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Habilitationsschrift

THE ORIGINS OF THE ASTHMA EPIDEMIC
MINING THE HYGIENE HYPOTHESIS THROUGH TEN QUESTIONS

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List of abbreviations

SES = socioeconomic status
RAST = radioallergosorbent test
CAP = capture
HAV = hepatitis A virus
ELISA = enzyme linked immunosorbent assay
TG = Toxoplasma gondii
HP = Helicobacter pylori
GI = Gastrointestinal
Treg = T regulatory cells

Summary

The allergy epidemic has been attributed to changes in the interactions between humans and the microbes of their ecosystem consequent to a “western lifestyle”. Microbes that coevolved with mammals influenced the evolution of their immune structures and functions along the zootic scale up to primates. Partially deprived of these microbial stimuli, some components of the human immune system (TH2-like activities) are no longer adequately regulated by other components. Therefore, in modern societies, allergies spread among new cohorts according to gradients dictated by hygiene and by the individual degree of genetic predisposition to atopy. Collectively, these concepts are known as the “Hygiene Hypothesis”.

This thesis reports on a series of investigations on the Hygiene Hypothesis suggesting: A) that the sibshio-size and birth order effect on the development of hay fever is mediated by an impact on allergen-specific IgE responses; B) that this effect cannot be observed in the presence of poor hygienic standards; C) that atopic sensitization and allergic diseases are inversely related to the exposure and acquisition of foodborne and fecal oral infections in western societies; D) that in westernized societies the epidemic trend of hay fever and asthma can be observed only in the fraction of the population not exposed and acquiring hepatitis A virus infection; E) that, among the infections of the GI tract, mild intracellular pathogens but not commensals are the best candidates to exert an atopy protecting effect.

All this information has been then analysed in the context of other epidemiological and experimental studies, most of which support the seroepidemiological observations reported here. The implications of these studies on the elaboration of new strategies for prevention and therapy of allergic diseases are also discussed. A new model has been elaborated to evaluate in an historical perspective the whole phenomenon of the allergy and asthma epidemic in western societies.

1. Introduction

The rising trend in cases of asthma is paralleled by coordinated efforts to identify the causes of this worldwide epidemic. Although the question why asthma is increasing remains unanswered, advances made over the last decades have led to a better characterization of the phenomenon, consequent to which, some hypotheses are now seen in a different perspective and new ones have been formulated.

1.1 The epidemiologic trend of asthma

In the nineties, the evidence for a worldwide increase in asthma cases is so strong and consistent that the trend cannot be considered artefactual [reviewed in 1]. Several studies reported an increase of the current prevalence of asthma estimated with the same methodology in cohorts born in different years in Europe (fig. 1,a) [2-8] and in other continents (fig. 1b) [9-19].

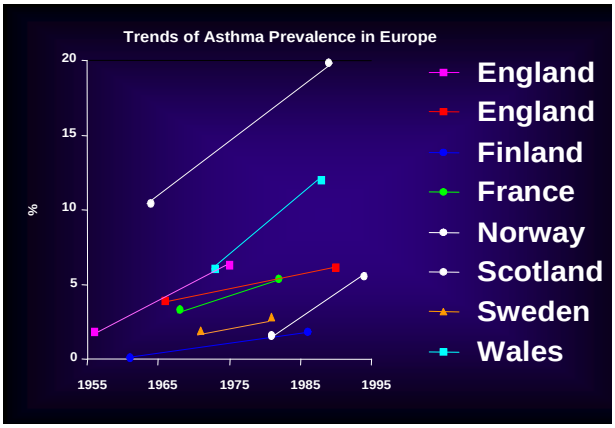

Figure 1a – *Trend of asthma in Europe*

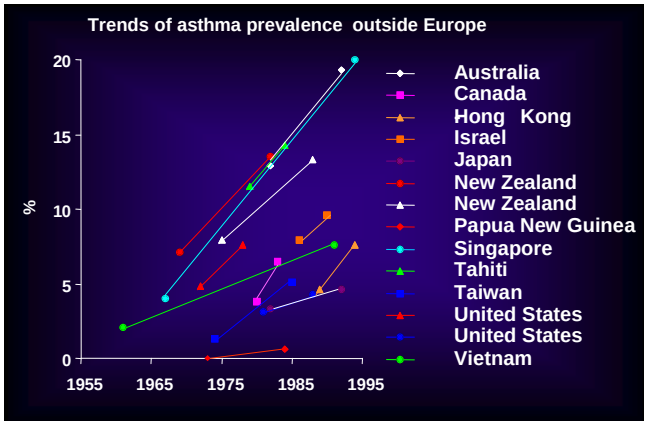

Figure 1b – *Trend of asthma outside Europe*

It is unclear when the epidemic started. In the UK, asthma was considered very infrequent in the nineteenth century, while the earliest reports of an established rising trend date back to the 1960s. Therefore, it could be argued that in the UK the epidemic started during the first half of the twentieth century. A pattern of geographical distribution began to emerge from international cross-sectional studies such as the European Community Respiratory Health Survey (ECRHS) [20] and the International Study on Asthma and Allergies in Childhood (ISAAC) [21] that provided comparable data on the prevalence of asthma in different parts of the world: with some exceptions, the highest prevalence of asthma was observed in westernised countries where English is the main language, intermediate values were found in other western countries (e.g. central and southern Europe), and the lowest prevalence occurred in most developing countries and in eastern

Europe. Therefore, the prevalence of asthma is higher in countries where a transition from a traditional to a westernised lifestyle occurred earlier, suggesting that a given population experiences a progressive increase in asthma cases during the process of westernisation. This hypothesis is being investigated in prospective studies in which the prevalence of asthma is being monitored in areas where westernisation is just starting (developing countries) or accelerating (Eastern Europe).

Similarly, we do not know if the epidemic will come to an end, at least in those countries where it started earlier, or if the rising trend will continue among westernised populations so enhancing an already high prevalence. Interestingly, already ten years ago there was substantial evidence suggesting that the incidence of episodes of asthma may have reached a “plateau” in the UK [22] and this was confirmed later in other westernized countries. These data would imply that in some westernised countries, exposure to unknown causes of the epidemic is no longer increasing (or it may even be declining).

1.2 One or more causes?

It is important to identify the causes underlying the asthma epidemic in order to find strategies for primary prevention and new therapeutic approaches. The hypotheses made in the nineties to explain the epidemic trend fall into two main groups: one that points to increasing exposure to aggressive factors; and the other that implicates decreasing exposure to protective factors. The most cited aggressive factors are airborne indoor or outdoor pollutants [23], high salt intake [24], indoor allergens [25], drugs (e.g. contraceptive pills) [26], and vaccines [27]. The principal proposed protective factors are antioxidants [28], microbial burden [29,30], and physical exercise [31].

At one extreme, the whole phenomenon of the asthma epidemic might be attributed to a single causative factor (pollution or hygiene or increased allergen exposure, etc.). At the opposite extreme, the epidemic might be considered a problem so complex that its solution should be at least equally complex. In this case, multiple causes would underlie the asthma epidemic, and no single factor would be sufficient, *per se*, to explain it.

The multiple cause approach is encouraged by the fact that asthma is itself defined a “multi-factorial disease” with a large series of causative, inducing, triggering and aggravating factors, each of which help shape the disease phenotype in the single patient by interacting with the expression of her/his unique genetic background at a given age. Accordingly, a model based on a single major cause of the epidemic could be rejected as being too simplistic. On the other hand, one should distinguish between factors associated with asthma at a given time and in a given population sample, and factors responsible for the epidemic. For example, active and passive smoking is clearly an important risk factor for asthma and it plays a major role in the natural history

of this disease in the single patient [32]. But tobacco smoke is not a causative factor in the epidemic trend of asthma, because the disease is increasing despite a decrease in smoking at the population level. Accordingly, the rather long list of factors that may be considered causes of or contributors to the epidemic becomes much shorter when factors that do not change with westernisation and on a global scale are eliminated. Given these premises, the non genetic risk factors of asthma may be divided in two major categories: those that determine most of the overall prevalence of asthma in a given population (primary risk factors), and those that determine who, in that population, will be asthmatic (secondary risk factors).

The characteristics of the increase in asthma cases in the various developed countries are rather similar: the incidence is rising mainly (but not exclusively) in new cohorts, it is strongly associated with atopic sensitisation (see below) and it parallels westernisation processes. It can reasonably be assumed, therefore, that the primary causes of the epidemic are similar in different parts of the world and that they are to be sought among known risk factors whose variation (increase if aggressive, decrease if protective) has been unidirectional and progressive over time during the twentieth century and whose trend is being reproduced in populations, in any area of the world, when they acquire a westernised lifestyle [33].

Consequently, not all types of studies can identify primary cause(s) of the asthma epidemic: it may be too late to search for these causes within urban areas of developed, English-speaking countries whose westernisation started earlier than in the rest of the world. In these cities, exposure to primary causes of asthma may have already spread to the whole urban population, including the more disadvantaged classes. In the absence of variability for exposure to primary causes, only the effect of exposure to secondary causes of asthma would be observed. Similarly, serial studies performed over a short period (e.g. <10 years) in the same area will be informative only if during that time there is a dramatic change in lifestyle or environmental exposure, which is actually unusual. Differently, studies of populations or communities that display a broad range of asthma prevalences (urban vs. rural areas of developing countries), or that abruptly change their environment and lifestyle (immigrants, refugees, adoptees, etc.) would have a much better chance of identifying primary causes, especially if the confounding role of secondary causes is also accounted for.

1.3 An epidemic of “asthma” or an epidemic of allergic eosinophilic inflammation?

It is difficult to discriminate between the trend of asthma and that of allergy, because the two disorders are closely interrelated. Many studies have demonstrated that the increase in asthma cases observed in developed countries is an indicator of a more complex phenomenon moving on a larger scale. ISAAC phase I studies demonstrated a significant correlation between the prevalence of asthma in a given country and that of allergic rhinoconjiunctivitis and atopic eczema

in the 155 examined centres [21]. Studies examining cohorts at least 10 years apart showed an increased prevalence not only of asthma, but also of allergic rhinitis and atopic eczema: among Aberdeen (Scotland) schoolchildren (8-13 y) the prevalence of asthma was 4.1% in 1964, 10.2% in 1989 and 19.6% in 1994, compared with 3.2%, 11.9% and 12.7% for allergic rhinitis and 5.3%, 12.0% and 17.7% for atopic eczema [34-35]. The excess in prevalence of asthma in westernised countries is almost totally accounted by an excess in atopic sensitisation: in 1992 asthma was more frequent in children from West Germany with respect to East Germany, but the difference was almost totally due to a marked difference in the prevalence of atopic sensitisation [36]. Among Scottish adults the prevalence of asthma has increased more than two-fold in 20 years, but subjects "responsible" for an excess rate were also affected by hay fever [37]

An increase in atopic sensitisation has been demonstrated in several studies [reviewed in 38]; perhaps the most striking is a study of student girls (13-14 years of age) in Akita (Japan) where serum IgE against airborne allergens (mites, and grass and tree pollen) occurred in 21.4% in 1978, 25% in 1981, 35.5% in 1985 and 39.4% in 1991 [39]. The foregoing data strongly suggest that the so-called asthma epidemic is merely an aspect of a rising trend of the propensity to produce IgE responses toward airborne allergens (“atopy”) and mucosal or cutaneous eosinophilic imflammation leading to organ-specific symptoms (involving bronchial and/or nasal mucosae and/or skin).

Based on these premises, the reason for the increased incidence of asthma cases might emerge from investigations of the primary causes of the underlying atopy epidemic. Unfortunately, objective markers of mucosal eosinophilic inflammation or of an increased propensity to Th2 immune responses that is easy to use in epidemiological studies and relatively inexpensive are still not available. Epidemiologic studies still rely upon less specific markers of atopic disease, namely skin sensitisation and testing of serum IgE. IgE sensitisation is not *per se* a disease and it may be just a down-stream event in asthma and not necessarily the major pathogenetic aspect of the disease. Nevertheless, given the strong association of atopic sensitisation with eosinophilic inflammation, atopy testing (by SPT, RAST or multi-RAST assays) is the best tool currently available with which to investigate why the propensity to develop eosinophilic inflammation is increasing worldwide.

Here again, however, the epidemic trend of atopy and eosinophilic inflammation might itself be not only an immunologic phenomenon, but the expression of still more complex modifications in the structure and physiology of our body surfaces exposed to the external environment (skin and mucosae) involving epithelia, fibroblasts, and other components closely interacting with the innate and adaptive immune system. Consequently, the potential role of an increasing prevalence of non-specific bronchial hyperreactivity independent from eosinophilic inflammation should be also investigated.

1.4 What requisites should a hypothesis fulfil?

In discussing seasonal allergic rhinitis, John Elliotson (1791-1868) noted "that some of the nobility of the very highest order have it" [40]. Obviously, aristocrats had easier access to doctors and perhaps they perceived symptoms that less advantaged people would have overlooked [40,41]. John Bostock noted in 1828 that *catarrhus aestivus* only occurred "in the middle or upper classes of society, some indeed of high rank" [41]. George Miller Beard (1839-1883) also reputed hay fever a rare disease of the privileged classes [40]. The ISAAC study has shown that today atopic diseases are diseases of privileged countries [21]. Moreover, an interesting pattern emerges from studies of the increase in atopy in populations that are changing from a traditional lifestyle to a western lifestyle. In a series of studies of two large British cohorts born in 1958 and 1970, atopy was more frequent among higher socioeconomic classes [33]. Similarly, among adults examined in the Second Nutritional and Health American National Examination Survey, atopic sensitisation was associated with higher income and education [42]. In all these studies, the strong association between indicators of socioeconomic status and atopy was independent of residence and other known sociodemographic factors. It is fascinating to think that a lifestyle associated with wealth may induce atopy and atopic diseases nowadays, exactly as it did among British aristocratics of the nineteenth century, well before the introduction in our daily life of a multitude of substances or habits acquired only in the twentieth century.

The propensity to develop atopy and atopic diseases was found to be a function of the family structure. In the British cohort studies cited above the risk of having hay fever was inversely related to the overall number of siblings (sibship size effect). Even the position in the sibship is relevant, because hay fever was less frequent in the presence of older versus younger siblings (birth order effect). Both sibship size and birth order were related to the occurrence of atopic eczema during the first year of life [33]. These associations have been confirmed in several other studies.

Another consistent finding is that farmers rarely suffer from atopic diseases, including allergic asthma. This was first observed by Charles Blackley in the last century, and has been investigated in greater depth more recently. Children growing up on Swiss farms [43] were less likely to be sensitised to common aeroallergens and to suffer from hay fever than children living in the same villages but in nonfarming families. These associations persisted after adjusting for a family history of asthma and allergies, parental education, number of siblings, maternal smoking, pet ownership, indoor humidity and heating fuels. Interestingly, children of full-time farmers were even more protected from atopy than those of part-time farmers [43]. In conclusion, hypotheses explaining the epidemic of asthma should have not only a very strong biological basis and evolutionistic plausibility, but should also explain why atopy and atopic diseases do not spread at random in a population but rather follow precise gradients and rules that, especially in Europe, seem to protect people with a low socioeconomic status, large family size and a farming lifestyle.

1.5 Pollution, allergen exposure, diet, hygiene: do they fulfil the requisites for a plausible hypothesis?

Among the long list of hypotheses previously reported only four have been investigated in depth. A complete analysis of these hypotheses is far beyond the scope of this chapter. Here we will just schematically review what they may explain and what major criticisms may be raised against them.

Pollution. Atopic diseases affect mainly the airways, which explains why airborne pollutants were first implicated in the asthma and allergy epidemic [23]. Animal models and *in vitro* studies suggested that certain pollutants (e.g. diesel exhaust particles) can influence the way an allergen, once inhaled, is processed by antigen-presenting cells, so facilitating TH2 responses [44]. The "pollution hypothesis" explains why atopic diseases are more frequent in urban and industrialized areas but not why they are less frequent in large and disadvantaged families or in heavily polluted cities of Eastern Europe or the Republic of China. Moreover, pollution has declined in many countries in Western Europe, displaying an opposing trend to the epidemic. The widely accepted notion that outdoor pollution (as well as smoke) can exacerbate already existing asthma and trigger new asthma attacks in affected patients does not imply that pollution is the major cause of the asthma epidemic.

Nutrients. Westernisation is characterized by profound changes in dietary habits, so it is conceivable that they may have caused changes in disease patterns. Accordingly, the increasing trend in allergy and asthma cases has been attributed to: a) increased salt intake [24]; b) increased consumption of vegetal oils (margarine) [45]; c) decreased consumption of anti-oxidants (e.g. vitamin C) [28]; and d) bottle feeding practices [46]. All these hypotheses have been discussed in detail elsewhere [47]. These "nutrient" hypotheses, however, do not explain the sibship size and birth order effect. In addition, dietary composition still varies very much among different areas of the western world, which runs contrary to the rather uniform increasing trend of atopic diseases.

Indoor allergens. Perhaps the risk factor with the strongest association with asthma in developed countries is exposure to indoor allergens [48]. Current high exposure to indoor allergens is associated with both sensitisation and asthma symptoms; high exposure to indoor allergens early in life is also associated with sensitisation later in life. In Australia, an increase in asthma cases was related to increasing concentrations of mites in dwellings. Moreover, the prevalence of asthma is very high in countries that have the highest mite allergen levels in the world (UK, New Zealand and Australia) [25]. In areas where mites are virtually absent, allergic asthma is associated with sensitisation to other indoor allergens (pets) [49]. Thus, the asthma epidemic could be at least partially due to increasing exposure to these allergens [25]. On the other hand, there is still no firm

evidence that indoor allergen exposure has increased all over Europe in the second half of the twentieth century. In Leipzig (former East Germany), where allergic asthma was rather infrequent, houses had mite allergen concentrations similar to those found in former West Germany, where the prevalence of allergic asthma was significantly higher [50]. Finally, also this hypothesis fails to explain why atopy is less frequent in large families, among farmers, or among the disadvantaged and it has no evolutionistic background.

Hygiene. The allergy epidemic has been attributed to changes in interactions between humans and the microbes of their ecosystem consequent to a “western lifestyle” [30]. Therefore, in modern societies, allergies spread among new cohorts according to gradients dictated by hygiene and by the individual degree of genetic predisposition to atopy. Collectively, these concepts are known as the “Hygiene Hypothesis” [29,30]. This theory, unlike others (pollution and increased allergen exposure), explains the epidemic in evolutionistic terms [51] and its biological plausibility is supported by an overwhelming amount of *in vivo* and *in vitro* evidence. Microbes that coevolved with mammals influenced the evolution of their immune structures and functions along the zootic scale up to primates. Partially deprived of these microbial stimuli, some components of the human immune system (TH2-like activities) are no longer adequately regulated by other components (TH1-like activities) [52,53]. This hypothesis explains all the gradients observed in the distribution of atopy, considering that hygiene spread earlier with westernisation in UK and in other English speaking countries and among the advantaged, and considering also that infections are acquired less frequently and later in life in small families and in the first born [33]. It also explains why farmers are protected by atopic diseases, farms being the environments where "modern" humans are more exposed to microbes, especially bacteria.

In the following 10 sections the hygiene hypothesis will be examined through ten research questions, corresponding in all to ten publications. Their sequence is established by a step-by-step (non-temporal) process: each tentative answer generates the next question. Additional publications on this topic are quoted in the appendix.

2. Ten research questions elaborated out of own works

2.1 Are the sibship-size and the birth-order effect on hay fever due just to reporting bias or are they mediated by their objective impact on atopic sensitization?

P-1 *Matricardi PM, Franzinelli F, Franco A, Caprio G, Murru F, Cioffi D, Ferrigno L, Palermo A, Ciccarelli N, Rosmini F. Sibship size, birth order, and atopy in 11,371 Italian young men. J Allergy Clin Immunol 1998; 101:439-44. [54]*

In his seminal paper, David Strachan reported that in a British population the risk of having hay fever was directly related to the socio-economic status (defined by the father's occupation) and inversely related to the overall number of siblings ("sibship size effect"); he also noted that hay fever was less frequent in the presence of older rather than younger siblings ("birth order effect") [29]. Assuming that infections were acquired more frequently in large, less affluent families and earlier in the presence of many older siblings, Strachan proposed that exposure to common infections, especially very early in infancy or even through the mother in utero, may "protect" from hay fever [29]. As a corollary, he hypothesized that the decline in cross infections within young families due to decreasing in family size and to improvement in hygienic standards is, among the set of characteristics of the WLS, the one mostly responsible for the increase of atopy prevalence (Strachan's original "Hygiene Hypothesis") [29]. (Fig 2)

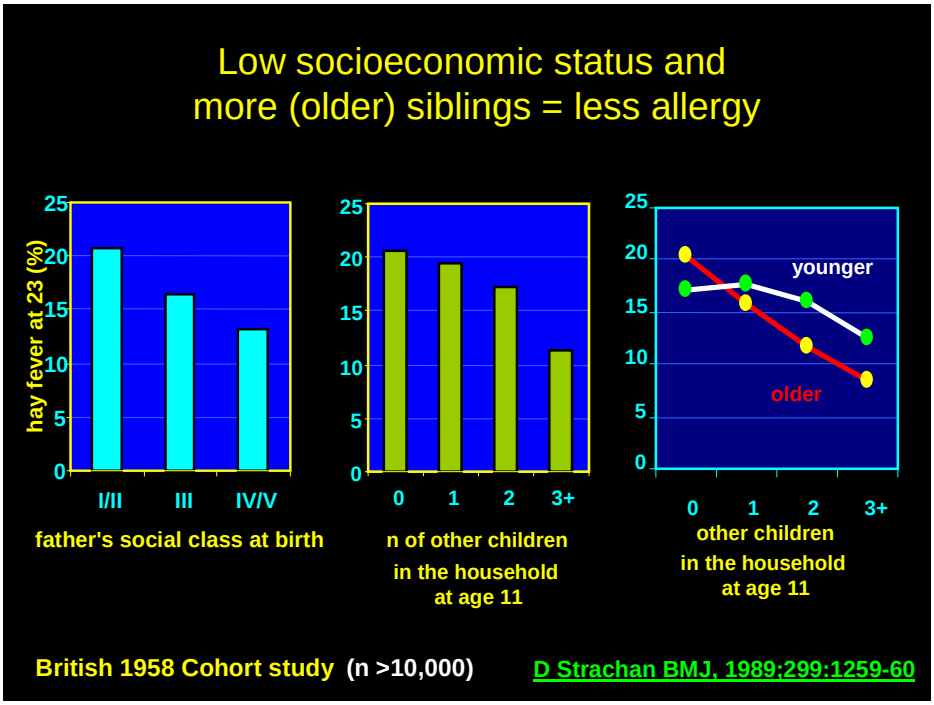

Figure 2 – *The data at the origin of Strachan's original formulation of the "Hygiene Hypothesis"*

At the time of the first study, the relevance of sibship size on atopy and atopic diseases had been confirmed repeatedly in studies performed in Britain and in Germany. On the contrary, the association of birth order with atopy or hay fever has been confirmed in two other population samples7, 8 but was not found in another survey among British adult women. Thus the necessity of further confirmation has been emphasized. This demand, however, clashed with the requirement of very large population samples, combined with an objective evaluation of atopy, to distinguish with enough precision the intriguing but rather weak relation of birth order with atopy.

The aim of our first study was to investigate the association of both sibship size and birth order with atopy in a large population of over 11,371 Italian young men. To this end, we used sociodemographic data collected by compilation of standard forms together with data on serum IgE sensitization toward common airborne allergens routinely obtained by an immunoenzyme multiallergen assay according to validated methodology.

This was a retrospective survey young men, 18 to 24 years old, all candidates for enrollment in the Italian Air Force. Demographic data had been collected by a standard questionnaire. Specific IgE for locally relevant airborne allergens had been tested by a multi-RAST assay (CAP-Phadiatop). We found that the prevalence of atopy (defined as a high level of specific IgE against inhalants [cut-point >1.2 log RU]) was inversely related to the total number of siblings (25% in those with no siblings and 9% in those with five or more siblings), with a mean of a 3% decrease in prevalence for each added sibling. This relation persisted after adjustment for relevant variables such as father's education and rural and southern residence. An independent association between birth order and atopy was also observed because the decrease in atopy prevalence with increasing numbers of older siblings was significantly steeper than that found with the number of younger siblings. (Figure 3)

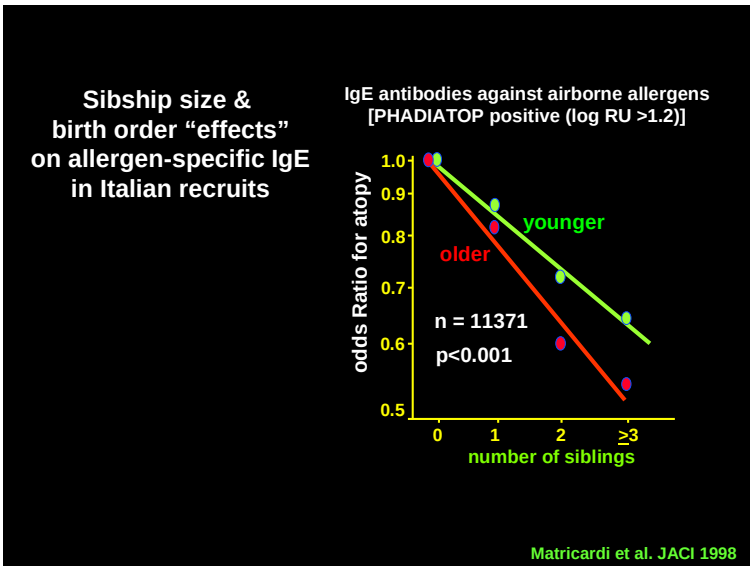

Figure 3 - *Sibship order and IgE sensitization in 11,371 Italian young males*

We concluded that in a very large and homogeneous population sample of a Mediterranean country, not only sibship size but also birth order was significantly associated with an objective measure of atopy. This observation further highlighted the role of family structure in the development of atopy and supported the hypothesis that cross-infections acquired early in infancy

or in later childhood might prevent development of atopy later in life. We did not exclude, however, a mother's immune system already "primed" by many pregnancies might interact in a qualitatively slightly different way, with her fetus tuning the first immune response toward environmental allergens. Nevertheless, in utero events obviously cannot explain the inverse association of the number of younger siblings with atopy.

2.2 Is atopy less frequent in subject living with a less hygienic lifestyle?

P2 *Matricardi P.M., Rosmini F., Ferrigno L., Nisini R., Rapicetta M., Chionne P., Stroffolini T., Pasquini P., D'Amelio R. Cross-sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis A virus. BMJ 1997;314:999-1003. [55]*

High recirculation of infectious agents is associated with environmental conditions such as overcrowding, poor hygiene in handling food, and infrequent washing. One of the most reliable markers of being brought up unhygienically or having been exposed to an infectious environment was considered in southern Europe to be the presence of antibodies to hepatitis A virus. We tested therefore the hygiene hypothesis by analysing the relation between the presence of hepatitis A antibodies and atopy in a population of 1659 Italian military students whose family structure, residence, father's education, atopy, and hepatitis A serology were already available. In this study, young men with antibodies to hepatitis A virus showed a lower prevalence of atopy and atopic respiratory diseases; interestingly, this was independent of the number of older siblings and other relevant risk factors. Accordingly, the prevalence of atopy among seropositive subjects was always low, whatever the number of older siblings was, but among seronegative subjects was only low when they had three or more older siblings. This suggested that common infections acquired either early in life due to the presence of many older siblings (among seronegative subjects) or due to unhygienic living conditions (among seropositive subjects) may have reduced the risk to develop atopy. This study also suggested for the first time that faecal contamination of the environment - a condition which facilitates transmission of the hepatitis A virus - may protect from atopy.(Figure 4)

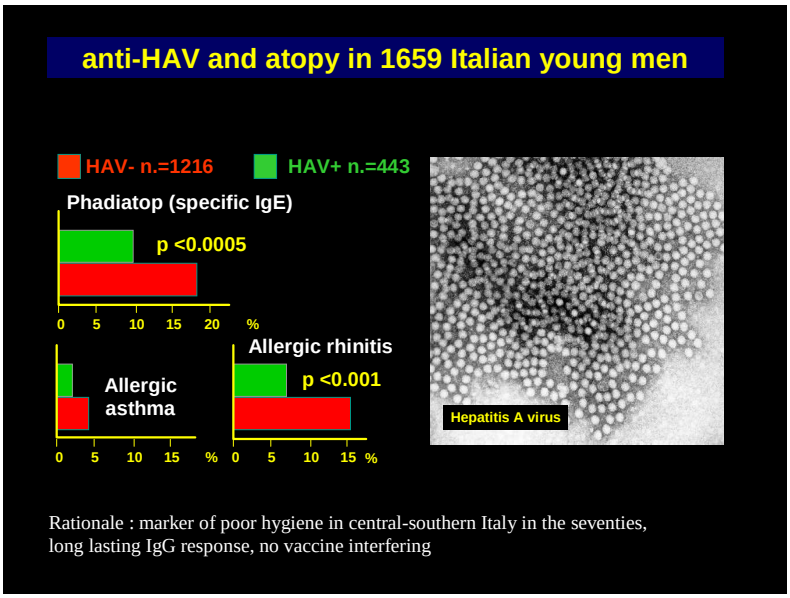

Figure 4 – *The inverse association between respiratory allergies, atopic sensitization and Hepatitis A virus seropositivity in Italian young men*

2.3 Is the HAV-atopy inverse association reproducible in other populations?

P 3 *Matricardì PM, Rosmini F, Rapicetta M, Gasbarrini G, Stroffolini T. Atopy, hygiene and anthroposophic lifestyle. Lancet 1999;354:430. [56]*

The HAV-atopy inverse association we found was rather intriguing, but before taking it as a indirect demonstration of the hygiene hypothesis, we wanted to test it again in other settings. After the initial study in military recruits, we tested therefore the putative HAV-atopy inverse relation in another setting. We took advantage of a previous survey on HAV seroprevalence in the adult general population of the Republic of San Marino, an independent state with 26 000 inhabitants (in 1998) that is completely surrounded by Northern Italy. Serum samples were collected during 1990–91 by a random-sample procedure (1527 men and women aged 20 years) and HAV antibodies were tested by ELISA. In 1509 (98,8%) of the serum sample, IgE against common airborne allergens were measured by a multi-radioallergosorbent assay as previously described. We found that atopy was strongly inversely related to HAV seropositivity: only 61 (6,23%) individuals were atopic among 979 HAV-seropositive participants, compared with 75 (14,15%) among 530 HAV-seronegative participants (p<0,001).

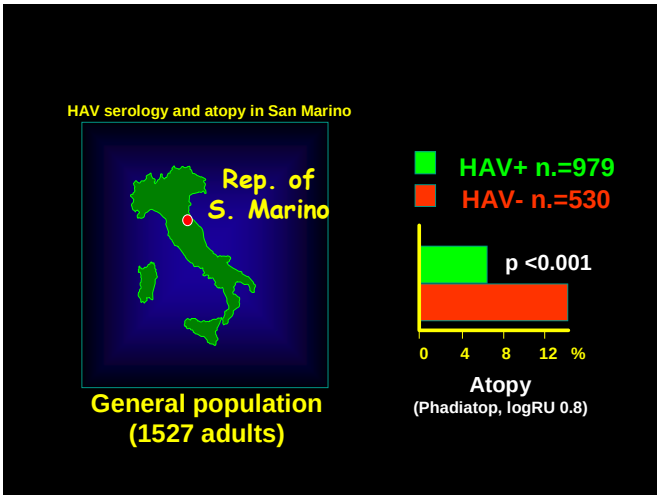

Figure 5 *– Atopic sensitization and Hepatitis A virus seropositivity in the general population of San Marino*

The inverse association between HAV and atopy remained significant after adjustment for age and sex by multivariate analysis (odds ratio 0,59 [95% CI 0,38–0,92]). Stratification of data by year of birth showed an increasing trend of atopy onwards. This trend is almost totally accounted for by individuals never exposed to HAV, because younger participants were less likely to be atopic if they had antibodies against HAV. (Figure 5)

At the time of our study, it was still believed that acquisition of measles infection early in life could protect from atopic sensitization. We considered, however, that since 1960 an economic "boom" started in the Republic of San Marino, when lifestyle changes produced also a sharp improvement in hygienic standards. Conversely, mass immunisation programmes against measles started in this area (and all over Italy) only in the late 1980s so that measles epidemiology did not substantially change until early 1990s. Our data therefore not only confirmed an inverse association in Italy between exposure to HAV and atopy, but also indicate that factor(s) specifically related to a faecal contamination of the environment, together with unhygienic food handling, may protect from atopy by an influence on the overall pattern of commensals and pathogens that stimulate the gut-associated lymphoid tissue.

2.4 Can the HAV-atopy inverse association justify the epidemic trend of allergic disaeses?

P 4 *Matricardi PM, Rosmini F, Panetta V, Ferrigno L, & Bonini S. Hay fever and asthma in relation to markers of infection in the United States. J Allergy Clin Immunol 2002; 110:381-7. [57]*

In the years following these studies, it was argued that the emergence of allergic asthma in unsanitary inner-city areas in the United States is irreconcilable with the hygiene hypothesis. It became therefore important to test whether the HAV-atopy association could be found also in that country. Should our interpretation of the Italian studies be correct, markers of exposure to a higher microbial burden, such as positive serology to HAV should be independently associated with less allergy and asthma in the United States, as they are in Europe. A chance to test this hypothesis came from the public availability of the data of the Third National Health and Nutrition Examination Survey, 1988-1994 (NHANES III). This study examined a large national sample of Americans who responded to questionnaires on allergic and respiratory diseases and who underwent allergy skin testing and blood testing for markers of infections. This public database provided a unique opportunity to investigate whether hay fever and asthma are indeed correlated with serology for HAV and other markers of infection in the US general population. We sought to test this hypothesis by examining the relationship of hay fever, asthma, and atopic sensitization with markers of infection in a large general population sample of the United States. To this end, we analyzed the data of 33,994 US residents recorded in a public database of a nationally representative cross-sectional survey (Third National Health and Nutrition Examination Survey, 1988-1994). The variables examined were sociodemographic information, lifetime diagnosis and age at first diagnosis of hay fever or asthma, current skin sensitization to hepatitis A virus and other infections.

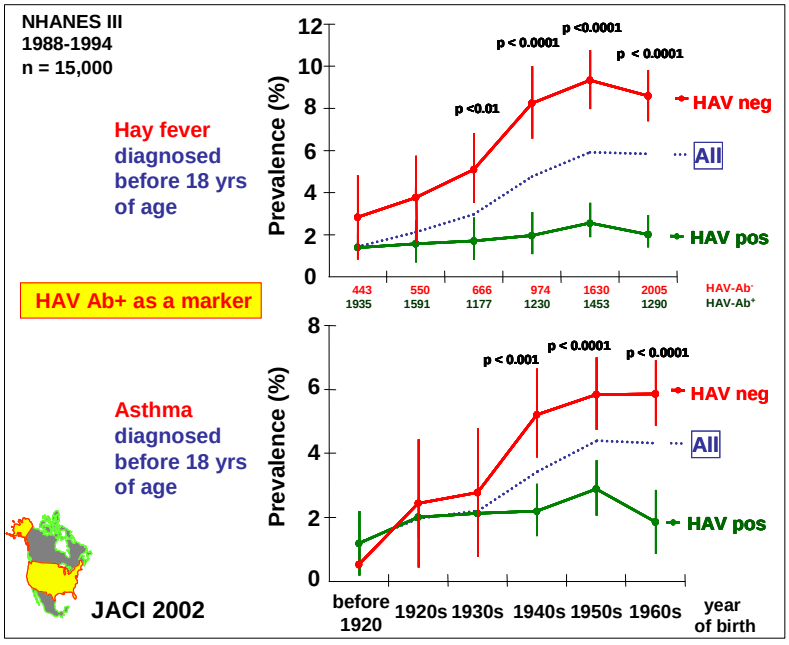

Figure 6 – *Trend of respiratory allergies (retrospective analysis) by serology for HAV antibodies in the general population of the United States*

We found that hay fever and asthma were less frequent in subjects seropositive for hepatitis A virus (HAV) after adjusting for age, sex, race, urban residence, census region, family size, income, and education. Skin sensitization to peanut and to all the airborne

allergens examined, except for cockroach, was less frequent among HAV-seropositive versus HAV-seronegative subjects younger than 40 years of age. The prevalence of hay fever and asthma diagnosed at or before 18 years of age in HAV-seronegative subjects increased progressively from 2.7% (95% CI, 0.7%-4.7%) and 0.4% (95% CI, 0.1%-1.6%), respectively, in cohorts born before 1920 to 8.5% (95% CI, 7.3%-9.7%) and 5.8% (95% CI, 4.8%-6.8%), respectively, in cohorts born in the 1960s, whereas they remained constant at around 2% in all cohorts of HAV-seropositive subjects. (Figure 6)

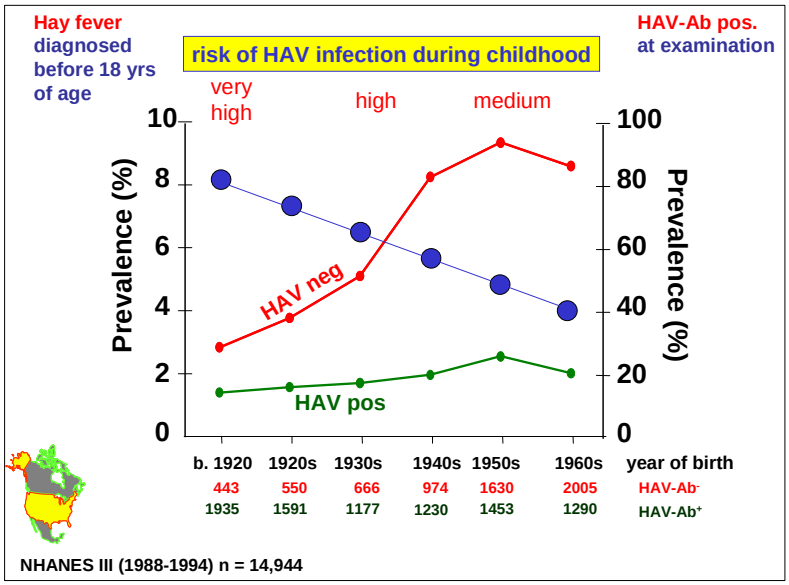

Figure 7 – *The declining trend of HAV seropositivity and its influence on the rising trend of hay fever in a general population sample of the United States*

Interestingly, the declining trend of HAV seropostivity was in close temporal connection with the increasing trend of hay fever in the HAV seronegative fraction of the American population.(Figure 7)

We concluded that the Third National Health and Nutrition Examination Survey data support the hypothesis that hygiene is a major factor contributing to the increase in hay fever, asthma, and atopic sensitization also in USA, as in Europe and other westernized countries.

2.5 In the context of the hygiene hypothesis, is a positive serology for HAV a marker of exposure to more infection in general or to certain infections in particular?

P 5 *Matricardi PM, Rosmini F, Riondino S, Fortini M, Ferrigno L, Rapicetta M, Bonini S. Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. Brit Med J 2000;320:412-7.[58]*

At the time we published the fifth papers, the theory that some infections in early childhood may prevent atopic sensitisation (the "hygiene hypothesis") was still hotly debated. Actually, initial evidence that some airborne infections exert a "protective" effect was not reproduced. These inconsistencies may have reflected differences in population samples and methodologies, or the infections that prevent atopy may include others not examined in those studies. Our report that atopy in Italian military cadets was inversely related to seropositivity for hepatitis A virus, a marker of high exposure to orofecal microbes was consistent with the hygiene hypothesis and with experimental models suggesting that adequate stimulation of the gut associated lymphoid tissue is necessary to avoid atopic sensitisa- tion to environmental allergens.

We reasoned that if this was true then other markers of orofecal and foodborne infections, besides hepatitis A virus, rather than markers of airborne viral infection should be inversely associated with atopy at population level. To test this working hypothesis we extended our survey on military cadets by examining the relation of atopy, con- centration of total IgE, and respiratory allergy with seropositivity to eight other microbes—two microbes mainly carried by food or transmitted by the orofecal route (Toxoplasma gondii, Helicobacter pylori) and six viruses transmitted by other routes, mainly airborne (measles, mumps, rubella, chickenpox, cytomega-lovirus, and herpes simplex virus type 1).

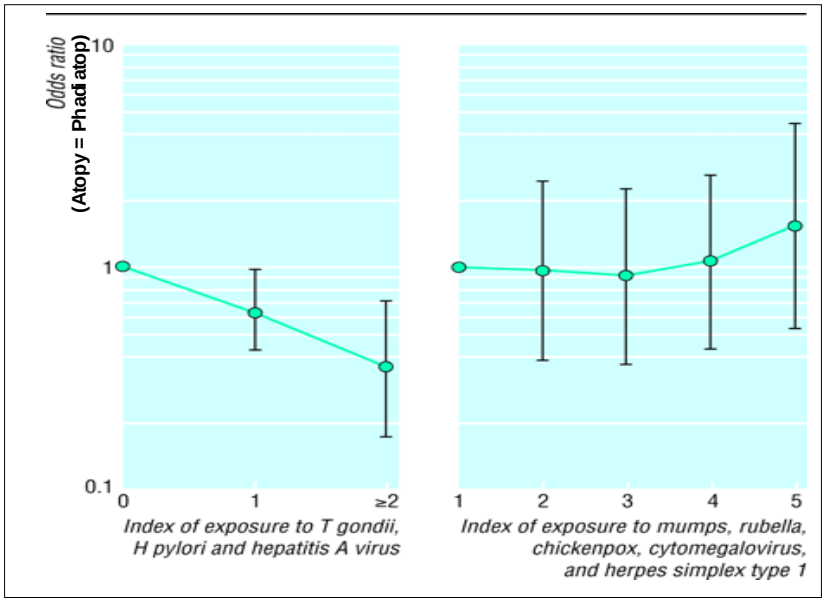

Figure 8 – *Atopic sensitization is inversely associated with foodborne and faecal-oral infections, but not with infections transmitted through other routes*

We designed therefore a retrospective case-control study on 240 atopic cases and 240 non-atopic controls from a population sample of 1659 participants; all Italian male cadets aged 17-24. The study setting was the Air Force School in Caserta,

Italy. Main outcome measures were the serology for Toxoplasma gondii, Helicobacter pylori, hepatitis A virus, measles, mumps, rubella, chickenpox, cytomegalovirus, and herpes simplex virus type 1; skin sensitisation and IgE antibodies to relevant airborne allergens; total IgE concentration; and diagnosis of allergic asthma or rhinitis. We found that, compared with controls there was a lower prevalence of T gondii (26% v 18%, P = 0.027), hepatitis A virus (30% v 16%, P = 0.004), and H pylori (18% v 15%, P = 0.325) in atopic participants. Adjusted odds ratios of atopy decreased with a gradient of exposure to H pylori, T gondii, and hepatitis A virus (none, odds ratio 1; one, 0.70; two or three, 0.37; P for trend = 0.000045) but not with cumulative exposure to the other viruses. Conversely, total IgE concentration was not independently associated with any infection. Allergic asthma was rare (1/245, 0.4%) and allergic rhinitis infrequent (16/245, 7%) among the participants (245/1659) exposed to at least two orofecal and foodborne infections (H pylori, T gondii, hepatitis A virus). (Figure 8)

We concluded that respiratory allergy is less frequent in people heavily exposed to orofecal and foodborne microbes. Hygiene and a westernised, semisterile diet may facilitate atopy by influencing the overall pattern of commensals and pathogens that stimulate the gut associated lymphoid tissue thus contributing to the epidemic of allergic asthma and rhinitis in developed countries. We proposed also that foodborne and orofaecal infections, through stimulation of gut-associated lymphoid tissues (GALT), Peyer's patches, and mesenteric lymph nodes, was protective against allergic diseases, autoimmune diseases and other immune-mediated disorders on the raise in the developed countries. This hypothesis did not completely exclude that airborne viruses may play a major role in regulating the development of atopy. Nevertheless, it suggested that the gut mucosa is a critical site among other sites where microbes may contribute to inhibit Th2-screwed immune responses against allergens that otherwise show deleterious effects in other mucosal areas (bronchial, nasal, conjunctiva). This hypothesis was in line with the concept that a proportion of the lymphocytes homing to the nasal, bronchial and enteric mucosa belongs to the same recirculating pool.

2.6 Do commensals of the GI tract protect from allergies?

P 6 *Adlerberth I, Strachan DP, Matricardi PM, Ahrné S, Orfei L, Aberg N, Perkin MR, Tripodi S, Hesselmar B, Saalman R, Coates AR, Bonanno CL, Panetta V, Wold AE. Gut microbiota and development of atopic eczema in 3 European birth cohorts. J Allergy Clin Immunol. 2007;120:343-50.[59]*

It is known since the seventies that mice reared under sterile conditions do not develop a fully functional immune system and that they are not susceptible to the induction of oral tolerance [31]. The relevance of these observations for the understanding of atopy in humans was for a long time overlooked. At the time when we planned the sixth study (late nineties), a “commensal” hypothesis emerged when Lactobacilli and Eubacteria were observed more frequently in the intestinal microflora of 1-year-old infants living in a country with a low prevalence of atopy (Estonia), while Clostridia were more frequent in age-matched infants living in a nearby country with a high prevalence of atopy (Sweden). It had been therefore proposed that the gastrointestinal microflora of westernised children may predispose to atopy because of the stable predominance of bacteria stimulating TH2 activation (e.g.: clostridia) or because of the absence of bacteria that stimulate TH1 activation (e.g. Lactobacilli). Accordingly, it could be speculated that atopy may be prevented simply by substituting one colonising species with another. However, it is evolutionary unlikely that one single bacterial species has the important task of protecting mammals from atopy. Moreover, translocation through the epithelial barrier and subsequent potent stimulation of the local immune system by a bacterium are only transient and limited to initial colonization phases, and they are soon prevented by an IgA response, otherwise, we probably could not tolerate our own microflora. A few observational studies had investigated the “gut commensals” hypothesis. In a Swedish cross-sectional study, it was found in stools of allergic participants increased levels of i-caproic acid, a marker of colonisation by Clostridium difficile, and lower levels of propionic, i-butyric, butyric, i-valeric and valeric acid. The authors concluded that the composition of gut microflora in allergic subjects may be causally related to their disease. In a small longitudinal study, a reduced ratio of bifidobacteria to clostridia was found in the stools of infants later developing atopy, compared to those remaining non-atopic. The Authors concluded that differences in the neonatal gut microflora precede the development of atopy, suggesting a crucial role of the balance of indigenous intestinal bacteria for the maturation of human immunity to a non atopic mode.

We designed therefore a quite large birth cohort study performed in three European cities. The qualitative and quantitative composition of the faecal microflora of over 300 infants born in London, Rome a Goteborg was monitored seven times throughout the first year of age and related to the appearance of sensitization against food allergens and atopic eczema at 18 months of age. (Figure 9)

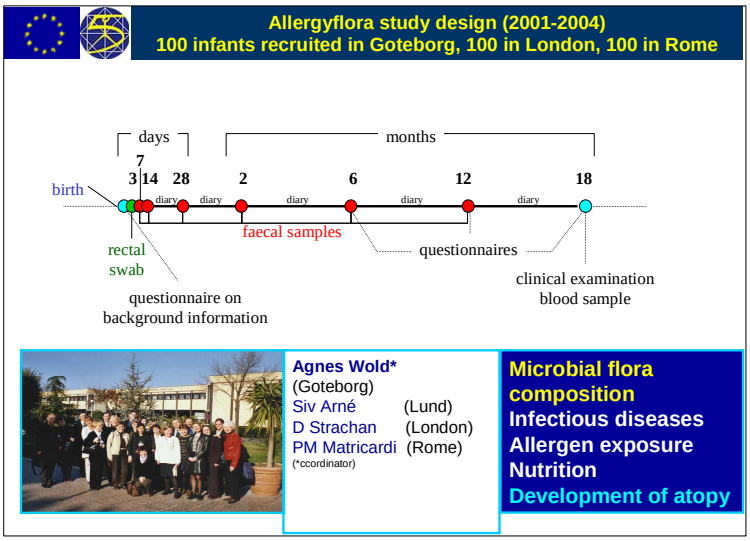

Figure 9 – *Study protocol of the "Allergyflora" multicenter project*

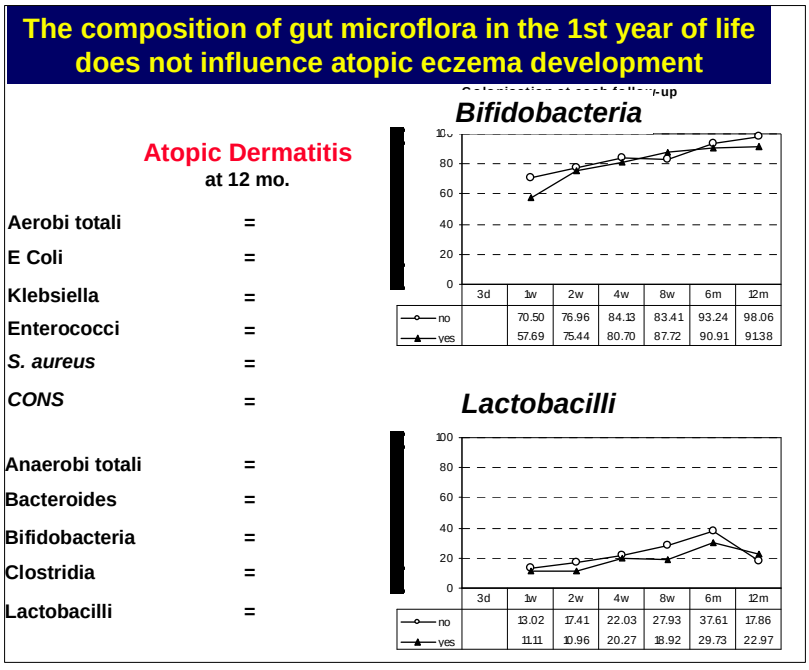

Figure 10 - *Lack of association between gut commensals and atopic dermatitis in the first years of life*

Neither atopic eczema nor food-specific IgE by 18 months of age were associated with time of acquisition of any particular bacterial group.

We concluded that this study did not support the hypothesis that sensitization to foods or atopic eczema in European infants in early life is associated with lack or presence of any particular cultivable intestinal commensal bacteria. (Figure 10)

However, a rather small nested case-control study of the same population, suggested that a reduced diversity in the early faecal microbiota of infants in the first week of life may be causally linked with atopic eczema appearing during the first 18 months of life.

2.7 Do mild pathogens of the GI tract protect from allergies?

P 7 *Pelosi U, Porcedda G, Tiddia F, Tripodi S, Tozzi AE, Panetta V, Pintor C, and Matricardi PM. The inverse association of Salmonellosis in infancy with allergic rhinoconjunctivitis and asthma at school age: a longitudinal study. Allergy 2005;60:626-630.[60]*

We wanted to investigate the interesting alternative hypothesis, that GI microorganisms, in order to protect from allergy, must stimulate the immune system with a pathogenic effect without causing a fatal disease. This category of microbes would include, by definition, mild pathogens. The analysis of the factors so far reported in the literature and summarized in the previous paragraphs may be of some help in identifying a good candidate to exert an allergy-protective effect within such category. Actually, atopy has been found to be less frequent among subjects who acquire faecal-oral and foodborne infections, in those exposed to stables and to high endotoxin concentrations, and consuming unpasteurized milk.Therefore, it was reasoned that an example of atopy-preventing infection may be found among gram-negative bacteria transmitted by contaminated food and the faecal-oral route, and by animals typical of farming environment. A number of infectious agents share these properties; among them, non-thyphoid Salmonellae were ideal candidates for an observational study since they cause diseases that are easily diagnosed even in early childhood. The hypothesis that acquisition of infection with Salmonella in the first 4 years of life may counteract the development of respiratory allergic diseases later in childhood was therefore tested with a longitudinal study design. In this study, the incidence of hay fever and asthma was compared in a group of Sardinian children (age 6–18 years) who had been hospitalized at preschool-age (at age <4 years) with salmonellosis (n=148) to that of age-matched children who had been hospitalized (at age <4 years) with acute enteritis of non-bacterial aetiology (n=168). This study showed that children who had been

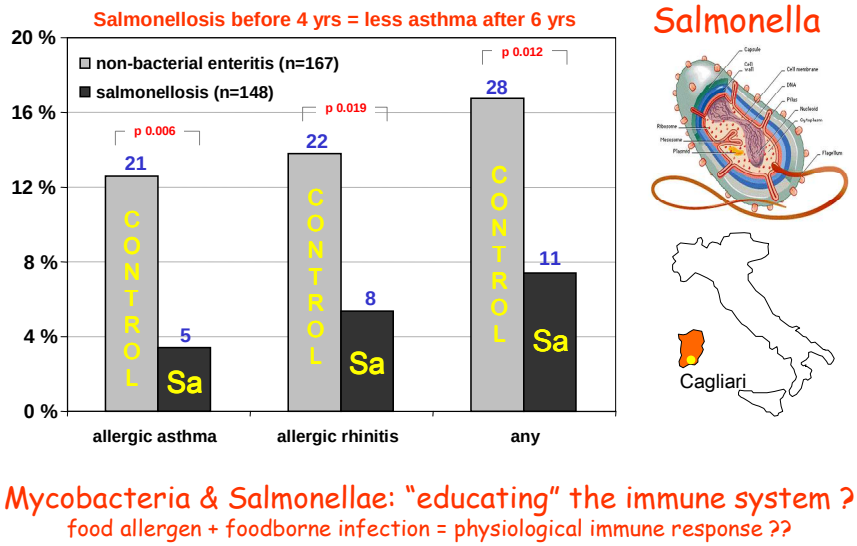

Figure 11 - *Pre-school children hospitalized with salmonellosis develop less frequently respiratory allergies than their peers hospitalized with gastroenteritis due to non-bacterial infections.*

hospitalized with salmonellosis had a lower prevalence of allergic rhinoconjunctivitis or asthma than controls (Figure 11).

The proportional hazard of salmonellosis for asthma was as low as 0.23 (95% CI: 0.08–0.67; P < 0.01) and for allergic rhinoconjunctivitis was 0.40 (95% CI: 0.17–0.95; P = 0.04), after adjusting for a list of relevant confounders. We speculated that acquisition of infection early in life by Salmonella may inhibit the development of atopic diseases and in particular of allergic asthma. We also concluded that Salmonella may contribute to prevent the development of respiratory allergies through a range of mechanisms acting on the innate immune system, in a critical period for the maturation of immune response against ubiquitous allergens. Salmonellae, similar to mycobacteria, grow into the endosomes of macrophages and induce a strong activation of CD4+ T helper-1 lymphocytes, which are required to enhance intracellular killing and clearance. This process is highly dependent on production of γ-interferon and interleukin-12, so that patients with selective deficiencies of these cytokines or their receptors develop fatal infections by Salmonella as well as mycobacteria. In addition, Salmonella play a transient regulatory role on adaptive immunity. Finally, the immune response to Salmonella is controlled by the natural resistance-associated macrophage protein 1 (Nramp-1) gene, which plays also a major role in regulation of atopic responses and airways allergic inflammation in rodents. These properties or other unknown mechanisms provide biologic plausibility to the hypothesis that salmonellosis contributed to protect from allergic asthma and rhinoconjunctivitis the children we examined.

2.8 Do helminths protect from allergies?

P 8 *Lau S, Matricardi PM. Worms, asthma, and the hygiene hypothesis. Lancet. 2006;367:1566-8 [61]*

Helminths were since the seventies proposed to protect from allergy and asthma either through the saturation of high affinity IgE-receptors on mast cells and basophils (by polyclonal IgE) or by induction of blocking IgG antibodies. Therefore we reasoned about the hypothesis that a decline in helminthic infections could underly the rising trend of allergy. We started from the evidence that helminthic infections were associated with asthma in a few studies, but not in others. This area re-emerged when the immunosuppressive properties of helminths were re-evaluated by combining two new concepts: the “anti-inflammatory network” (cytokines produced by regulatory T cells: IL-10, transforming growth factor-β) and the “hygiene hypothesis”. Helminths may prevent allergy and asthma by stimulating the anti-inflammatory network, and the allergy epidemic was attributed to the sharp decline of helminthic infections. Indeed, chronic intestinal helminth infection has been shown to protect children from atopic reactivity in a variety of settings in developing countries. Similarly, worm infestation early in life (mainly Ascaris, Oxyuris) was negatively associated with subsequent eczema in a large population of East German children. In contrast, transient, delayed or milder forms of helminthic infections in children were positively associated with atopic disorders. To explain these new inconsistencies, it was speculated that early, heavy, and chronic helminthic infections protect children in endemic countries against allergies, e.g, by stimulating regulatory T cells and cytokines. This observation was supported by studies in experimental models showing strong induction of regulatory activities subsequently inhibiting allergic immune responses and development of airway inflammation in mice treated with Helminths or helminth products. In contrast, sporadic, delayed, or transient infection may potentiate allergy in sensitised children through bystander TH2 stimulation. Cross-sectional studies are clearly insufficient to answer whether helminths promote or suppress allergy. Theoretically, such a link could be experimentally investigated by monitoring either allergic child during a new helminthic infection or children chronically infected by helminths during de-worming treatment. Indeed, the latter approach saw that Venezuelan children chronically infected mostly with A lumbricoides and T. trichuria developed or increased their atopic reactivity to mites after successful treatment with antihelmintic medication. Similarly, Gabonese children chronically infected by A lumbricoides and T trichuria transiently increased their reactivity to airborne allergens during a 3-year follow-up after antihelminthic treatment. In contrast, an antihelminthic programme in Ecuadorian schoolchildren reduced helminthiases without promoting atopic sensitization, allergic symptoms and exercise-induced bronchospasm during a 1-year follow-up.

2.9 Can we learn from infections how to prevent and treat allergies?

P 9 *Matricardi PM and Bonini S. Mimicking microbial "education" of the immune system: a strategy to revert the epidemic trend of atopy and allergic asthma ? Respiratory Research 2000;1:129-132.[62]*

As a consequence of the hygiene hypothesis, we theorized in 2000 a new category of intervention strategies based on the use of microbial products for allergy prevention and therapy.[68] We speculated that if we understood how microbes ‘educate’ our immune system, we could perhaps learn to mimic safely their beneficial effect. Programmed ‘Immuno-Education’ would consist in the administration, by the correct route and at the correct dose and time schedule, of the right variety of microbial stimuli required by the mucosal immune system during its development and necessary to maintain later an appropriate equilibrium between its components. This strategy may be helpful to prevent atopy with a more "physiologic" stimulation without the need of immunizing against all the allergens potentially encountered during a whole lifetime. However, the immune system homeostasis is so complex and microbial exposure so diversified that, at the present state of our knowledge, this goal is far beyond our reach. Meanwhile, more feasible and specific immunologic therapies or prophylactic measures may emerge from the ongoing studies mentioned above. Ultimately, although poor hygiene will never cure asthma, the hygiene hypothesis will have favoured new strategies in the fight against the allergy and asthma epidemic.

Consistent with the "hygiene hypothesis", research on immunotherapy of allergic diseases centres also on bacterial-derived molecules (e.g.: DNA-immunostimulatory sequences) as adjuvants for allergen-specific type 1 immune responses. If we understood how certain microbes physiologically ‘educate’ our immune system to interact safely with environmental non-microbial antigens, we may then learn to mimic their beneficial action. A programmed ‘Immuno-education’ would consist in the safe administration by the correct route, dose and timing, of those microbial stimuli necessary to ‘train’ the developing mucosal immune system and to maintain later an appropriate homeostatic equilibrium between its components, thus obtaining an overall prevention of atopy not limited only to some specific allergens. Perhaps utopian, this strategy may revert the epidemic trend of atopy and allergic asthma without jeopardising the fight against infectious diseases.

In the same years, probiotics, oral bacterial extracts, mycobacteria, LPS derivatives, immunostimulatory sequencies of oligodeoxynucleotides (ISS-ODN), and products derived from helminths had been already proposed in this area. Some of these approaches (oral bacterial extracts, probiotics) have so far given negative or inconsistent results, others (ISS-ODN) seem more promising, but have not reached a level of evidence for efficacy high enough to be recommended by international guidelines. We also promoted a Task Force to produce a position paper in this emerging area of interest.

The one approach that has been most frequently investigated and promoted by the Industry is the attempt to prevent or treat allergic diseases with probiotic bacteria, such as lactobacilli or bifidobacteria. Notwithstanding the lack of a strong rationale, the hygiene hypothesis has also been abused as a rationale to “invent” probiotic functional food for patients with allergic disease. Trials have been performed to evaluate the putative preventive or therapeutic effects of probiotics in allergies. Initial studies, although promising, have been highly criticized for their insufficient design or other weaknesses in data handling or data interpretation. We have taken a strong position both at individual and collective level against the risk that the hygiene hypothesis were used as an alibi to promote products for prevention and therapy of allergic diseases in the total absence of evidence. Actually, the evidence provided so far was still insufficient to generally advise the use of probiotics for primary prevention or therapy of allergies, and consequently this approach is considered an experimental one by a Task Force of EAACI, the GINA group, and individual opinion leaders.

2.10 Can conflicting data be reconciled with the hygiene hypothesis?

P 10 - *Matricardi PM. 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: controversial aspects of the 'hygiene hypothesis'. Clin Exp Immunol. 2010;160:98-105.[63]*

The hygiene hypothesis has been criticized for some apparently weak points. Questions raised in the recent years include the following ones: (1) why allergic asthma is on the rise in “unhygienic” American inner-cities; (2) why allergic diseases are less prevalent among migrants children living in European big cities; (3) why infections with airborne viruses do not “protect” from allergic sensitization; (4) why the association between some infections (e.g. hepatitis A virus) and allergic diseases has been reproduced in some populations, but not in others; (5) why probiotics are not effective in the prevention and therapy of allergic diseases.

These challenging questions are useful starting points to improve our understanding of the hypothesis, and to identify among the infectious agents those really responsible of a protective influence against atopic and autoimmune diseases. We therefore wrote a review in which each of these points has been examined, and it has been propose, on the basis of the data available in the literature, how each of these arguments can be easily reconciled with the hygiene hypothesis. Some of these questions are re-analysed in the next section.

3. Discussion

The results we obtained between 1990 and 2005 substantially support the hygiene hypothesis and have provided specific novel information about the mode of transmission and the nature of the infections that protect from allergic diseases. Our central results is that atopy and respiratory allergies were inversely related to a gradient of exposure to orofecal and/or foodborne infections (TG, HAV and HP), but not to viruses transmitted through other routes, i.e. mumps, rubella, chickenpox, HSV1 and CMV. The power of our studies to detect an association between atopy and measles was limited by the extremely high prevalence of this illness. However, it follows that virtually none of our atopic subjects had been "protected" against atopy by measles. In addition, it is unlikely that the observed associations were confounded by low socioeconomic status, because they persisted after adjustment for paternal education, which is strongly inversely associated with atopy in Italy.

The real meaning of a positive serology for HAV antibodies should be cautiously interpreted. HAV is a typical faecal oral infection, and is also acquired from contaminated food and water. On the other hand, TG is acquired mainly through ingestion of meat containing tissue oocysts and of unwashed, raw vegetables contaminated by faeces of infected mammals (mainly cats). Finally, HP has been cultured from human faeces, from house flies and from sheep milk, and intrafamilial, early cross-infection through the faecal-oral, oral-oral route or by ingestion of contaminated food and water have also been suggested. By contrast, measles, mumps, rubella and chickenpox are highly infectious airborne viruses whose recirculation is less affected by hygiene. HSV and CMV are acquired mainly through close prolonged person-person contacts. We have therefore presented the first epidemiological evidence that faecal-oral and/or foodborne microbes are better candidates than airborne respiratory viruses as determinants of an atopy "protective" effect.

3.1 The site where microbes may inhibit atopy and their pathogenic power

Our studies also suggested that the gut-associated lymphoid tissue is the site where immune deviation in the response to common airborne allergens is influenced by adequate exposure to microbes. Animal models lend biological plausibility to this interpretation: gut flora is essential in the mouse post-natal preferential enhancement of TH1 immunity toward environmental antigens; intestinal bacteria regulate IgE isotype switching in rats; TH2 responses of germ-free mice are not susceptible to oral tolerance induction, and reconstitution of intestinal microflora or oral administration of microbial substances (LPS) restore this susceptibility so preventing atopy.

Consistent with these animal models, our data suggest also that microbes need not cause disease to exert a protective effect against atopy. For example, most cases of postnatal acquisition of TG are subclinical, but TG strongly stimulates dendritic cells to produce in vivo IL-12, a key molecule in

the deviation of T-cell responses toward the TH1 phenotype. However, we do not rule out that airborne bacteria which induce disease such as M tuberculosis, or inhaled bacterial substances (endotoxins) may help to prevent respiratory allergy by stimulating other sites (e.g. bronchial-associated lymphoid tissue, Waldayer's ring, and related lymph nodes).

3.2 The effects of diet and animals on atopy

Our data shed light on the role of diet in the allergy and asthma epidemic. They support the hypothesis that daily ingestion of traditionally processed food, not treated with antimicrobial preservatives and not subjected to hygienic procedures, may help to prevent atopy. A traditional or “unhygienic” diet may act either by providing adequate daily microbial stimulation of the mucosal immune system (e.g.: mycobacteria spp.), or by favouring gut colonisation and high turnover of appropriate commensals (e.g.: enterobacteriaceae, lactobacillus spp.).

Our results also impinge on the controversial debate as to whether close contact with domestic animals (i.e. dogs and cats) affords protection against allergy. The inverse relation between TG and atopy may imply that higher exposure to microbes and their antigens released by animals may prevent atopy, a hypothesis borne out also by studies of farmer's children. However, caution should be exercised because early exposure to pets in a hygienic context can facilitate specific IgE sensitisation to their allergens in predisposed subjects.

3.3 The time-frame of balance between infections and atopy

Although the infections examined are usually acquired in infancy, in our studies it was not possible to determine how early the cadets became infected. However, we do not necessarily have attributed a direct, causal role to HP, HAV and TG in the observed lower risk of atopy. Rather, we considered that seropositivity to these microbes is a very reliable proxy of being reared in an environmenedt that entails a higher exposure to many other orofecal/foodborne microbes that may exert atopy-preventing effects. Our data suggest that appropriate microbial stimulation in subjects exposed to TG, HAV and/or HP may have prevented atopy completely in early infancy, or it may have acted later to prevent a low subclinical sensitisation, which started during childhood, from developing during adolescence and triggering allergic respiratory symptoms.

Thus, we hypothesized that prevention of the atopic tendency may not be dichotomous (a yes/no event), or limited exclusively to a certain "window" period (very early in life), rather it may be a dynamic and quantitative process, extending at least to adolescence, and subjected to genetic factors that regulate how early and intense must a continuous microbial exposure be to afford permanent protection from atopic sensitisation or to delay its onset.

We proposed therefore that the decline of orofecal and foodborne infections and changes in the overall pattern of commensals and pathogens that stimulate gut-associated lymphoid tissue may be strong determinants of the epidemic of allergic rhinitis and asthma in developed countries.

While further studies are required to verify this conclusion, it is not inconceivable that we may soon use certain microbes or their molecules to prevent atopy without causing infectious disease.The current status and future of the Hygiene Hypothesis

The future of the hygiene hypothesis will be based on three main directions: first it is necessary to reconcile the different epidemiological aspects in one overall scenario. Then it will be essential to understand the immunological mechanisms underlying the associations found at epidemiological level. This is an essential step in order to better design new strategies for intervention, which should not be empirical, but scientifically driven. In this discussion we briefly elaborate on these three points.

3.4 Understanding the epidemiology – converging trend in the hygiene hypothesis

The evidence accumulated by our research and from that coming from other work is quite convincing. We actually see that our research line, developed through a sero-epidemiological approach, has led to converging results when compared to the research line focusing on the farming environment and to the research line focusing on experimental models.

A – Immunoepidemiological studies (presented in this thesis)

1. Sibship size and birth order was shown to affect directly the IgE response against common allergens, and not only disease manifestation and/or their reporting [54]
2. This “effect” was shown to be dependent on the level of exposure to Hepatitis A virus [55], initially considered a marker of poor hygiene and faecal contamination of food and environment
3. Hepatitis A virus was then shown to be a more specific marker of exposure to GI, foodborne, faecal-oral infections: such as TG, HP and HAV.[58] The link between these infections and atopy has been repeatedly confirmed in several populations undergoing epidemiological transition [56, 57]
4. A birth cohort study showed that commensals of the GI tract probably do not have an atopy protective influence [59]
5. A longitudinal retrospective study showed that Salmonellae prevent the development of respiratory allergies [60]

B - Studies in the farming environment

1. First it has been shown that children raised in a traditional farming environment are protected from allergies [43]
2. This “effect” was shown to be linked to many factors, but especially to stables and livestock, i.e.: to faecal contamination of the environment [64]
3. The stronger protective factor in and outside farming environment is the ingestion of unpasteurized milk, i.e.: a source of food-borne infections [65]

C - Studies in animal models

1. Several studies in animal models have shown that the ingestion of microbial products and the stimulation of GALT are able to prevent or even suppress atopic sensitization and its consequences. [66]

2. Most of the microbes ("old friends") [67] which play a major atopy protective role in animal models are mild intracellular pathogens. However, all the collected data should be interpreted from an historical perspective.

3.5 Arguments apparently at variance with the hygiene hypothesis

The Hygiene Hypothesis has been challenged by conflicting data on the alleged atopy-protecting role of measles and other airborne viruses, and by the not infrequent occurrence of severe allergic asthma and atopic sensitisation in unhygienic American inner cities, in rural Africa and among adult immigrants who in their infancy were exposed to a traditional, unhygienic lifestyle. These inconsistencies, which seemingly confute the hygiene hypothesis, may, on the contrary, cast further light on the theory.

Measles. An initial report that measles virus may protect from atopy, has not been confirmed. Indeed, it seems unlikely that evolution relied on transient infection by a single virus to down-regulate TH2 responses in mammals. Moreover, measles has only recently become a worldwide disease: natives of South America, for example, were not exposed to measles until the arrival of Europeans in the 15th century. If measles prevented atopy or asthma, most native Americans would have been atopic and asthmatic.

Airborne viruses. The prevalence of atopy and asthma was inversely related to childhood infections by airborne viruses in some studies, but not in others. From an evolutionistic and epidemiologic perspective, these inconsistencies are not surprising. First, airborne viruses that infect humans have not been constantly present throughout mammalian evolution. Second, some of them even induce or exacerbate asthma thereby introducing a potent confounding effect in epidemiologic studies addressing the Hygiene Hypothesis. Finally, most respiratory viruses are so infectious that hygiene and declining family size could hardly reduce their circulation among humans (as compared, for example, to infections transmitted through other routes). Therefore, although we have not enough data either to refute or to accept the hypothesis that certain viruses may protect from atopy, both epidemiologic background and evolutionistic arguments indicate that the hygiene hypothesis should be examined mainly in the light of exposure to other microbes.

Inner city asthma. Most asthma cases among children living in American inner cities are due to atopic sensitisation to indoor allergens, including mites and cockroaches. Hence it was extrapolated that atopic sensitisation in inner cities is more frequent than in more affluent and

hygienic urban areas, which is in clear contrast to the hygiene hypothesis. It is tempting to speculate, however, that children living in 'unhygienic' inner-city areas may be indeed not so heavily exposed to a wide array of bacteria compared with children that have access to natural soil, eat only food not industrially processed, or live in areas endemic for many different orofecal infections. Thus, it seems that the words 'dirty' or 'hygiene' are too generic to label environments facilitating or protecting from allergy. American inner cities represent very interesting areas for further epidemiologic reasearch on the hygiene hypothesis.

Atopy in developing tropical areas. Inhabitants of rural, unhygienic areas of developing countries, not rarely have serum IgE responses toward airborne allergens. However, severe helminthic infestation, very frequent in such areas, can potentiateTH2 responses through bystander effects, which may counteract the downregulating effects exerted by other microbes. These atopic responses are rarely associated with allergic disease, suggesting that severe helminthic infestation may stimulate biological activities (e.g., by competing for IgE binding sites on effector cells) that may prevent the inflammatory consequences of exposure to allergens. Interestingly, eradication of helminths from Venezuelan patients produced a decrease in total serum IgE level,paradoxically associated with increasing skin reactions to airborne allergens and onset of respiratory allergic symptoms.

Onset of atopy in adulthood. IgE sensitisation to common or occupational allergens not infrequently starts in immigrants or adult workers who in their early years were exposed to a traditional, unhygienic lifestyle. Therefore, atopy does not appear to be programmed exclusively in early infancy and infections acquired within a "window period" early in life may not provide lifelong protection. Downregulation of atopic responses may rather be a dynamic and quantitative process, extending at least to adolescence and probably to adulthood, subjected to genetic factors that dictate how early, intense and prolonged continuous microbial exposure must be to afford protection from atopic sensitisation or to delay its onset.

3.6 A historical perspective of the allergy and asthma epidemic

At the same time we should try to see the whole epidemiological evidence around the hygiene hypothesis in a historical perspective. This approach can help us to understand the whole evolution of the phenomenon of allergic diseases and can be helpful in the interpretation of the status of the allergic and infectious diseases in different countries in a dynamic model (figure 11).

We believe that in a western country, when income and education improve dramatically (as it has been the case in Western Europe in the wake of the Second World War), the borders of low socioeconomic status remain fluid and relative. Historically, the least affluent of one generation end up adopting the lifestyle of the middle classes of the previous one. The rising trend in respiratory allergies among the poorest could be therefore seen as the natural continuation of epiphenomena that affected the richest socio-economic strata of the United States population in the first half of the

20th century to reach the middle classes in the 1950s and '60s and eventually cascade down to affect the least-advantaged Americans in the inner cities from the 1970s onwards. In this context, it can be speculated that the primary cause of the epidemic of allergic asthma in American inner-cities is the consequence of the deprivation of factors linked to infections which confer protection from atopy and airways allergic inflammation. Emerging cases of allergic asthma among the least affluent African American and Hispanic communities are likely more severe because atopic susceptibility encounters concurrent, chronic exposure to secondary risk factors typical at any time of poor urban environments (high exposure to cockroaches, smoking, damp and inadequate access to health care). Inner-city asthma may thus be the final stage of a class-driven urbanization and westernization that started two centuries ago in the United States and that is only now coming full circle (Figure 11).

3.7 A tentative unifying model aimed at reconciling apparently alternative hypotheses

In the light of speculation and hypotheses about the asthma epidemic, a third approach may be attempted, which is alternative to those proposing a single cause for the epidemic and to those claiming that pollution, diet, allergen exposure and hygiene all contribute to a roughly similar extent to the epidemic.

Hygiene would be the major (not necessarily the only) primary cause of the epidemic of atopy and atopic diseases, including allergic asthma. It would facilitate, in genetically predisposed subjects, those type 2 responses against non-microbial antigens normally found in all environments. A westernised diet would contribute to this phenomenon, not because of its different content in nutrients, but because it deprives the organism of daily, diversified microbial stimulation. Indeed, a characteristic of a westernised diet the world over is a reduced quantity and variety of bacteria.

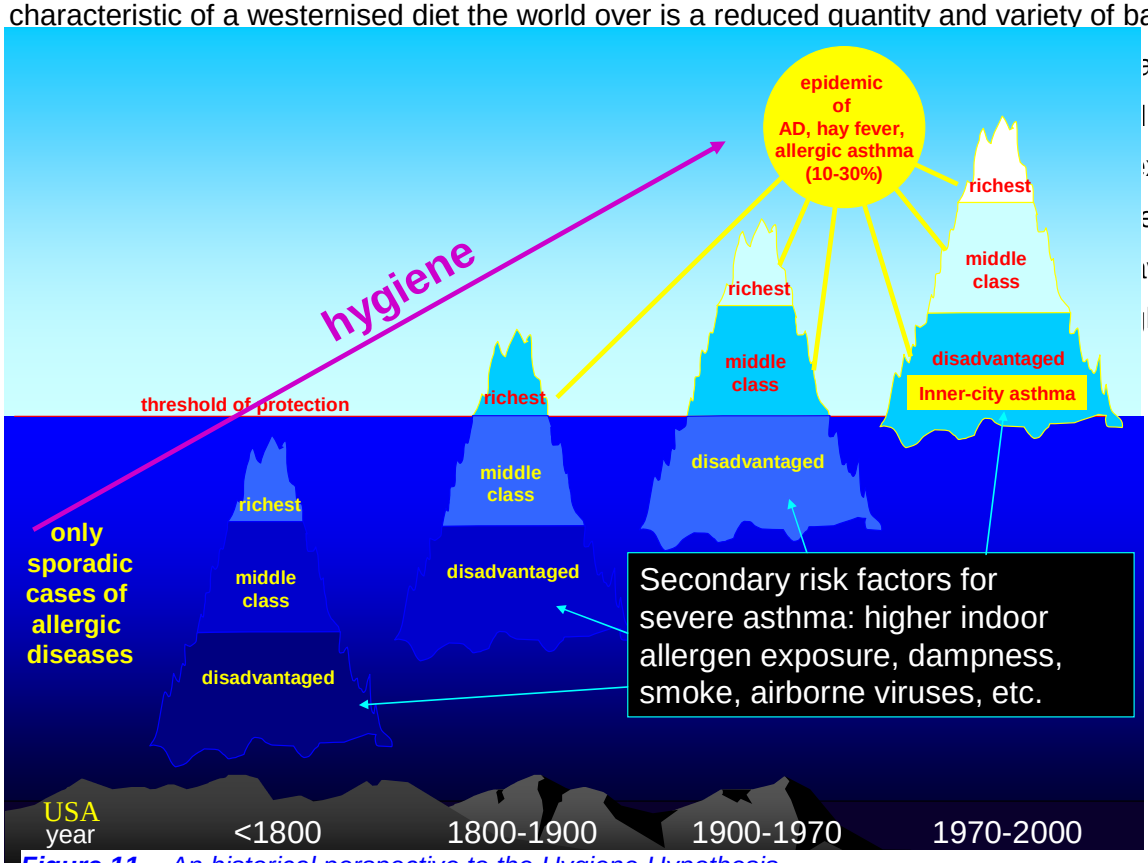

Figure 11 - *An historical perspective to the Hygiene Hypothesis*

3.8 Speculations on the underlying mechanisms

The hygiene hypothesis has been explained mostly with two apparently alternative immunological models, based on the TH1/Th2 paradigm and on T-regulatory cells, respectively. We have tried to integrate the two models in one. A unifying explanatory model for the hygiene hypothesis would consider the role of infections in influencing both the induction and the effector phase of the immune response against allergens. A strong regulatory network stimulated by chronic infections not only bacterial and viral (Th1 skewing) but also including those from helminths (Th2 skewing), may be the predominant allergy protective mechanism among populations living in developing countries, but their relevance may be lower in countries whose populations are much less exposed to chronic infections, including helminthic infections. Under these circumstances, a persistent microbial stimulation of primarily Th1 type immune responses that influences the induction phase may be the predominant atopy-protective effect. This might be the most important mechanism among populations living with a western lifestyle, whose subsets still protected from atopy are nowadays limited to traditional farming families. More research is needed to resolve whether Th1 responses are present to allergens both at the T and the B cell level in protected subjects and more importantly what level of Th1 responses are safe. It is also concievable that a graded mixture of Treg, Th1 and Th2 responses determine the outcome of allergies in a) high Th1and Th2 pathogen load, b) Th1 pathogen load and c) low pathogen load environments (Figure 1). Similarly, the existence of a “window” period in early life for infection-driven protection from atopy would become more and more important as a population advances from a traditional to a westernised lifestyle.

For a careful examination of the immunological mechanisms that may explain the hygiene hypothesis, it is important to highlight the fact that allergic disorders can be divided into two phases, the sensitization phase and the effector phase. The sensitization phase is considered to be the stage when Th2 responses are first induced, preparing the way for increased downstream allergic inflammation. The effector phase encompasses the reactions that follow the expansion of the initial Th2-mediated reactions. In this phase, allergen reacts with IgE on mast cells, eosinophils become involved, and pro-inflammatory mediators are released, attracting additional inflammatory cells into the affected organs; some of these mediators act on surrounding tissues to enhance blood flow and they can increase smooth muscle contraction and mucus secretion. The effector phase is responsible for the expression of allergic inflammation and thus allergic disease.

With respect to the sensitization phase of allergy, few studies examining the hygiene hypothesis have reported changes in IgE antibody levels over time. It is very likely that throughout the period when helminth infections were highly prevalent in the presently industrialized world, the infected population had strong Th2 responses. However, serial serum samples obtained some time during

that period and later when these infections had disappeared in that part of the world are not available, so it is not possible to determine whether and how antibody responses to allergens might have changed in response to the disappearance of the parasites. Nevertheless, a few data are available. In a study of adolescent Japanese female students, serum IgE antibody against both mites and pollen was present in 21.4% in 1978, 25% in 1981, 35.5% in 1985 and 39.4% in 1991. Similarly, in a study carried out in Greenland with sera collected in 1987 and again in 1998, a two fold increase in prevalence of IgE sensitization to allergens, most frequently to grass pollen, was recorded over that period. The level of exposure to this allergen is not likely to have increased significantly over this 11-year period, as has been argued for exposure to house dust mite. These results point to a clear change in how the immune system recognized this allergen over the period of a decade. Therefore, an important question is whether the immune system of these individuals was able to recognize these allergens at all, but then not with an IgE response. It was recently proposed that, in the case of cat allergy, a modified Th2 response to the allergen may be associated with lower allergic reactivity in those individuals with high exposure to cats, and this idea may be relevant to the above-mentioned studies. Did the Greenlanders make anti-grass IgG or IgG4 rather than IgE in 1987? If the answer turns out to be positive, then there could still be a role for the anti-inflammatory response to explain the increase in IgE reactivity to common allergens in western populations. For example, IL-10 has been shown to be a switch factor that enhances IgG4 production while inhibiting IgE production. Thus, in populations with chronic parasitic infections and a strong regulatory network characterized, among other factors by high IL-10 production, the abundance of this cytokine could bias antibody responses towards IgG4. However, if no immune responses were made to environmental allergens, not even IgG, at times prior to the beginning of the allergic march, then the role of infections in allergen ignorance by the immune system would have to be investigated.

With respect to the effector phase, the increase in allergic disorders, measured by skin prick tests, airway responsiveness or clinical scores of allergy needs to be mechanistically linked to lower exposure to infections. A recent report showing that IL-10 can inhibit mast cell degranulation provides a possible mechanism by which an anti-inflammatory response, stimulated by parasites and characterized by increased production of IL-10 could prevent allergic reactions. However, suppressor cytokines such as IL-10 and TGF-β can also inhibit allergic inflammation by suppressing the influx of granulocytes into affected tissues or by inhibiting antigen presenting cells, leading to down regulation of T cell responses. The question of whether these cytokines can directly inhibit smooth muscle cell contraction or goblet cell function is as yet unanswered. In animal models of airway inflammation, it has been shown that dendritic cells that release IL-10 can suppress airway hyperreactivity, which would support the notion that regulatory networks stimulated by persistent pathogens could suppress allergic reactivity.

The explanation of the hygiene hypothesis through the Th1/Th2 paradigm implied initially that increases in IFN-γ should be associated with decreased allergic diseases. However, later studies showed that i) high levels of IFN- γ can be found in the bronchial lavage cells of asthmatic subjects, ii) T cells producing IFN- γ can exacerbate airway inflammation in animal models and iii) protective measures to suppress airway inflammation such as CpG or Mycobacteria vaccae administration are effective via mechanisms not mediated by IFN- γ. Thus, with regard to the effector phase of the allergic response, there is considerable evidence that the anti-inflammatory network may be an important mechanism to down regulate inflammation resulting from a Th2 response.

3.9 Perspectives for novel intervention strategies

Research lines investigating the Hygiene Hypothesis by using different models (farming environment, military recruits, general population samples, animal models, etc.) converged to identify the gut associated immune system as a potential target of an allergy preventive effect of foodborne and orofaecal infections. The infectious agents that may induce such a protective effect through this route are mostly in the category of the mild intracellular pathogens, such as non-thyphoid Salmonellae, Toxoplasma gondii, and Mycobacteria. These infectious agents are included in the broader category of the so called "old friends" microbes, and they share many characteristics: They are mild intracellular pathogens, stimulate TH1 and T regulatory immunity, are widely spread in the environment and in food/water, and suppress allergy in experimental models. It is to be hoped that from this quite solid basis, new observational and experimental studies will be conceived and designed in order to better define the mechanisms of an allergy protective role of these infections. Only afterwards, strategies for the safe use of these microbes for the prevention and therapy of allergic diseases can be designed. Until then, any other attempt to prevent allergies with products lacking of a solid rationale has to be intended as a doubtful, empiric and perhaps non ethic enterprise.

Anyhow, a large number of new molecules and preparations have been tested for the prevention or treatment of allergic diseases. Unfortunately, the outcomes of many attempts are quite discouraging, while some approaches (CpG ODN) seem more promising. The reason for disappointing effects might be our lack of clear understanding of all the events preceding and causing allergic diseases. All these new therapeutic strategies in allergy target the immune system and have been tested in adults. IgE mediated allergic diseases start with an immune dysfunction predisposing to IgE mediated sensitivity; require an end-organ related dysfunction and exposure to appropriate environmental factors which facilitate the expression of these genetic inclinations. Targeting only the immune system might not be enough as immune dysfunction might be only one of the features defining allergy. Successful prevention or cure might require also identification and

treatment of the end organ dysfunctions. Furthermore, preventative or curative therapeutic strategies based on microbial products may need to be initiated very early in life, before the occurrence of the irreversible changes linked to chronic allergic inflammation. It is clear that until we will get a more precise idea of what the causes of allergy are, we will not be able to design products with a high probability rate of success.

Can we make a better use of the epidemiological evidence? It has been consistently shown that consumption of unpasteurised milk is the most relevant atopy protective factor in the farming environment. Could this information be useful for intervention studies? Unfortunately, “unpasteurised” milk is neither a standardised nor a totally “safe” product and intervention trials with unpasteurised milk from traditional farms would pose ethical issues and safety concerns. However, if we consider that farmer children “protected” by the ingestion of unpasteurised milk grow up as healthy as their peers, we may also hope to find novel and safe therapeutic methods to mimic the effect of unpasteurised milk in a standardised and safe way. Studies investigating which microbial components or nutrients contained in the unpasteurised milk are useful are in progress and this type of studies are crucial for the development of future translational research originated from the hygiene hypothesis.

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Appendix – list of the publications enclosed *in extenso*

P-1 *Matricardi PM, Franzinelli F, Franco A, Caprio G, Murru F, Cioffi D, Ferrigno L, Palermo A, Ciccarelli N, Rosmini F. Sibship size, birth order, and atopy in 11,371 Italian young men. J Allergy Clin Immunol 1998; 101:439-44. [43]*

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P 10 - *Matricardi PM. 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: controversial aspects of the 'hygiene hypothesis'. Clin Exp Immunol. 2010;160:98-105.*

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