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Abstract

Crohn's disease (CD) is an inflammatory bowel disease of unknown etiology. During the last decades, significant technological advances led to development of -omic datasets allowing a detailed description of the disease. Unfortunately, these have not, to date, resolved the question of the etiology of CD. Thus, it may be necessary to (re)consider hypothesis-driven approaches to resolve the etiology of CD. According to the cold chain hypothesis, the development of industrial and domestic refrigeration has led to frequent exposure of human populations to bacteria capable of growing in the cold. These bacteria, at low levels of exposure, particularly those of the genus *Yersinia*, are believed to be capable of inducing exacerbated inflammation of the intestine in genetically predisposed subjects. We discuss the consistency of this working hypothesis in light of recent data from epidemiological, clinical, pathological, microbiological and molecular studies.

Key words:

Crohn Disease, Yersinia, Causality chain, macrophages, autophagy, cold, refrigeration, plague, mucosal immune response, gut inflammation, mesenteric lymph nodes, creeping fat, food products, enteral nutrition, exclusion diet.

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) that affects several million people worldwide [1]. It most often begins in young adults and lasts a lifetime. It requires long-term, often aggressive and costly medical and surgical management. It can therefore be considered as a public health problem.

While it is recognized that environmental and genetic factors are involved in the development of the disease, the etiology of CD remains largely unknown. In recent decades, significant technological advances have led to development of -omic datasets which have made it possible to describe the disease in detail. Unfortunately, they have not, so far, enabled us to resolve the issue of the etiology of CD. Since hypothesis-free approaches are unconvincing, it may be necessary to (re)consider hypothesis-driven approaches to resolve the etiology of CD.

Scientific hypotheses may be weighed up and compared using several criteria (https://en.wikipedia.org/wiki/Hypothesis). First, a hypothesis must be testable by the scientific method. In other words, it must be corroborated by all available observations on the disease. A single observation overtly incompatible with the theory would reject its validity. Second, the hypothesis must be parsimonious. Ideally, a single idea must provide a complete chain of causality with as few gaps as possible. Third, the hypothesis must have a broad scope explaining a large number of phenomena, if possible from several sources and at different scales of observation, from the molecule to the population. Finally, it must be useful with practical implications for the future.

In 2003 we proposed a working hypothesis on the etiology of CD: the cold chain hypothesis [2]. According to this, the development of industrial and domestic refrigeration has led to frequent exposure of human populations to bacteria capable of growing in the cold. These bacteria, at low levels of exposure, particularly those of the genus *Yersinia*, are believed to induce exacerbated inflammation of the intestine in genetically predisposed subjects. Today, we would like to update this theory and to review whether recent data from epidemiological, clinical, pathological, microbiological and molecular studies have further substantiated it or, on the contrary, have pushed it out of the realm of possibility.

To construct the theory, we started from four major, indisputable, disease-specific observations that require full explanations: 1) CD is related to the modern western lifestyle. 2) Some nutritional interventions reduce intestinal inflammation at least temporarily. 3) Inflammation of the small intestine and/or colon is focal with transmural lesions, thickening

of mesenteric fat and sometimes granulomas. 4) A set of genes involved in innate immunity play a role in CD susceptibility.

Below, we will develop and discuss the chain of reasoning in support of the cold chain hypothesis with respect to the above-mentioned observations and in light of recent publications (table 1).

Are epidemiological data consistent with the cold chain hypothesis?

CD is definitively associated with the occidental modern way of life [3]. If the cold chain hypothesis is valid, the emergence of CD must be parallel to the development of industrial and domestic refrigeration. Figure 1A compares the annual incidence of the disease and the household equipment rate in several countries. There is a temporal correlation between these variables. It should be noted, however, that in a given country the disease begins to be detectable not at the time when refrigerators begin to be sold, but only when about 50% of households are equipped. This observation is counter-intuitive.

Results from mathematical modeling can provide an explanation [4]. In this approach, a disease is modeled by a small number of key biological functions. For a given individual, depending on his/her genetic and environmental background, these biological functions are permissive or, on the contrary, protective against the occurrence of the disease. Applied to CD (and other complex genetic disorders), it has made it possible to reproduce age-dependent incidence curves. Looking at the effect of environmental changes applied to the population, the model showed that the increase in CD incidence only began to be readily detectable when about 50% of the general population was exposed to the risk factor (Figure 1B from ref 4). Since the number of refrigerators sold is a marker of exposure to refrigerated foods, the temporal evolution of the incidence of CD and the development of domestic refrigeration appear to be consistent with the cold chain hypothesis.

A few years after the publication of the hypothesis, two studies investigated the link between individual exposure to domestic cold and CD. In a study performed in Wales, where CD first appeared in the 1960s, elderly patients had a refrigerator in their homes at a younger age than the control group [5]. Another study was conducted in Iran, where CD first appeared in the 1990s. Again, patients were exposed to a refrigerator earlier in life than controls [6]. Although these epidemiological studies have significant methodological limitations and do not formally demonstrate the role of refrigeration in CD (an association is

necessary but not sufficient to prove a causality), their results are consistent with the working hypothesis.

What do nutritional treatments for CD teach us?

Nutritional treatment of CD was first proposed by Voitk in 1973 [7]. The treatment consists of administering a specific liquid formula for 6-8 weeks by oral and/or enteral routes (for a review see [8]). Many formulas have been proposed, including elementary, semi-elementary or polymeric diets based on proteins from various origins (table 2) [9]. In all cases, they are exclusion diets, i.e. the patient needs to stop his/her usual diet and replace it with an exclusive intake of the dietary product.

Nutritional treatments are effective [10]. In children, their efficacy is comparable to that of corticosteroids [11]. In adults, they are generally less effective, probably due to non-compliance with the exclusive liquid diet and/or a more advanced disease phenotype. Within a few days, nutritional treatments decrease digestive and extra-digestive symptoms, systemic inflammatory response and lead to mucosal healing. Thus, nutritional treatments can arguably induce deep remission. The *sine qua non* of their effectiveness is, however, that the exclusion diet is strictly followed. As soon as the usual diet is resumed, even in small quantities, gut inflammation returns.

The mechanisms of action of exclusive enteral nutrition remain unknown. It has been suggested that formulas may have direct anti-inflammatory properties (e.g. by modulating the TGF-β or NF-κB pathways) but this explanation does not explain the efficacy of the multiple products with various compositions. The impact of formulas on the intestinal microbiota has also been put forward to explain its mechanism of action, but in practice their effect is opposite to that expected, in particular by reducing microbial diversity [12]. The role of specific food allergens seems unlikely, since the formulas are based on proteins of various origins [9]. Furthermore, no specific foodstuff has been incriminated in CD and combining enteral nutrition with a highly controlled oral diet was recently shown to be effective [13].

To explain the link between food and CD, an unbalanced diet associated with the modern Western way of life is often cited. To illustrate this concept, mice genetically susceptible to colitis have been fed a high-fat diet instead of normal kibble [14]. The animals had modified bile acids composition, intestinal dysbiosis and a higher risk of colitis. In

another study, mice on a high sugar diet developed more severe experimental colitis than control mice [15]. The animals had fewer short-chain fatty acids, lower microbial diversity and higher intestinal permeability. Thus, animal models provide evidence that an unbalanced diet can lead to an over-risk of colitis.

From this point of view, the efficiency of nutritional treatments would be related to their ability to rebalance the diet. Because the modern Western diet is usually characterized by a higher amount of fats, refined sugars and animal proteins but less fibers, the roles of these food groups have been questioned in CD (for review see [16,17]). Rapid sugars have been suspected but not confirmed in a randomized clinical trial [18]. Fibers, particularly fruit, have been inversely associated with the risk of CD in America but not in Europe [19,20]. In addition, enteral nutrition products do not contain fibers. Animal proteins were suspected to be a risk factor of CD occurrence but their role in the risk of relapse was not established in a clinical trial.[2122] The total amount of fat ingested does not seem to have a major role either and enteral nutrition solutions, in particular those rich in fatty acids, still have a beneficial effect [9,23]. Alcohol has no significant role identified [24]. Researchers have also investigated for risky dietary patterns. A “prudent diet” rich in fruits, vegetables and fish was found to be protective in one study but not in another [25,26].

In fact, the macronutrient composition of enteral nutrition formulas used to treat CD display substantial variations with regard to both quality and quantity (table 2). Furthermore, the macronutrient balance of enteral nutrition therapy resembles that of spontaneous diets of patients [9]. Hence, clinical observations provide little support for the need to equilibrate the diet to treat CD.

In the absence of conclusive roles for specific aliments or nutrients, the role of industrialized food has been questioned (for a review see [27]). Indeed, ultra-processed food accounts for 16% of food weight and 33% of the calories consumed daily in developed countries.

Food additives which are increasingly used in industrial food production are likely candidates to carry a pro-inflammatory effect. Microparticles such as titanium dioxide and aluminum silicates are capable of accumulating in tissues, increasing intestinal permeability and promoting local inflammation [28,29]. However, in clinical trials, reducing the intake of microparticles had a limited impact [30,31]. Other groups of additives are Emulsifiers and thickeners. They include products like lecithin, carrageenans, carboxymethylcellulose and

polysorbate-80, which have repeatedly been associated with a risk of colitis in animals [32,33,34]. Food additives could thus play a role in inflammation by modifying the intestinal microbiota or by altering the intestinal barrier.

Although food additives are likely candidates, it should be noted that most enteral nutrition formulas used for the treatment of CD contain such additives. Among the 61 products studied by Logan et al. [9], all contained several food additives (median 11, range min: 6, max: 16). Modified starch, including maltodextrin, was present in all formulas, carrageenan was present in 12/55 (22%), carboxymethyl cellulose was present in 7/55 (13%) while sucralose and polysorbate 80 were present in 3/55 (5%) formulas [9]. In addition, all enteral nutrition products had approximately the same efficacy in treating CD, regardless of their additive content, suggesting that there is no need to exclude them from the diet [9]. Finally, a recent prospective cohort study found no association between ultra-processed food and CD [26]. Nevertheless, industrial food is becoming an increasingly topical issue and many diets offered to patients today exclude industrial food suggesting that it may be related to CD risk (e.g. CD exclusion diet [13], specific carbohydrate diet [35], paleolithic diet[36]….).

Is CD related to refrigerated foods?

Industrialization involves not only the processing of foodstuffs, but also the packaging of products and methods for transporting and preserving food. In that case, domestic and industrial refrigeration may explain the link between food and disease. Indeed, more than half of our food is refrigerated at some point including both natural and manufactured products. Of note, under this hypothesis, not only is the link between food categories and the disease difficult to identify, but even the link between processed and non-processed food becomes tenuous, as observed for CD.

The role of refrigeration, if any, cannot be linked to a change in the nutritional quality of food. Rather, refrigeration should be seen as promoting exposure to an exogenous risk factor. The most plausible candidates are psychotrophic bacteria. These bacteria multiply at best at temperatures above 30°C but they are still able to grow at temperatures near or below 0°C. Among them we can mention *Listeria monocytogenes*, *Pseudomonas fluorescens* and *Yersinia* species. These bacteria have been proposed to have a role in CD but only *Yersinia* has been effectively investigated in CD.

Species of the genus *Yersinia* are present in the environment, particularly in freshwater rivers and lakes. However, the main source of contamination in humans is food [37]. Typically, *Yersinia* is found in meat, poultry, raw vegetables, fish and seafood, pastries, raw milk, etc. [38,39]. Sample contamination rates are high, ranging from 10% to 75% depending on detection methods and food products. It can therefore be concluded that we are probably all exposed to *Yersinia* on a regular basis. The foodstuffs most commonly contaminated with *Yersinia* are meat products. However, exposure to *Yersinia* depends on the method of production, transport, storage and preparation of the food. For example, salted, dried or smoked meat is not contaminated and cooking easily kills bacteria. Under these conditions, it is very difficult to link CD to a particular food group and to evaluate the exposure of a given person to *Yersinia* species.

Yersinia are able to survive and proliferate at refrigeration temperatures and in vacuum packaging. Thus, contamination of food during the industrial production chain, transport or home storage appears to be very common. It is favored by factory farming, the world food trade, the production of industrial dishes and thus the modern western lifestyle as a whole. However, little is known about the actual exposure of the general population to *Yersinia,* as these bacteria are difficult to identify by culture. PCR methods are more sensitive but most often look for pathogenic bacteria, while the genus *Yersinia* comprises 18 different species, most of which have low pathogenicity. In addition, legislation does not require mandatory food monitoring in most countries. Where information of *Yersinia* contamination is available, it is for products traditionally monitored by the food industry but little data is available on manufactured food, delicatessen products or catering. Information on the sources of *Yersinia* is therefore very incomplete and the number of contaminated foods is probably seriously underestimated.

It should be noted, however, that enteral nutrition effectively excludes refrigerated foods. Formulas are based on sterile products or powders that are reconstituted extemporaneously or kept in the refrigerator only for short periods of time. Similarly, the exclusion scheme proposed by Levine et al. for the treatment of CD prohibits industrial foods, excludes frozen products and requires peeling of fruit and vegetables, thus drastically limiting *Yersinia* contamination [13]. Food handling that has been proven to be effective in CD therefore effectively excludes contact with products potentially contaminated by *Yersinia*.

Are Yersiniae present in the intestine of CD patients?

If *Yersinia* is involved in CD, a link between the bacteria and the disease must be established. The clinical similarity between CD and yersiniosis has been known for decades. More importantly, numerous reported cases indicate a possible progression from *Yersinia* infection to CD. Conversely, stigmas of immune responses towards *Yersinia* have been observed in CD patients [40].

The presence of the bacteria in CD lesions has only been reported by two independent teams [41,42]. In contrast, most groups did not find *Yersinia* species in CD lesions. This naturally raises questions about the validity of the link between CD and *Yersinia*.

It is obvious to everyone that CD is not due to bacterial multiplication and invasion of the gut by the bacteria. Thus, large quantities of bacteria are not expected to be found in the intestinal tissues. On the contrary, the role of *Yersinia*, if any, should be to trigger an excessive immune response. Given the intense immune response, the number of viable bacteria present in the lesions is expected to be very small, and therefore *Yersinia* could probably not be detectable by conventional microbiological culture methods. Similarly, analyses of the intestinal microbiota are doomed to failure because they do not reliably detect very minor bacteria present in the digestive tract.

In an attempt to explore this question, we developed a PCR detection method for seven species of *Yersinia*: *Y. aldovae*, *Y. bercovieri*, *Y. enterocolitica*, *Y. intermedia*, *Y. mollaretii* and *Y. pseudotuberculosis* [43]. We tested ileal samples from surgical specimens or biopsies from patients with CD, ulcerative colitis or non-inflammatory digestive diseases. 10% of the 338 participants had a positive PCR for at least one *Yersinia* species. This rate was the same regardless of the patient group or clinical presentation of CD. The most frequently positive samples were Peyer's patches, lymph nodes and ileal surgical specimens. Positivity was estimated in the range of 1 to 100 bacteria per sample tested. Finally, the larger the number of tissue samples examined in a given person, the greater the likelihood that this person would be positive. Thus, considering the very small sizes of the tested samples, it is credible that the bacteria is present in almost everyone.

From this work, we conclude that *Yersinia* are present in very small quantities in the human ileum, pathological or not, and this probably in a very common way. The distribution of the *Yersinia* species in CD patients was the same as that observed in contaminating food [38]. At the first glance, this suggests that these are "ordinary" bacteria that could be

considered as innocent bystanders. However, since *Yersinia* are more frequent in deep tissue and Peyer's patches, it is difficult to consider them as simple commensal bacteria of the intestinal lumen.

What are the intestinal consequences of exposure to Yersinia?

Yersinia species are generally low pathogenic bacteria, as shown by the high rate of exposure compared to the limited number of reported infections. In fact, most strains have few or no virulence factors and *Yersinia* infections are mainly caused by *Y. enterocolitica* and *Y. pseudotuberculosis*.

The reservoir of the bacterium is in the cecum and terminal ileum in mice. Bacteria are capable of colonizing Peyer's patches of the small intestine and isolated lymphoid follicles of the colon in mice and humans [44]. Several studies have shown that these sites are also the sites of the very initial aphtoïd lesions in CD [45,46]. *Yersinia* can lead to the local formation of epithelioid and gigantocellular granulomas, which are also hallmarks of CD. The bacteria then colonize the intestinal lymphatic tissue up to the draining lymph nodes. This dissemination occurs through uptake by macrophages and dendritic cells present in the intestine [47]. Indeed, a defect in bacterial clearance within the macrophage is associated with CD and may promote local dissemination of the bacteria (see below). Finally, it has been shown that modulation of T cells by *Y. pseudotuberculosis* leads to a strong alteration in T-regs induction and differentiation towards Th17 cells [48], both of which are also present in patients with CD.

In mice, a moderate dose of *Y. pseudotuberculosis* results in the resolution of intestinal infection within three weeks. Despite effective control of the bacterium by infiltration of infected tissues by polynuclear cells and macrophages, 70% of mice develop chronic mesenteric adenolymphitis, which is also seen in humans [49]. Lymph node hypertrophy can persist as long as 9 months after infection. The lymph nodes do no longer contain *Yersinia* but they are not sterile. They contain various microbes, mainly lactobacilli. Furthermore, chronic inflammation is partially reversible with antibiotic treatment, demonstrating the unspecific role of the intestinal microbiota in perpetuating the post-infection abnormalities.

Mice with chronic mesenteric adenolymphitis have a defect in response to antigens affecting T-reg cells, Th17 cells and IgA+ B cells [49]. The observed post-infectious effect is related to structural abnormalities of the lymph nodes and a defect in the migration of

dendritic cells from the epithelium to the lymph node. Of note, compartmentalized drainage of lymph node in the gut dictates adaptive immune responses in mice [50]. If true in human, this finding could explain why CD lesions are focal and relapse at the same place in case of recurrent exposure to microbial challenge.

In mice, post-infectious changes also include an increased permeability of the lymphatic vessels and accumulation of lipids in the mesenteric tissue that persists up to 10 months after acute infection. The enlarged mesenteric adipose tissue is infiltrated with inflammatory cells secreting Th1 cytokines (IFN-γ, TNF-α and IL-1β). It contains numerous dendritic cells as if these had escaped into the fat tissue before reaching the lymph node. CD8 memory lymphocytes of *Yersinia* infection are also located in mesenteric fat [51].

Increased lymph vessel density and lymph drainage abnormalities have also been shown in CD [52,53,54,55] or in a Crohn-like model in dogs [56]. The lymphatic network in CD is characterized by the presence of tertiary lymphoid follicles and granulomas, both related to lymphatic vessels [55,52]. A hallmark of CD is also that fat around the lymphatic vessels expands and moves up on to the intestinal wall, giving it the name “creeping fat”. This fat is a carrier of inflammation [57]. As in mice, its origin could be the permeability to the chylomicrons transported by the lymph.

Mice deficient for *Tlr1* were followed for 70 days after acute *Y. enterocolitica* infection [58]. Despite the infection was resolving without persistence of the bacterium in the tissues, mice showed poor weight growth, proximal colitis, dysbiosis, and increased immune response to commensal bacteria. Here too, experimental data are similar to those observed in CD, where dysbiosis with reduced microbial diversity and presence of alloantibodies against commensal bacteria or yeast are well demonstrated.

Overall and at the opposite of non-specific animal models of colitis, the immune response toward *Yersinia* mimics an enterocolitis consistent with CD in all respects. However, while everyone is exposed to *Yersinia* species, only a few people develop chronic gut inflammation.

Why are CD patients more susceptible to *Yersinia*?

Genetic studies in IBD have identified more than 200 susceptibility polymorphisms [59]. Most of these are common to CD and ulcerative colitis and sometimes to other inflammatory conditions. Few of polymorphisms specific to CD are mutations that alter the structure and function of proteins with a proven biological impact [60]. Of these, mutations in

the *NOD2* gene are the most specific and strongly associated with CD [61,62]. *NOD2* codes for an intracellular protein capable of recognizing products derived from the peptidoglycan, a component of the bacterial cell wall. NOD2 can activate several pro-inflammatory pathways: MAP Kinases and NF-κB; caspase 1 with IL-1β production and autophagy. Loss of function mutations have also been reported for *IRGM*, *ATG16L1*, *LRRK2* and *TPTN22* which participate in autophagy. To this list should be added genes that give rise to Mendelian disorders resembling CD, which are most often encountered in young children: *XIAP* [63], genes participating in the NADPH complex [64] and the *NPC1* gene involved in Nieman Pick type C1 disease [65]. Of note, most products of these genes are able to interact with NOD2 which thus appears as a hub in the CD susceptibility gene network.

All these genes whose function is specifically impaired in CD or related diseases provide a comprehensive view of the main biological consequences of genetic anomalies. What they have in common is that they all contribute to "xenophagy" which allows the handling and clearing of invading bacteria by phagocytic cells. Specifically, NOD2 is part of a plasma membrane-associated complex that is formed in contact with pathogenic bacteria [66]. It recruits ATG16L1, which initiates the formation of a double-membrane vesicle allowing the internalization of the bacteria. The autophagy machinery then starts up and the internalized bacterium is degraded by activation of the NADPH complex and addressing to the lysosome. Mutations in *NOD2*, *XIAP*, *NPC1*, *IRGM*, *ATG16L1* and NADPH complex all alter this biological function, leading to a defect in bacterial clearance [65]. It could be caricatured that the specific genetic defects of CD lead to macrophage indigestion of invading bacteria.

Mutations in the SCL39A8 zinc transporter gene have also been associated with CD [6768]. SCL39A8 regulates the intracellular concentration of zinc. In line, low zinc intakes have been associated with CD in two prospective cohorts [69,70]. Zinc deficiency was also predictive of a shorter time to subsequent relapse in a prospective syudy[71]. Zinc plays a key role in autophagy and bacterial clearance, thus pointing toward the same biological defect [72]. Furthermore, chronic stimulation of NOD2 induces metallothioneins expression of in macrophages, leading to increased intracellular zinc levels [73]. Co-stimulation of Toll Like Receptors 5 or 9 produces a synergetic effect. This finding can be related to the anecdotical case of a man carrying both *TLR5* and *NOD2* mutations who developed severe chronic yersiniosis [74]. Overall, the observations on zinc homeostasis also support a key defect in autophagy and bacterial clearance in CD.

Several bacteria, including *Salmonella typhimurium*, *Shigella flexneri*, *Listeria monocytogenes*, group A *Streptococcus*, *Francisella tularensis*, *Mycobacterium tuberculosis* and *Yersinia* species are all engulfed by the phagocytes via xenophagy (for review see [75]). Is *Yersinia* special in regard to CD susceptibility genes?

NOD2 is involved in the innate immune response toward a very large number of bacteria [76]. For all pathogens studied, Nod2 mutations associated with CD are deleterious *in vitro* or *in vivo* and lead to more severe infections [77]. To our knowledge, the only exception is *Y. pseudotuberculosis*. Mice that are invalidated for *Nod2* or carry a mutation homologous to the human 1007fs mutation associated with development of CD are resistant to oral infection with *Y. pseudotuberculosis* [78]. This effect is due to an increased immune response at the site of entry of the bacteria. At the molecular level, it is likely mediated by fine tuning of IL-1β secretion which is controlled by the bacterial virulence factors YopJ and YopM [79]. YopJ subverts Nod2/RICK/TAK1 signalling and promotes activation of caspase 1 and IL-1β secretion [80]. No link has been demonstrated between YopM and Nod2, but both molecules are structurally close, with leucine-rich domains, and both interfere with caspase 1. Thus, CD associated *Nod2* mutations appear to result in a more intense response to oral *Y. pseudotuberculosis* infection.

NOD2 mutations are common in human populations of European ancestry but are rarely present in Asian and African people [8182]. In Europe, up to 10% of healthy individuals carry one or more CD associated *NOD2* mutations. This particularly high frequency suggests a beneficial effect for mutation carriers that may outweigh the deleterious effects of mutations in response to pathogens and the development of CD [59,83]. As an example, such a mechanism has been proposed for a mutation in the hemoglobin gene that confers protection to malaria in heterozygotes but causes sickle cell anemia in homozygotes [84]. Since *Y. pseudotuberculosis* is genetically very similar to *Y. pestis* [85], we hypothesized that *NOD2* mutations may have provided a survival advantage to mutation carriers in the past during plague epidemics [86]. As supposed, the current frequencies of CD-associated *NOD2* mutations (i.e. in the offspring of plague survivors) are correlated with the intensity of past *Y. pestis* epidemics in European and Mediterranean countries. This finding further supports a link between *NOD2* and *Yersinia*.

To our knowledge, among the other CD susceptibility genes, a relationship with *Yersinia* infection has been investigated only for *ATG16L1* [87]. Human monocytes carrying the

CD at-risk mutation have a clearance defect with respect to *Y. enterocolitica*. The same observation was made with mouse macrophages carrying the mutation homologous to the human one. These macrophages secreted more IL-1β in the presence of the bacterium. Finally, mice mutated for *Atg16l1* developed an exacerbated immune response with production of IL-1β, TNF-α and IL-6 in their mesenteric lymph nodes. Thus, as with *NOD2*, *ATG16L1* mutations that are associated with CD are characterized by an exacerbated inflammatory response in the gut following exposure to *Yersinia*.

Conclusion.

In summary, data from recent years provide additional elements in favor of the cold chain hypothesis which is now supported by a large number of independent observations from epidemiological, clinical, anatomopathological, microbiological and molecular studies (figure 2A). With time, the hypothesis thus becomes more and more valid in proposing a comprehensive theory to explain the causes of CD. But as no single experiment can definitively confirm the theory, we must continue to test it with additional works. Among them (and even if insufficient to definitively validate the hypothesis), a randomized clinical trial comparing patients with low versus high exposure to *Yersinia* would be an important step.

Above all, if the cold chain hypothesis is exact, it would have important practical consequences for the management of CD. Several non-mutually exclusive interventions could be proposed to prevent and treat CD (Figure 2B). The first is to implement food surveillance of *Yersinia* species in food products and to revise rules of good practice in food industry. The second is to reduce patient exposure to *Yersinia* through precautionary measures. These measures would necessarily be very restrictive. Fruit and vegetables should be washed, peeled or cooked before consumption. Meat, fish and dishes should be cooked or reheated to above 70°C. Certain products should be prohibited such as ice creams, prepared salads, cold cuts, some dairy products, etc. In general, foods prepared at home with controlled products are preferable. Finally, the third intervention could be to reduce *Yersinia* colonization of food and surfaces by using phages specific to the bacteria [88].

Table 1. Overview of the cold chain hypothesis and key findings supporting it.

Causality chain	**Key findings.**	**References**
The development of refrigeration….	CD is associated with the modern Western way of life. The expansion of domestic cold parallels the outbreak of CD in US, Europe and China. CD patients are exposed earlier to domestic cold.	3 4, 90 5, 6
increased the exposure to Yersinia species.	Yersinia species are common in refrigerated food. They can be found in ileal tissues of CD and controls. While enteral nutrition products contain all kinds of macronutrients and additives in various proportions, exposure to refrigerated foods is drastically reduced in all CD diets.	13, 36-38 40-42 9, 10
The host reaction against enteric Yersinia mimics CD in all respects	Key findings during the acute infection: ° Lesions centered by intestinal lymphoid follicles ° Epithelioid and gigantocellular granulomas. Chronic lesions observed after the infection: ° Decrease of Tregs and increase of Th17 lymphocytes ° Mesenteric adenolymphitis and alteration of the lymphatic network ° Inflammatory mesenteric adipose tissue ° Dysbiosis and increased reactivity toward commensal bacteria	43-45 47, 48, 50, 57
especially in genetically at risk people.	Mutations specifically associated with CD are characterized by a defect of intracellular bacterial clearance. NOD2 mutations are associated with an increased inflammatory response toward Yersinia in mice. ATG16L1 mutations are also associated with an exacerbated response to Yersinia. NOD2 mutated people have probably been protected during plague outbreaks in the past.	60-65. 78 85 84

Table 2. Nutritional analysis of 61 products with reported efficacy for induction of clinical remission in CD patients (from Logan M. et al. Ref 9).

Diet	Polymeric: 39 Semi-elemental: 16 Elemental: 6
Main sources of macronutrients	**Proteins**: milk, soy, pea, meat, egg. **Carbohydrates**: maltodextrin, sucrose, glucose syrup, starch of diverse origins, corn syrup, rice flour, dextrins. **Fat:** sunflower oil, canola oil, soybean oil, rapeseed oil, fish oil, corn oil, palm oil, coconut oil, salflower oil, milk fat, arachidonic acid, DHA, none. **Fibres:** fructo-oligosaccharides, inulin, gum arabic, pectin, resistant starch, cellulose, guar gum, none.
Proportions of nutrients.	Carbohydrates: from 22,8% to 89,3% Protein: from 7,8% to 30,1% Fat: from 0% to 52,5% Saturated fat: from 0% to 28,6% n-6:n-3 fatty acid ratio: from 0,25 to 46,5
Additives (proportion of products containing the additive)	Modified starch (60/60) Inorganic phosphates (49/54) Maltodextrin (47/60) Soy lecithin (38/55) Carrageenan (12/55) Carboxymethylcellulose (7/55) Sucralose (3/55) Polysorbate 80 (3/55)

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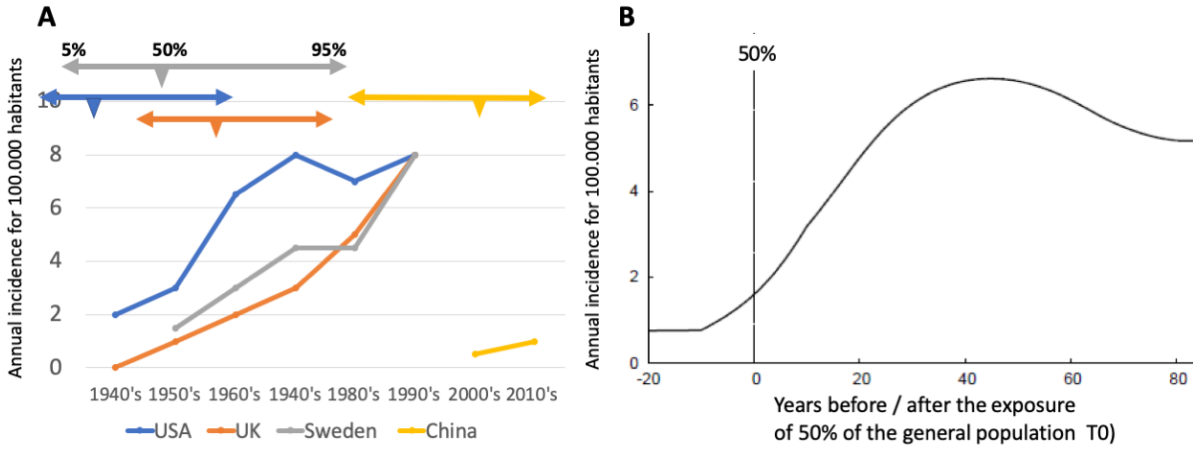

Figure 1. Observed (A) and modelled (B) annual incidence of Crohn's disease in relation to exposure to domestic refrigeration A. Approximate values of the annual incidences of CD in USA [89], Sweden [90], UK [91] and China [3] for the indicated decades. Arrows show the periods during which domestic refrigeration has expanded. The ends of the arrows correspond respectively to the approximative times when 5% and 95% of households own a refrigerator. Arrowheads indicate when about 50% of the population owns a refrigerator (from ref. 92). B. Values calculated from a mathematical model predictive of disease risk (from ref.[4]). T0 corresponds to the time point when 50% of the population is exposed to the environmental risk factor (here supposed to be domestic refrigeration).

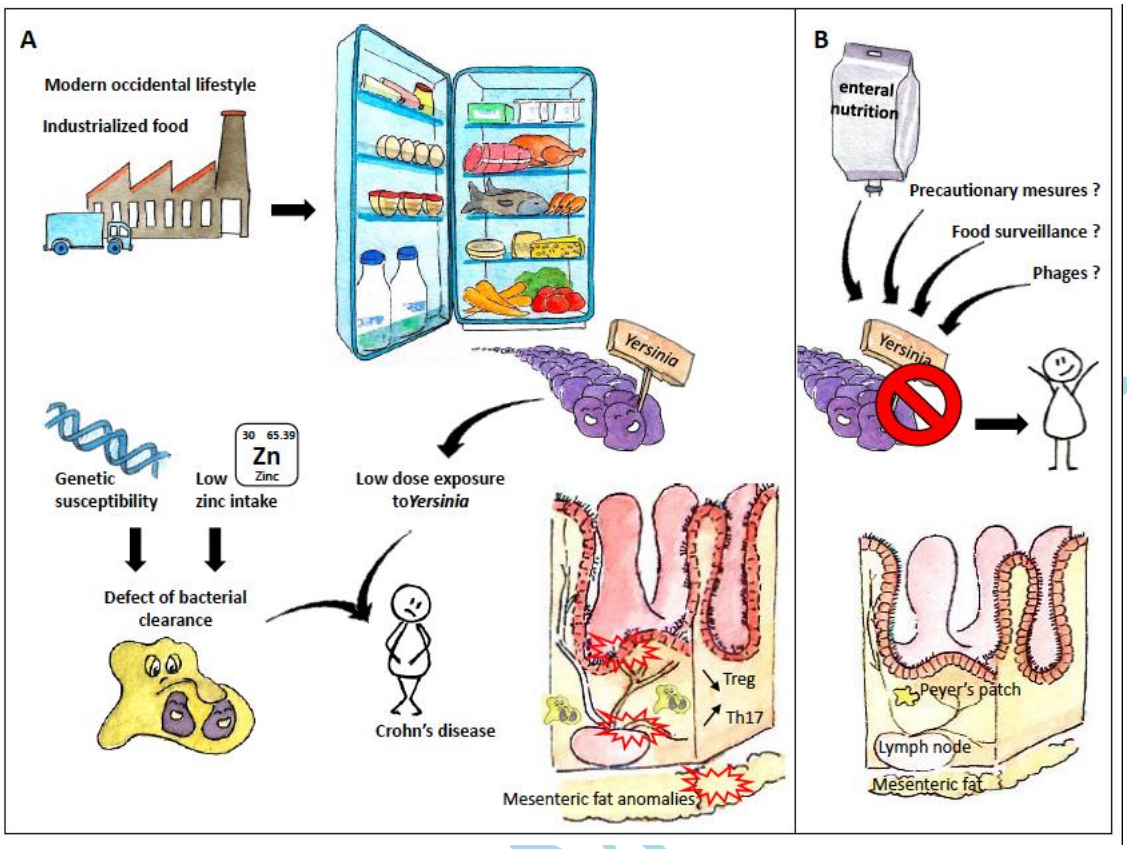

Figure 2. Graphical abstract of the cold chain hypothesis. A. Chain of causalities proposed to explain Crohn's disease occurrence. B. Suggested preventive actions to limit Crohn's disease incidence (in healthy people) or relapses (in patients).