renal involvement	Yes	No
Clinical Course		
-remission and relapse	Yes	Yes

6 Not seen in sheep

Table 5 Result from literature: PCR detection of Map in human tissue samples

Target	# CD patients	Total samples	Inflamed samples	Site of collestion	Positive Patients	# UC	Collection	Inflamed	Positive	# Contr. Pat.	Site of collection	Positive patients	Sample storage	Remarks	Reference
IS900 nested PCR	30	24 resections 10 biopsy	17	12 Peyer's pathes or lymph follicles 20 involved aereas 2 uninvolved	0	14	12 resections 2 biopsy	10	0	3	3 biopsy	0	-70°C	Japanese patients	Chiba *et al.*, 1998 [30]
IS900 nested PCR	272	Resected bowel and lymph nodes from surgical and outpatients		normal and pathologic bowel tissue	US: 15/175 (*8.6%) tissues & 9/30 (30%) patients DK: 10/312 (3.2%) tissues & 6/49 (12.2%) patients	167	normal and pathologic bowel tissue		US: 21/212 (9.9%) tissues & 6/49 (12.2%) patients DK: 7/158 (4.4%) tissues& 4/26 (15.4%) patients	275		US: 1/98 (1%) tissues & 1/18 (5.6%) patients DK: 2/91 (2,2%) tissues & 2/30 (6.7%) patients		0% of US and 78-88% of Danish patients = BCG vaccinated. In DK IBD patients: 33% of non-BCG vs. 9% of BCG = PCR+ None of the 181 patients tested both by PCR and ELISA was positive in both tests.	Collins *et al.*, 2000 [39]
Multiple x IS900 and MP2 single PCR	10	Terminal ileum biopsies	All involved areas		0	6	Biopsies	All involved areas	0	21	Irritable bowel syndrome biopsies	0	-70°C	Kuwaiti patients	Al-Shamali *et al.*, 1997 [2]
IS900 single PCR	40	Resections from ileum and colon	26/40 (65%)			23	1/23 (4.3%)			40	Cancer & ulcers from colon	5/40 (12.5%)	-20°C	UK patients	Sanderson *et al.*, 1992 [193]
IS900 and IS1311	18	Paraffin resections with granulosas	All granulomas	Ileum, colon, lymph nodes	0	0				0			FFPE	23 samples from 18 patients. No control patients	Baksh *et al.*, 2004 [5]

Target	# CD patients	Total samples	Inflamed samples	Site of collection	Positive Patients	# UC	Collection	Inflamed	Positive	# Contr. Pat.	Site of collection	Positive patients	Sample storage	Remarks	Reference
IS900 nested PCR	37	biopsies	All inflamed tissues	Ileum or colon or ileum + colon	34/37 (92%)	0				34	Colitis, cancer, screening	9/34 (26%)	Fresh, ambient temp.	2-8 samples per patient. From 21/43 PCR+ patients both duplicates from all sites were + (49%), and from 13/43 both duplicates from at least one site were positive (30%), and from 9/43 (21%) one duplicate from a single site was + UK patients	Bull *et al.*, 2003 [242]
LCM and IS900 nested PCR	15	Paraffin Granulomatous resections	granulomas		In LCM samples 6/15 (40%) In whole tissue 3/15 (20%)					12	Non-CD granulomatous resections	0/12	Paraffin	Two consecutive tissue sections: one = granuloma dissected by LCM for DNA and 2nd = whole tissue for DNA. PCR was performed twice with >93% concordance Irish patients	Ryan *et al.*, 2002 [192]
Single PCR and ISH on IS900	36	Biopsies and resections: 15 granulomas 22 non-granulomas	Granulomas and non-granulomas	Colon	PCR: 3/14 granulomas (21.4%) 6/22 non-granulomas ((27.3%) ISH: 6/15 (40%) granulomas (3 biopsies and 3 resections) 1/22 (4.5%) non-granulomas 3/15 both PCR and ISH +	21	Colon		PCR: 3/21 (14.3%) ISH: 2/21 (9.5%)	22	Resections from colon	PCR: 0/22 ISH: 0/22	Paraffin	+ signals only in myofibroblasts and macrophages of lamina propria. No signals within granulomas 4/6 CD and 3/5 UC samples were + for MAComplex US and Finnish patients	Hulten *et al.*, 2001 [103]

Target	# CD patients	Total samples	Inflamed samples	Site of collection	Positive Patients	# UC	Collection	Inflamed	Positive	# Contr. Pat.	Site of collection	Positive patients	Sample Storage	Remarks	Reference
ISH and PCR on IS900	33 patients, 48 samples	Paraffin Resections	Granulomas in 25 samples	22 ileum, 14 colon, 6 rectum, 1 stomach, 5 duodenum	ISH: 35/48 (73%) of samples PCR: 0/48 ISH+ in 18/25 (72%) granuloma samples and 17/23 (74%) non-granuloma samples	20			0/20	20		0/20	Paraffin	One slide was acid-fast stained and one slide was for ISH. No acid-fast bacteria detected ISH + signal was localized within granulomas Italian patients	Sechi *et al.*, 2001 [198]
Nested PCR on IS900	13	Resections		5 ileum, 8 colon	0/13	14	Colon resections		0/14	13	Colon resections	0/13	-70°C	Japanese patients	Kanazawa *et al.*, 1999 [107]
Single PCR and culturing	21	Biopsies	Areas of active disease		1/21	5		Areas of active disease	0/5	11	Normal mucosa	0/11	-70°C	US patients	[35] Wendell *et al.*, 1998
IS900 Nested PCR	47	33 resections from 22 patients and biopsies from 25 patients	Resections: 22 samples from inflamed wall and 11 lymph nodes	Inflamed wall and lymph nodes and mucosa	0/47	27	11 resections 15 biopsies		0/27	20	14 resections 9 biopsies	0/20	-80°C	31 CD patients (67%) had granulomas. French patients	Cellier *et al.*, 1998 [26]
Single IS900 PCR (and culturing)	32	Stool samples	Clinical active	Stool	15/32 (46.8%)	27			9/27 (33.3%)	41		1/41 (2.5%)		Italian patients	Del Prete *et al.*, 1998 [48]
Singel PCR followed by probe hybridisation	37	30 patients with oro-facial granu-lomatitis 7 patients with also gut CD	Non-caseating granulomas	Oral samples	0/37					12		0/12	Paraffin	Orofacial samples UK patients	Riggio *et al.*, 1997 [178]

Target	# CD patients	Total samples	Inflamed samples	Site of collection	Positive Patients	# UC	Collection	Inflamed	Positive	# Contr. Pat.	Site of collection	Positive patients	Sample storage	Remarks	Reference
IS900	59	118 biopsy samples (2 samples per patient)	40% of samples is inflamed	Ileum and colon	4/59 (7%)	25	52 biopsy samples (2 samples per patient) from colon	13% of samples is inflamed	2/25 (8%)	79	164 biopsy samples from ileum and/or colon	4/79 (5%)	Fresh	Dutch patients	RIVM 2004; This report Appendix 2

Table 6 Result from literature: detection of Map in human tissue samples using culture

Method	# CD patients	Samples	Inflamed	Collestion site	Positive patients	# UC	Collection	Inflamed	Positive	# Control patients	Collection site	Positive patients	Remark	Reference
BACTEC	2263 cultures from 83 US patients & 1722 cultures from 108 DK patients	Resected bowel and lymph nodes		Normal and pathologic tissue	0	167	Normal and pathologic tissue		0	275		0		Collins *et al*., 2000 [39]
MGIT and BACTEC followed by IS900 PCR	27	7 resections 20 biopsies			10/27 (37%): 6/7 (86%) resections and 4/20 (20%) biopsies					36	33 biopsies 3 resections	2/36 (5.6%) all from biopsies	1 year culturing	Schwartz *et al*., 2000 [197]
MG3 medium followed by PCR	18	13 resections 5 biopsies			6/18 (33.3%): 1/5 biopsies and 5/13 resects	7	2 resections 5 biopsies		0	6		1/5 (20%)	2-6 years culturing followed by PCR	Moss *et al.,* 1992 [151]
MGIT followed by IS900 nested PCR	33	Biopsies	All inflamed	Ileum or colon or ileum + colon	14/33 (42%)	0				33	Colitis, cancer, screening	3/33 (9%)	Up to 18 months culturing	Bull *et al.,* 2003 [242]
Culturing 7H10/11 (and PCR)	21	biopsies	Areas of active disease		0/21	5		Areas of active disease	0/5	11	Normal mucosa	0/11	US patients	Wendell *et al.,* 1998 [35]
Culturing on L-J (and single IS900 PCR)	24	Stool samples	Clinically active	Stool	8/24 (33.3%)	22		Stool	5/22 (22.7%)	40	Stool	1/40 (2.5%)		Del Prete *et al*., 1998 [48]
MGIT and BACTEC	49	biopsies	29% of samples is inflamed	Ileum and colon	13/49 (27%)	16	colon	19% of samples is inflamed	3/16 (19%)	61	Colon	17/61 (28%)		RIVM, 2004; This report, Appendix 2

Table 7 Result from literature: antibody responses against Map in humans

Method	# CD patients	Samples	Positive patients	# UC	Positive	# Control patients	Positive patients	Remark	Reference
IDEXX antibody ELISA IFN-g & IL-5 assays	142 US 119 DK		US: 19/142 (13.4%) DK: 11/119 (9.2%)	65 US 99 DK	US: 1/65 (1.6%) DK: 9/119 (7.6%)	212 US 107 DK	US: 6/212 (2.8%) DK: 6/107 (5.6%)	Pre-absorption on M.phlei None of the 181 patients tested both by PCR and ELISA was positive in both tests. No significant differences in IFN-g and IL-5 production	Collins *et al*., 2000 [39]
Antibody response against Map AhpC, AhpD, 14kD (MPP14) and PPD-J	10		Significantly elevated Ab levels against MPP14	10				The significantly elevated Ab levels against MPP14 were negatively correlated with the duration of the disease. No significant differences in IFN-g and IL-10 production	Olsen *et al*., 2001 [164]
P36 immunoblotting	89		77/89 (86.5%)	27	4/27 (14.8%)	8 TBC sera 10 Leprosy 15 non-IBD 50 healthy	6/8 (75%) TBC sera 10/10 Leprosy 1/15 (6.7%) non-IBD 10/10 healthy BCG vaccinated 4/40 (10%) healthy non-BCG vaccinated		El-Zaatari *et al*., 1999 [58]
Antibody ELISA	13	10 patients active disease 3 patients non-active	5/13 (38%) sera had significant higher IgG response (all active disease)	20	2/20 (10%)	4	0/4	No difference in IgA and IgM levels TBC patients were used	Suenaga *et al*., 1999 [215]
P35 and p36			p35: 40/53 (75%) p36: 79/89 (89%) p35 & p36 39/53 (74%)		p35: 1/10 (10%) p36: 4/27 (15%) p35 & p36: 1/10 (10%)		p35: 5/35 (14%) p36 7/50 (14%) p35 & p36: 0/35	Sera with no TBC or Leprosy history and no BCG vaccination. Only CD sera react to both p35 and p36.	Naser *et al*., 2000 [160]
IDEXX antibody ELISA	283		37.8%	144	34.7%	402	33.6%	No correlation between *Map* serology of nonaffected siblings and IBD affected siblings	Bernstein *et al*., 2003 [11]

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