A comparative genetic study of tuberculosis and asthma susceptibility

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Abstract

The worldwide rates of asthma have been increasing in the past twenty years, coinciding with a decreasing prevalence of tuberculosis in the major asthma endemic regions. The hygiene hypothesis proposes that in countries with better hygiene, reduced exposure to pathogens is responsible for the rising prevalence of allergic diseases. *Mycobacterium tuberculosis*, the cause of tuberculosis (TB), is thought to be a critical effector of the hygiene hypothesis since it can skew the immune system away from developing into an atopy associated immune profile. Hence, absence of exposure to *M. tuberculosis* might remove suppression of asthma development.

Based on the hygiene hypothesis, we reasoned that genetic factors that predispose to TB should be protective for asthma / atopy and vice versa. Consequently, we tested genetic variants for 1) asthma risk based on known genetic effects in TB susceptibility, and 2) TB risk based on known genetic effects in asthma susceptibility. To identify genetic variants for asthma risk, we tested for nonrandom transmission of genetic variants of the Vitamin D Receptor (*VDR*) and the Natural Resistance Macrophage Protein 1 (*NRAMP1*) genes, two known TB susceptibility genes, in an asthma family-based cohort of French Canadian. To identify genetic variants for TB risk, we tested for distribution differences of genetic variants of several asthma associated genes: interleukin 4 (*IL*-4), IL-4 Receptor A (*IL4RA*), tumour necrosis factor A (*TNFA*), lymphotoxin A (*LTA*) and *VDR* in TB cases and non-TB controls recruited in Mexico. Only *VDR* variants showed a risk modulating effect and where analyzed in more detail in the present thesis.

In a North – Eastern Québec asthma family-based cohort, of the 12 *VDR* variants tested 6 genetic variants located between intron 2 and 3' UTR we found

to be strongly associated with asthma ($0.0005 < p < 0.01$) under an additive model. Haplotype specific genetic effects were also observed for asthma ($0.0004 < p < 0.01$). By contrast, no variants of the *NRAMP1* gene were observed to be associated with asthma in this French Canadian asthma cohort. In the final set of experiments, I tested asthma associated genes for their impact on risk of TB disease. In a Mexican case-control study, 14 *VDR* variants tested, we observed 2 genetic variants at the 5' end of the gene to be associated ($p < 0.05$) with either susceptibility to TB, *M. tuberculosis* infection or disease progression. No associations were observed between genetic variants of *IL4RA*, *TNFA* and *LTA*, and TB or TB related phenotypes.

These findings clearly identified *VDR* as asthma and TB susceptibility gene. However, since the genetic variants associated with asthma differ from those associated with TB it remains unclear how these variants influence the immune system to promote asthma but not TB, and vice versa. Although TB has been proposed as a suppressing force for asthma, other than *VDR,* we could not detect any genetic effect of TB susceptibility genes in the asthma study, nor effects of asthma susceptibility genes in the TB study. We interpret these findings as evidence against counter – selection of asthma and TB susceptibility gene variants.

Résumé

Le nombre de cas d'asthme a augmenté à travers le monde au cours des vingt dernières années, coïncidant avec une prévalence de la tuberculose (TB) à la baisse dans les principales régions endémiques de l'asthme. L'hypothèse de l'hygiène stipule que dans les pays avec une meilleure hygiène, la réduction de l'exposition aux pathogènes serait responsable de la prévalence accrue des maladies allergiques. On soupçonne *Mycobacterium tuberculosis* (*M. tuberculosis)* ,la cause de la TB, d'être un effecteur critique de l'hypothèse de l'hygiène parce qu'il peut détourner le système immunitaire afin de l'empêcher de développer un profil immunitaire associé à l'atopie. Ainsi, l'absence d'une exposition à *M. tuberculosis* pourrait enlever la suppression du développement de l'asthme.

À partir de l'hypothèse de l'hygiène, nous avons supposé que les facteurs génétiques qui prédisposent à la TB devraient être protecteurs pour l'asthme/atopie et *vice versa*. Par conséquent, nous avons testé des variantes génétiques pour 1) le risque d'asthme basé sur des effets génétiques connus de susceptibilité à la TB, et 2) le risque de TB basé sur des effets génétiques connus de susceptibilité à l'asthme. Afin d'identifier les variantes génétiques pour le risque relié à l'asthme, nous avons testé la transmission non aléatoire des variantes génétiques des gènes du récepteur de la vitamine D (*VDR*) et de la protéine de résistance naturelle du macrophage 1 (*NRAMP1*), deux gènes de susceptibilité à la TB connus, auprès d'une cohorte familiale de Canadiens-Français asthmatiques. Afin d'identifier les variantes génétiques reliées au risque de TB, nous avons évalué la différence de distribution des variantes génétiques de plusieurs gènes reliés à l'asthme: l'interleukine 4 (*IL-4*), le récepteur A de l'IL-4 (*IL4RA*), le facteur onconécrosant A (*TNFA*), la lymphotoxine A (*LTA*) et le *VDR* auprès de cas de TB et de témoins sans TB

recrutés au Mexique. Seules les variantes de *VDR* ont montré un effet modulant le risque et ont été analysées plus en détails dans la présente thèse.

Dans une cohorte familiale d'asthmatiques du Nord-Est du Québec, parmi les 12 variantes de VDR testées, 6 variantes génétiques situées entre l'intron 2 et la portion 3' non codante (3'UTR) se sont avérées être en forte association avec l'asthme ($0,0005 < p < 0,01$), dans un modèle additif. Des effets génétiques haplotype-spécifiques ont également été observés pour l'asthme ($0,0004 < p < 0,01$). Par contre, aucune variante du gène *NRAMP1* n'a été observée en association avec l'asthme dans cette cohorte d'asthmatiques Canadien-Français. Dans la cohorte cas-témoin de TB, sur les 14 variantes de *VDR* testées, nous avons observé que 2 variantes génétiques situées à l'extrémité 5' non codante du gène étaient associées ($p < 0,05$) soit avec la susceptibilité à la TB en tant que telle, l'infection avec *M. tuberculosis* ou la progression de la maladie. Aucune association ne fut observée entre les variantes génétiques de *IL4RA, TNFA* et *LTA,* et la TB ou les phénotypes y étant reliés.

Ces résultats identifient clairement *VDR* comme un gène de susceptibilité à l'asthme et à la TB. Cependant, sachant que les variantes génétiques associées avec l'asthme diffèrent de celles associées avec la TB, ceci indique que la manière dont ces variantes influencent le système immunitaire à favoriser l'asthme mais non la TB et *vice versa* demeure incertaine. Bien qu'il fut proposé que la TB représenterait une force de suppression de l'asthme, outre *VDR*, nous n'avons pu détecter d'autres effets génétiques de gènes de susceptibilité à la TB dans l'étude de l'asthme, ni d'effets des gènes de susceptibilité à l'asthme dans l'étude de la TB. Nous interprétons ces résultats comme un argument à l'encontre de la contre-sélection entre les variantes génétiques de l'asthme et de la TB.

Preface

The work described in Chapters 1, 2 and 3 of this thesis has been published as follows:

Chapter 1 Poon A and Schurr E (2004), The NRAMP genes and human susceptibility to common diseases. In: Cellier M, Gros P (eds) The Nramp family. Landes Bioscience and Kluwer Academic/Plenum Publishers, New York, USA: pp 29-43.

Chapter 2: Poon, AH., Laprise, C., Lemire, M., Montpetit, A., Sinnett, D., Schurr, E. and Hudson, TJ. Association of Vitamin D Receptor Genetic Variants with Susceptibility to Asthma and Atopy. **American Journal of Respiratory and Critical Care Medicine**, November 1, 2004, 170 (9); 967 – 973. © The American Thoracic Society

Chapter 3: Poon, AH., Laprise C., Lemire M., Hudson JT., Schurr E. *NRAMP1* is not associated with asthma, atopy, and serum immunoglobulin E level in the French Canadian population. **Genes and Immunity** advance online publication, 30 June 2005; doi:10.1038/sj.gene.6364238. © Nature Publishing Group

In addition, at the time of the submission of this thesis, Chapter 4 is in preparation for publication.

Contribution of co - authors

The work described in Chapters 2 to 4 has been performed with the collaboration of co – authors as follows:

Chapter 2: I selected, genotyped and analyzed genetic variants of the *VDR* gene (variants *Fok*I C>T and *Taq*I C>T) in a subgroup of the cohort. Positive association led to the comprehensive coverage of the *VDR* gene for the entire study cohort in which I selected the genetic variants for genotyping and performed the statistical analysis. I participated in the writing of the manuscript. Thomas Hudson and Catherine Laprise provided the DNA samples and phenotypic data of the French Canadian study asthma cohort. Mathieu Lemire provided advice on the statistical analysis. Alexandre Montpetit participated in genotyping the samples by Orchid technology. Donna Sinnett produced all sequencing data. All co-authors provided comments on the manuscript. Thomas Hudson and Erwin Schurr provided supervision throughout this study.

Chapter 3: I selected 5 variants of the *NRAMP1* gene for investigation, developed high – throughput genotyping assays, genotyped 3 of the variants (variants 5'$(CA)_n$, D543N and 1729+delTGTG), and performed the statistical analysis on allelic and haplotype associations in the French Canadian asthma cohort. I participated in the writing of the manuscript. Y. Renaud participated in the genotyping of two SNPs. Thomas Hudson and Catherine Laprise provided the DNA samples and phenotypic data of the cohort families. Mathieu Lemire performed the power calculation. All co – authors provided comments on the manuscript. Erwin Schurr provided supervision throughout this study.

Chapter 4: I selected the 14 variants of the *VDR* gene for investigation in the Mexican adult and Texan pediatric TB cohorts. I extracted DNA from blood of all subjects provided by M.L. García García, A. Jiménez-Corona, M. Palacios-Martínez, J. Sifuentes-Osornio, A. Ponce-de-León, M. Bobadilla, M.Kato and Peter Small. I managed the phenotypic and genotypic data of the Mexican cohort. I performed association analysis and constructed LD patterns for both the adult and pediatric cohorts. Lastly, I participated in the writing of the manuscript. Neil Malik prepared and managed the pediatric TB DNA samples

provided by J.R. Starke, K.C. Smith, E.A. Graviss, J.M. Musser. All co – authors are presently providing comments on the manuscript. Erwin Schurr provided supervision throughout this study.

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Objectives of the Present Work

Epidemiologic studies have observed a rising prevalence of immune-mediated diseases in developed countries over the past decades. The hygiene hypothesis proposes a biological plausible idea to explain this rise of immune-mediated diseases. It proposes that the rise of immune-mediated diseases in developed countries is caused by the absence of childhood infections. Since the initial formulation of the hygiene hypothesis, the search for the infective agents exerting protective effects against immune-mediated diseases has been intensive but largely fruitless. In particular, the efforts aimed to identify *M. tuberculosis* as a protective agent against asthma and allergic diseases have provided inconclusive results. Hence, I propose to investigate this plausible relationship at the molecular level by:

1. assessing the genetic effects of known TB risk genes on asthma susceptibility in a French Canadian asthma population.
2. assessing the genetic effects of known asthma risk genes on TB susceptibility in a Mexican TB population.

I hypothesize that if the above protection from asthma by *M. tuberculosis* is correct, that persons who are for genetic reasons at increased risk of *M. tuberculosis infection* or tuberculosis disease should be protected from asthma and vice versa.

Chapter 1

Introduction and Literature Review

1.1 The hygiene hypothesis

Over the past two decades, developed countries have experienced striking changes in the prevalence of common diseases. Infectious diseases such as tuberculosis (TB), measles and hepatitis A have become less prevalent, whereas the prevalence of immune-mediated disorders such as allergic and autoimmune diseases has increased [1-2]. Anecdotal evidence in humans and established animal models of immune-mediated disorders showed that exposure (or lack of exposure) to pathogens is associated with decreased (or increased) manifestations of allergic or autoimmune diseases suggesting a causal relationship between these two classes of diseases. Measles and probiotics have been shown to reduce the severity of atopic dermatitis [3-4] while frequent administrations of antibiotics during infancy increased incidence of allergy and other atopic disorders compared children who had not received antibiotics [5-6]. In animal models, pathogen-free breeding environment has been shown to induce earlier and higher rates of autoimmune diseases in susceptible strains. For example, the incidence of diabetes is increased in diabetic prone rats that are delivered by cesarean and raised in pathogen – free conditions. [7]; and diabetes can be prevented in mice by infecting the animals with mycobacteria and viruses [8-9].

The inverse prevalence trends can be exemplified by two diseases of the respiratory systems: TB and asthma. Once given the names 'consumption' and 'the white plague', TB is caused by *Mycobacterium tuberculosis*, and this disease had killed 25% of the adult European population in the 17th and 18th centuries. Fortunately, since the discoveries of anti – TB drugs between the 1940s and 1960s, mortality and morbidity from TB have been declining tremendously in developed countries [1]. The prevalence of asthma, a disorder characterized by reversible obstruction of airways causing cough, wheezing, dyspnea and mucus over production, has been soaring in the past 50 years. As a consequence, in the United States (US), the prevalence rate of active TB has

dropped from 80/100,000 people in 1950 to <10/100,000 people in 1997, while that of asthma has reached 5380/100,000 people or 14 million people in 1994 from 3500/100,000 or 8 million people in 1981 (http://www.cdc.gov/asthma/asthmadata.htm). In Japan, the prevalence rate of active TB has dropped from 190.8/100,000 people in 1935 to 15 - 60/100,000 people in 1998 [10], while that of childhood asthma has risen from 1710/100,000 in 1971 to 6400/100,000 in 1996 [11]. The reverse trends are not observed in the less developed countries where infectious diseases remain heavy health burdens while allergic and autoimmune diseases are uncommon (Figure 1). The general trend observed is that in countries where TB prevalence is high, such as China (246/100,000), Ethiopia (533/100,000) and Russia (160/100,000), asthma prevalence is relatively low (2.1%, 3.1% and 2.2%, respectively); and in countries, such as Canada (5/100,000) and Australia (6/100,000) where TB prevalence is low, asthma prevalence is high (14.1% and 14.7% respectively) [12-13].

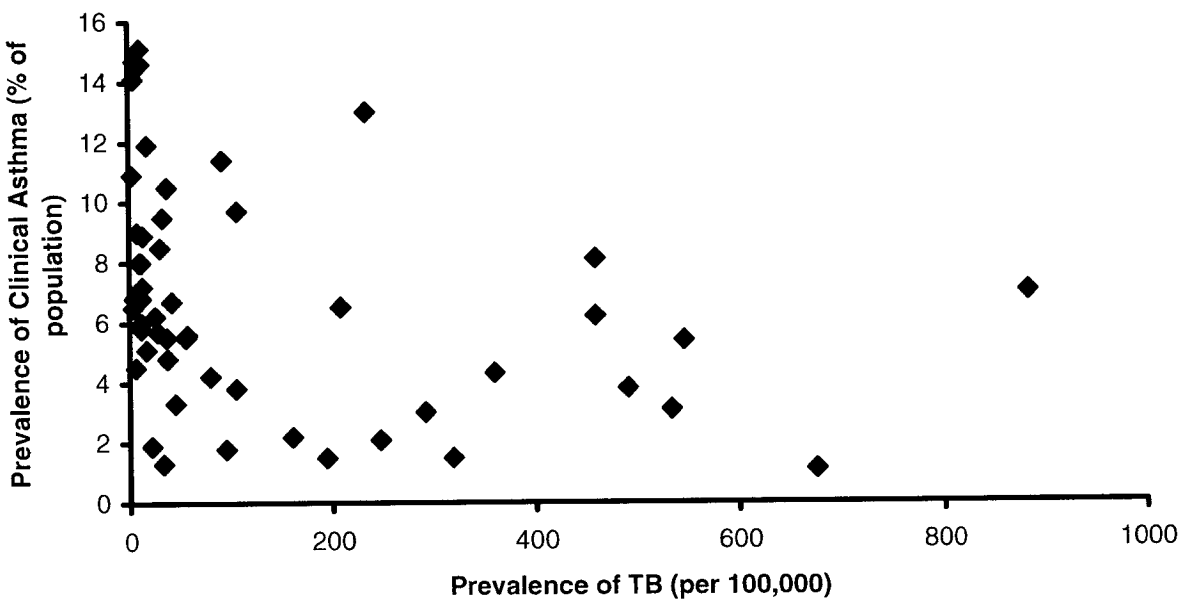
The decline in the prevalence of infectious diseases in developed countries has been attributed to the use of antibiotics, vaccination, improved hygiene and better socioeconomic conditions. A number of hypotheses have been proposed to explain the rise of allergic diseases, and the most biologically plausible and intensively studied is the *hygiene hypothesis* proposed by a British epidemiologist, David Strachan, in 1989. In a prospective study of 17414 British children from birth to 23 years of age [14], Strachan observed a statistically significant inverse correlation between the number of older siblings in the household and the prevalence of hay fever (an inflammatory response in the nasal passages to allergic stimulants) at age 11 years and 23 years. He proposed that declining family size, improved household amenities and higher standards of personal cleanliness have reduced infection in early childhood, often transmitted by unhygienic contact with older siblings, or acquired prenatally from a mother infected through a contact with her older children, may have resulted in a rise in allergic diseases. Other epidemiologic studies confirmed the inverse

association between 'infection prone' lifestyle such as having older siblings [15], living in farms [16-17], attending daycare [15,18-19,] , and atopic markers such as skin prick test positivity and specific immunoglobulin (Ig) E level. At the population level, reduced microbial infections may have increased the prevalence of atopic diseases in developed countries. The hygiene hypothesis has also been proposed to explain the rising prevalence of autoimmune diseases in developed countries. Over the past decade, there has been an intensive search for the microbes exerting protective effects against immune-mediated disorders by comparing the prevalence of surrogate markers for various specific infections in groups of individuals suffering from immune-mediated disorders with healthy controls. Pathogens such as measles, pertussis, mycobacteria, heptatitis A, schistosoma and helminthes, and microbial components such as lipopolysaccride have been investigated for their protective effects, but results are inconsistent and inconclusive (reviewed in [20-21]). In spite of the inability to identify the protective agent, the hygiene hypothesis offers a biological plausible and coherent explanation for the variation in immune – mediated disorders over time between countries and between individuals with different lifestyle.

1.1.1 The hygiene hypothesis at the molecular level – the Th1 / Th2 paradigm

One proposed mechanism to explain the hygiene hypothesis is the Th1 / Th2 paradigm [22-23]. It proposes that early - life encounters (either naturally through infections or manually through vaccinations) with microbes such as bacteria and intracellular pathogens promote the maturation of T helper type 1 cells (Th1) from T helper precursor cells in the presence of Th1 – inducing conditions (reviewed in [24-25]). Briefly, the present knowledge models the paradigm as follows: upon antigen presentation to the precursor cells, secretion of the Th1 cytokines interleukin (IL)-12 and interferon-γ (IFN-γ) by macrophages, dendritic cells and natural killer cells promotes and enhances the Th1 population

Figure 1. Global trends of TB and asthma prevalence. The scatter plot illustrates the global overall inverse trends of TB and asthma prevalence by plotting 50 countries of varying degree of prevalence. TB prevalence data is the most recent available (year 2003) in the WHO database (http://www.who.int/tb/country/global_tb_database/en/index.html). Clinical asthma prevalence data was obtained from the Global initiative for Asthma (GINA) Program (GINA) [13]. The 50 countries represented in the plot are (in the order from lowest asthma prevalence to highest): Indonesia, Albania, Romania, Nepal, Georgia, Greece, China, Russia, India, Ethiopia, Mexico Bangladesh, Morocco, Latvia, Pakistan, Italy, Portugal, Chile, Nigeria, Argentina, Iran, Saudi Arabia, Spain, Austria, Belgium, Philippines, United Arab of Emirates, Sweden, Thailand, Japan, France, Norway, Germany, Kenya, Lebanon, Czech Republic, Finland, South Africa, Kuwait, Barbados, Israel, Uruguay, Paraguay, Paraguay, Fiji, United States of America, Brazil, Costa Rica, Peru, Canada, Ireland, Australia, and New Zealand.


Prevalence of Clinical Asthma (% of population)
16
14
12
10
8
6
4
2
0
0
200
400
600
800
1000
Prevalence of TB (per 100,000)

expansion [26-27]. In the absence of Th1 inducing conditions, or the presence of either IL-4, precursor cells would differentiate into type 2 helper (Th2) cells [28-29]. Subsequently Th2 cells synthesize Th2 cytokines (IL4, IL5, IL9 and IL13) and enhance immunoglobulin (Ig) (IgE, IgG1) synthesis by B cells. Since Th1 responses are characteristic of infectious immunity to intracellular pathogens and Th2 responses are associated with atopic diseases, the mechanism is called the Th1 / Th2 paradigm [22-23]. Direct evidence of the paradigm has been shown to exist in animal models [30-31] and in humans *in vitro* [22,32]; however, the discovery of additional classes of regulatory T cells involved in infectious and allergic diseases implies that the Th1 / Th2 paradigm is too simplistic for explaining the hygiene hypothesis [33-34]. In addition, contrary to prediction Th1 cells exacerbate allergic disease [35] and are associated with several autoimmune disorders [1]. Hence, a more complex mechanism underlies the hygiene hypothesis (reviewed in [20])

1.1.2 A genetic approach to identify a protective role of *M. tuberculosis* against asthma / atopy

Since the proposal of the hygiene hypothesis, many pathogens have been studied for their protective roles against immune-mediated diseases, but the findings have been inconclusive (reviewed in [20-21]). To search for a relationship between pathogens and protection against immune – mediated diseases, one can compare prevalence of immune – mediated diseases among groups of individuals with presence of absence of prior exposure to specific pathogens [14,20]. An alternative approach involves investigating genetic variants that vary development of either infectious or immune-mediated diseases. Through this approach, Hepatitis A virus (HAV) has been shown to confer protection against atopy through its cellular receptor TIM-1 (T cell immunoglobulin domain, mucin-like domain) (reviewed in [36-37]). Two variants of the *TIM-1* gene, a six amino acid (methionine-threonine-threonine-threoine-valine-proline) insertion at residue 157 (157insMTTTVP) and a threonine deletion at codon 195 (195delT) confer

protection against atopy in those previously exposed to HAV [38]. The latter alleles may facilitate easier HAV entry into CD4+ T cells than wild type alleles, allowing the virus to inhibit Th2 differentiation of the cells, thus skewing the immune response away from the atopy associated Th2 profile [37].

1.2 Tuberculosis

1.2.1 Global distribution of TB cases

Development of effective anti – TB drugs between the 1940s and the 1960s has reduced the health threat posed by TB in developed countries [39], however, due to political and economical disadvantages, underdeveloped countries continue to be haunted by TB. According to the World Health Organization (WHO) in 2000, of the total 56 million deaths reported globally, TB was responsible for 2.2 million (4%) deaths [40]. In 2002, WHO recorded 8.8 million new cases of TB worldwide, and predicted a growth of 2.4% per year in the total number of cases [41]. Countries that ranked 1st to 22nd in terms of total estimated TB cases in 1998 are India, China, Indonesia, Bangladesh, Pakistan, Nigeria, the Philippines, South Africa, Ethiopia, Vietnam, The Russian Federation, DR Congo, Brazil, Tanzania, Kenya, Thailand, Myanmar, Afghanistan, Uganda, Peru, Zimbabwe, and Cambodia. In particular, TB prevalence has been rising quickly in eastern Europe (5% per year, 1997-2002), and in African countries with high HIV prevalence (eastern and southern African countries, 7% per year). Importantly, TB is the leading cause of death amongst HIV infected individuals (11%) [41]. If the present TB epidemic is not controlled, WHO estimated that between 2002 and 2020, 1000 million people will be infected, 150 million people will develop the disease and 36 million people will die from TB. (http://www.who.int/mediacentre/factsheets/who104/en/)

1.2.2 The etiological agent

M. tuberculosis, the causative agent of most TB cases, was identified in 1882 by Robert Koch [42]. This species belongs to the genus *Mycobacterium* that comprises over 50 species, including pathogens such as *Mycobacterium leprae*, (the causative agent of leprosy), and *Mycobacterium ulcerans* (Buruli ulcers) [43-44]. Within the genus *Mycobacterium*, *M. tuberculosis* belongs to the *M. tuberculosis* complex with four other phylogenetically related members: *M. africanum, M. canetti, M. bovis* and *M. microtti* [45]. Comparative genomic studies suggest that in the *M. tuberculosis* complex, *M. tuberculosis* is the most ancestral and *M. bovis* is the most recently evolved complex member [46-47].

Mycobacteria are rod-shaped and share a common property of a lipid-rich cell wall that retains Carbol Fuchsin dye even in the presence of acidic alcohol, thus they can be detected by acid fast staining [45]. The cell wall of *M. tuberculosis* consists of a cytoplasmic cell membrane and a peptidoglycan layer, structurally similar to the classical bacterial cell walls [48]. An unusual feature of the *M. tuberculosis* cell wall is the addition of an extra layer of complex lipids, glycolipids (mostly mycolic acids) and polysaccharides to the peptidoglycan layer. Importantly, most of these lipids and glycolipids are immunomodulators [48-49]. For example, among many proteins interspersed in the cell wall is lipoarabinomannan (LAM), which uses its mannose-containing oligosaccharides to bind to the mannose receptors of phagocytic cells such as macrophages to induce intake of the bacteria into the cells [50].

Many biological characteristics of *M. tuberculosis* have been revealed by the completion of the 4.4 Mbp genome of the clinical strain H37Rv. *M. tuberculosis* possesses one of the largest bacterial genomes, only the genomes of *Streptomyces coelicolor* and *E. coli* are larger than that of *M. tuberculosis* [51]. Of the *M. tuberculosis* genome, 90.8% contain protein encoding sequences, and a large portion of these genes is involved with lipid biosynthesis or lipid degradation, suggesting that *M. tuberculosis* uses lipids over glucose as carbon

source when necessary [51]. In addition to the large portion of the coding sequences devoted to lipid metabolism, approximately 9% of the *M. tuberculosis* genome encode two related families of genes involved in growth - the PE and PPE families. These genes have been proposed to contribute to antigenic variation of the bacteria and may help *M. tuberculosis* to evade host immune responses [51].

1.2.3 Molecular mechanisms of TB pathogenesis

To identify genetic factors affecting TB susceptibility, genetic studies must take into account the complex host – pathogen interactions responsible for a variety of epidemiologic and clinical features. TB is transmitted among humans by airborne droplet nuclei containing *M. tuberculosis* [52]. By inhaling such nuclei, *M. tuberculosis* can reach the alveoli within the lungs [52]. *M. tuberculosis* bacteria enter the alveolar macrophages via phagocytosis upon binding their bacterial cell wall proteins to surface receptors such as mannose receptors [50,53-54] and complement receptors [55], and then replicate within infected cells.

Once the host detects an infection, it mounts a cell - mediated immune response involving the production and secretion of various Th1 cytokines (IL-2, IL-12, IFN - γ and TNFα) [56-58]. Working in synergy, these cytokines promote (1) generation of reactive oxygen intermediates (ROI) and nitrogen intermediates (NRI) by infected macrophages that are toxic to the bacteria [59-61]; (2) lysis of the infected macrophages by cytotoxic T cells [62-64]; (3) apoptosis [61,65-66]; (4) granuloma formation and maintenance [67]; and (5) intoxication of *M. tuberculosis* with granulysin [68-69]. These microbicidal activities protect the host by killing the tubercle bacilli or by inhibiting *M. tuberculosis* replication [70].

M. tuberculosis has evolved mechanisms to counter host immunity and to survive inside macrophages. To avoid being degraded by hydrolytic enzymes stored in lysosomes, *M. tuberculosis* arrests the fusion between the pathogen-

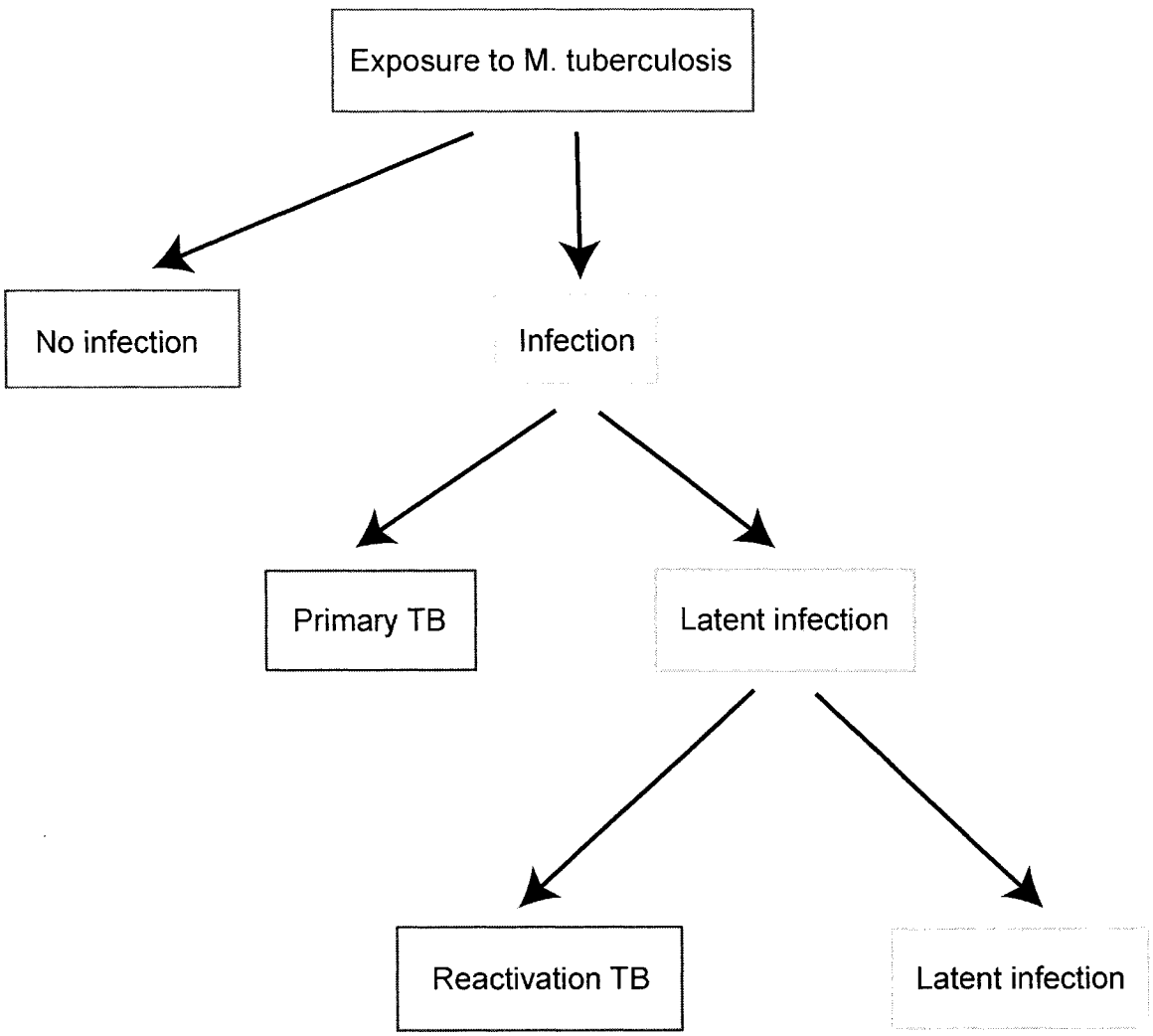
containing phagosomes and lysosomes [71-73]. To prevent the recruitment of T cells to the infected macrophages that promote microbial killing or growth inhibition, *M. tuberculosis* disrupt antigen presentation to T cells [74], inhibit IFN-γ signalling pathways in macrophages [75], and stimulate IL-6 production by infected macrophages to suppress T-cell proliferation [76]. To evade RNI toxicity by activated macrophages, *M. tuberculosis* has developed a redox machinery to catabolize this potent oxidant [77-79].

Depending on the host's immune competence, the bacteria can: (1) be killed by the initial host response, but without sterilizing immunity, (2) begin to multiply immediately after infection and progress to active TB (primary TB), (3) be walled off inside granulomas and become dormant (latent infection) or (4) revive from dormancy, replicate and progress to disease (reactivation TB) [45,80] (Figure 2).

1.2.4 Clinical features

Longstanding evidence indicates that among those infected with *M. tuberculosis*, approximately 5 to 10% cannot contain the pathogen and progress to TB within 2 years, termed primary TB [45,81-82;]. For those who can contain the pathogen initially, complete microbial eradication does not occur and a dynamic balance between bacterial persistence and host defence develops. The host triggers the formation of granulomas at the sites of infection [83-84]. These spherical aggregate structures of immune cells physically wall off the bacteria and prevent dissemination. In most infected individuals (approximately 90%), *M. tuberculosis* remains trapped within the granulomas that subsequently undergo calcification. The infection is arrested at this point and no disease develops. This state of TB pathogenesis is termed latent infection.

Figure 2. TB pathogenesis. Upon exposure to *M. tuberculosis*, depending on host – pathogen interactions, 6 main outcomes are possible in TB pathogenesis. Green boxes represent outcomes where infection is not successful, yellow boxes represent stages where infection has occurred but the host immune system is able to mount an efficient response to suppress disease development, and red boxes represent stages where infection has progressed to disease. Both latently infected and non-infected Individuals may be exposed to *M. tuberculosis* later in life and outcomes will once again depend on host – pathogen interactions.


Exposure to M. tuberculosis
No infection
Infection
Primary TB
Latent infection
Reactivation TB
Latent infection

Clinical manifestation of latent infection into reactivation TB occurs when the host can no longer maintain the integrity of the granulomas, e.g. due to a weakened immune system, allowing the dormant pathogens to replicate [85-87]. If untreated, the cavities containing replicating *M. tuberculosis* can erode pulmonary arteries with aneurysm (sac being formed on the arterial walls) with fatal bleeding and form bronchopleural fistula with empyema (collection of pus) [44,88]. The risk that an infection progresses to active TB for immunocompetent individuals is 10% for a lifetime, whereas for HIV infected individuals the risk increases to 8 - 10% per year [89]. If left untreated, a single TB case can infect 10-15 individuals annually [90-91].

People with TB exhibit a spectrum of outcomes. In non HIV infected individuals, 85% of TB occurs in the lung and the remaining 15% in other organs such as the lymph nodes [92], the brain [93] and the genitourinary tract [94-96]. Those with pulmonary TB and who are HIV negative almost always have abnormalities which can be detected on chest X-rays. Children under the age of 4 years are more likely to develop primary disseminated forms of the disease such as miliary TB and tuberculosis meningitis [45,97]. Although cavitations in the lungs are rare for children, they can suffer from partial or complete lung collapse caused by enlarged lymph nodes compressing the airways.

1.2.5 Diagnosis

TB is diagnosed by symptomatic, radiographic and / or bacteriologic characteristics. Although *M. tuberculosis* may be organ specific, symptoms are often systemic. Fever, loss of appetite, weight loss, weakness and night sweats are common systemic symptoms associated with TB [45]. Untreated, disseminated TB cases may also present organ specific symptoms such as enlargement of the liver and spleen [98-102]. Often on the chest X-rays of pulmonary TB and most disseminated TB cases, abnormalities such as cavities, tuberculous lesion scars,

calcified or non calcified nodules, infiltrate, granulomas and small evenly distributed nodules can be detected [45,88].

Depending on the sites of disease development, body fluids such as sputum, gastric aspirates, urine, cerebrospinal fluid, pleural fluid, bronchial washings and bone marrow are collected to detect *M. tuberculosis* [45]. The first evidence of *M. tuberculosis* infection comes from detection of acid-fast stained bacilli (AFB) [45]. AFB staining procedures are time and cost efficient, but they have low sensitivity in specimens containing low numbers of bacilli [103]. Hence, negative smears cannot preclude TB and the specimens should be grown in media for further examination [45,82]. In addition to the routine AFB smear staining and cultivation, amplification of the bacterial nucleic acid may be employed to confirm diagnosis of active TB [104].

Latent infection with *M. tuberculosis* can be detected by tuberculin skin testing [45,82]. In the widely used tuberculin test (also known as PPD test), a standard dose (0.1 mg/0.1 ml) of purified protein derivative (PPD) containing antigenic components of *M. tuberculosis* is administered intracutaneously in the forearm to induce immune response. The size of swelling at site of injection, and the occasional vesiculation / necrosis, are examined 48 to 72 hours after injection. Swelling at the site of injection is a surrogate marker for the delayed-type hypersensitivity reaction associated with *M. tuberculosis* infection [105]. When *M. tuberculosis* infects a host, the immune system generates memory T cells that recognize and respond to *M. tuberculosis* antigens. The tuberculin skin test triggers an intensive secondary response termed delayed - type hypersensitivity (DTH) [106]. The guidelines are to consider swelling > 5mm as positive for *M. tuberculosis* infection when an individual has been exposed to any of the following risk factors: in close contact of individuals with active TB, present abnormality indicative of old or healed TB on chest X-rays and is immunosuppressed [45,82]. In the absence of these risk factors, one needs to have a swelling > 15mm to be considered infected. For individuals with epidemiologic

(e.g. injection drug use) or clinical (e.g. diabetes mellitus, chronic renal failure) risk factors, the cut of point is 10mm. For children (< 4 years) unexposed to any risk factors, the cut-off size for positive result is 10 mm. For individuals with strong risk factors (e.g. immunosuppressive) induration > 5mm is considered PPD test positive [107].

1.2.6 Prevention

Millions of people globally have been vaccinated with Bacille Calmette - Guérin (*BCG*) as a means of preventing TB. *BCG* is an attenuated strain of *M. bovis* developed in 1921 by the French scientists Calmette and Guérin [108-109]. Ideally, an effective vaccine is one that can prevent infection and / or disease development in those vaccinated. *BCG* vaccines have been shown to protect against miliary TB and meningeal TB in newborns [110]; however, its protective efficacy against the pulmonary TB in children and adults varies from 0% to 80% [111-112], hence, it is considered too unreliable as compared to other established vaccines such as those for measles which have more consistent efficacies of > 80% [113-114]. In British school children, *BCG* vaccination has successfully prevented 80% of those vaccinated to develop TB [115], while in school children in Georgia, US no protection was observed [116]. Global variation in the protective efficacy of *BCG* vaccination has been attributed to factors including genetic variability of the subjects vaccinated, the exposure prevalence of mycobacteria, nutrition status and genetic variability of the *BCG* strains used [117-119]. The latter has been speculated as a result of different cultivating conditions in different laboratories, leading to loss of genomic elements which may be associated with over-attenuation [118,120]. These over-attenuated *BCG* strains may have failed to mimic *M. tuberculosis* to induce host immunity that could generate *M. tuberculosis* – specific Th1 cell immune response to subsequent *M. tuberculosis* infection [118,121-122]. Intensive vaccine development is underway to design more TB specific and less variable vaccines [123-127].

1.3 Asthma

1.3.1 Global distribution of asthma

Epidemiologic studies comparing the global prevalence of asthma observed an increase worldwide since the 1950s, with a higher prevalence in developed compared to developing countries [128-130]. Both adult and childhood asthma prevalence are increasing. According to the European Community Respiratory Health Survey that examined the prevalence of adult asthma in 22 countries, those most affected with asthma are the British Isles, New Zealand, Australia and the US (25-30% of the population suffered from asthma), followed by Italy, France, Belgium, and Germany (10-17%) and the least affected are India and Algeria (4%). For childhood asthma, a similar trend was observed by the International Study of Asthma and Allergies in Childhood that compared the prevalence across 50 countries. The highest prevalence occurs in the English-speaking countries and the lowest in developing countries [131]. For developing countries which are becoming more westernized, more asthma cases are emerging than previously observed [132-133]. For example, in a short 4 year period in Taiwan, the childhood prevalence of asthma has risen from 6.5% to 8.5% [134].

1.3.2 Molecular mechanisms of asthma development

The appeal to investigate the hygiene hypothesis in the context of TB and asthma comes from the biological plausibility highlighted by the molecular mechanisms of the two diseases. *M. tuberculosis* induces a Th1 immune response by the host, whereas an asthmatic response, inferred mainly from mouse studies, is dominated with Th2 characteristics [1,135]. An asthma response begins when inhaled allergens are taken up by the airway dendritic cells and their antigens are presented to T helper (CD4+) cell precursors. At this time, if the cytokine milieu is dominated by IL-4 (e.g. in the absence of any Th1 stimulant),

these precursor cells mature into Th2 cells that secrete IL-4, IL-5, IL-9 and IL-13 cytokines [135]. These Th2 cytokines activate many different cell types including fibroblasts, B cells, mast cells and eosinophils in the airway [136], to produce mediators responsible for the clinical features of asthma [137-138]. Most of these data have been obtained from animal models, however, similar disease pathogenesis may exist for humans since several studies have detected elevated mRNA expression and cytokine levels of IL-4, IL-5 and IL-13 in brochoalveolar lavages, sputa, bronchial biopsies, blood, peripheral mononuclear blood cells and serum of asthmatic individuals [139-147]. In addition, eosinophil, mast cell, T cell and macrophage counts tend to be higher in asthmatic than non-asthmatic individuals [148-149]. This complex pathogenesis of asthma implies that genetic control of asthma is likely to be equally complex and possibly conditional on specific gene – environment interactions.

1.3.3 Clinical forms and features

Unlike TB, asthma is not caused by a single agent. It is described as a chronic inflammatory disorder of the airways. Features of asthma include airway hyper responsiveness, excessive airway mucus production, airway inflammation and elevated serum immunoglobulin E (IgE) levels [45]. Airway inflammation manifests into many asthma symptoms including recurrent episodes of wheezing, breathlessness, chest tightness, coughing, airway hyper responsiveness and airflow limitation [150-151]. Airway inflammation also dictates disease chronicity [11,152]. Immunohistopathologic features of asthma include denudation of airway epithelium, collagen deposition beneath basement membrane, edema, mast cell activation and inflammatory cell infiltration (neutrophils, eosinophils and lymphocytes). The term asthma encompasses a wide range of subtypes such as atopic asthma, nonatopic asthma, childhood asthma, late-onset childhood asthma, adult-onset asthma, occupational asthma, seasonal asthma and many others [153]. Stimulants known to trigger asthmatic attacks include inhalant allergens (animal allergens, house-dust mites, cockroach allergens, pollens,

molds), irritants (tobacco smoke), exercise, medications (aspirin, non-steroid anti-inflammatory drugs, topical and systemic beta blocks), sulfite and a variety of health conditions (rhinitis, sinusitis, viral respiratory infection and gastroesophageal reflux) [11].

Often childhood asthma begins during the preschool years [154-155], and most symptoms will not persist into adulthood [156-157]. Even within the phenotype of childhood asthma, heterogeneities in age of onset, atopy status and persistence exist. Children with asthma may (1) wheeze during infancy up to the age of 3 years and show impaired lung function at birth, (2) wheeze beyond the age of 3 years and may or may not be atopic, (3) wheeze during the first 10 years of life while being atopic, and (4) exhibit asthma symptoms during or after puberty [158-159]. Whether children with asthma will continue to be asthmatic in adulthood depends on risk factors including early age of onset, female gender, atopy, eczema, impaired lung function, and bronchial hyper responsiveness [160]. Heterogeneity also exists for adult – onset asthma; it can be categorized into subtypes based on specific triggers, such as aspirin-induced asthma [161], asthma related to chronic infection with respiratory pathogens [162-164] or occupational asthma [160,165].

Atopy, the genetic predisposition to develop IgE-mediated responses to common environmental allergens, is the strongest predisposing factor identified so far for asthma development [166-167]. In general, individuals who are non atopic mount a low-grade immune response to allergens and they produce allergen-specific IgG1 and IgG4 antibodies [168]. Atopic individuals, however, upon exposure to allergens, mount an exaggerated response by producing allergen-specific IgE antibodies [23,169-170]. *In vitro* experiments have shown that T cells from non atopic individuals respond to allergens with a moderate degree of proliferation and production of IFN-γ by Th1 cells while T cells of atopic individuals display production of Th2 cytokines (IL-4, IL-5 and IL-13) [23,169-170]. Atopy is often assessed with skin prick tests; common allergens are deposited on

the skin (usually on the back of an arm) where previously pricked by a needle. Swelling associated with histamine released is observed if an individual is atopic towards the allergen applied [171].

1.3.4 Diagnosis

Clinical symptoms suggestive of asthma include hyper - expansion of the thorax, sounds of wheezing during normal breathing or a prolonged phase of forced exhalation, increased nasal secretion, mucosal swelling, nasal polyps and allergic skin reaction. Asthma is diagnosed by assessing lung function and detecting abnormalities such as limited airflow, variable airflow and airway hyper - responsiveness [172]. These lung abnormalities can be measured by (1) bronchoconstrictor challenge testing using methacholine or histamine, (2) peak expiratory flow variability before and after administration of a short acting bronchodilator and (3) ratio of the maximal volume of air forcibly exhaled in 1 second to maximal inhalation (forced expiratory volume in 1 second / forced vital capacity = FEV1 / FVC) [45]. Essential information needed in the diagnosis of asthma are family history, physical examination, symptoms and assessment of living environment (i.e. second hand smoke and dust mites) [150].

1.3.5 Treatment and Prevention

There are numerous therapeutic regimens that are being used in the clinical management of asthma. However, in light of the hygiene hypothesis, intensive research is underway to explore the efficacy of using mycobacterial products in asthma / atopy immunotherapy and prevention [173]. While findings from murine models are promising [174-175], efficacy in humans remains controversial [176-177]. Parameters such as dosages, treatment protocols, patient characteristics and the mycobacterial products used are being evaluated for prospects of such 'mycobacterial therapy'.

1.4 Genetics of complex traits

1.4.1 'Less' complex versus complex traits

Generally, the process of identifying genes and their variants causing specific traits, (e.g. increased susceptibility to common diseases) involves first establishing that the trait under study has a genetic component to its development. Twin and familial aggregation studies are often used for this purpose. Twin studies are based on the fact that monozygotic twins share identical genetic makeup while dizygotic twins share on average half of the genes. If a disease is manifested more frequently in monozygotic twins compared to dizygotic twins, then the disease can be concluded to have a genetic component in its development [178]. Similarly, in familial aggregation studies, if a disease is found more often in related individuals compared to its prevalence in the general population, then a genetic component in the disease development could be suspected [179]. Once a hereditary component has been established, the genetic mechanism (number of major loci, mode of effects and etc) can be assessed using segregation analyses [180]. With or without the knowledge of the genetic mechanism, the chromosomal regions where the genes that influence disease lie can be located by conducting parametric or non – parametric linkage studies using families with multiple affected individuals (reviewed in [181]). To further narrow down these linked regions to identify the disease risk or susceptibility gene(s), association studies comparing allele frequencies in unrelated cases and controls or comparing allele transmissions in from informative parents to affected children are conducted (reviewed in [182]). These approaches to investigate the genetics of disease have achieved great successes when clear genotype - phenotype correlations exist. In other words, if risk of disease is very strongly dependant on presence or absence of one gene variant.

However, the genetic contribution to most common human diseases is anything but simple. Common diseases such as cardiovascular diseases and diabetes have complex underlying mechanisms where disease developments are governed by both genetic variations and the environment. Complexities arise from the fact that many genetic variants are involved in disease manifestations (polygenic traits); that the same disease may be caused by different variants (genetic heterogeneity); or that genetic disease variants may reside in different locations in the same gene (allelic heterogeneity) [183]. These genetic disease risk variants may directly change the functional activity of the encoded protein (gain-of-function or loss-of-function), or they may modify disease risk by changing the mRNA and protein levels [184]. In addition, these genetic risk variants may need to be turned on or off by the environment (gene environment interaction) or other genetic variants (epitasis) in order to be relevant in disease development [185]. Findings from genetic studies on complex traits unraveled the fact that many human diseases have many genetic factors each exerting low to moderate effects. Often their effects are so low that they cannot be detected in linkage studies, and it can be shown that without knowledge of the genetic model association studies have greater power to detect genetic modulators of the risk of human diseases [186-187].

1.4.2 Association analysis

If the genetic effects are large enough to be detected by linkage studies, the genomic region of interest can only be narrowed down to the level of centi-Morgans or megabases. Association studies, however, can locate the trait or disease causing genetic variants with higher resolution. Genes that are tested in association studies, called candidate genes, are those that have evidence to support the hypothesis that they are involved in disease development. Evidence supporting the candidacy may include to reside in a disease linked chromosomal region, a biological function in humans or animal models consistent with the phenotype or to be associated with related diseases or with extreme cases of the

disease under investigation. Broadly, two types of study designs are available for association studies: population – based case control and family-based [186,188]. In the former, association can be concluded if the genetic variants (alleles, genotypes and / or haplotypes) are significantly unevenly distributed in the two groups of subjects. Genetic variants that are found to occur more commonly in patients would be considered risk factors, while those that occur more frequently in non-affected controls would be considered protective factors. Population – based case control designs, although easier for patient recruitment, are prompt to report false positive findings, since such designs strongly depend on proper selection of cases and controls and the absence of population substructures. Factors that can disturb allele frequencies that are being compared include immigration, emigration (population admixture) and nonrandom mating for at least several generations [189]. To prevent the detection of false associations due to substructures of the study cohorts, an alternative approach using non-transmitted alleles in family-based designs as pseudocontrols was proposed [190-191].

In the family – based association designs, instead of comparing frequencies of alleles, genotypes and / or haplotypes in cases and controls, it is the transmissions and non-transmissions of the genetic variants from informative parents to affected offspring that are being analyzed [186,188,191]. Association between the allele under study and disease can be concluded if the allele is over-transmitted or under-transmitted (i.e. if allele transmission deviates significantly from the expected 50:50 ratio from informative parents). Recent statistical advances allow family - based designs of any pedigree type, for dichotomous or quantitative phenotypes, for bi- and multi-allelic markers, and for various models of the mode of inheritance [192-196]. Although a family - based study design is more robust to population substructures, it is less powerful than the case control design since for each control pair, 3 subjects (2 parents and 1 affected child) need to be collected [188]. For both population - based case control and family-based studies, an association between a disease and a genetic variant may reflect that the

variant is the true underlying cause that manifests the phenotype (i.e. disease causing), or that it is associated with the trait causing locus, perhaps due to their close proximity on the chromosome [186,188].

1.4.3 Genetic Markers

In the past decade, the number of association studies has increased explosively, largely due to the discovery of abundant DNA markers called single nucleotide polymorphisms (SNPs). SNP refers to a position on the chromosome where alternative nucleotides may occur but is also used if a short deletion / insertion polymorphism exists at a given DNA position [197]. It has been estimated that 90% of the variation in the world's human population are SNPs with a minor allele frequency $\geq$ 1% [198]. Their abundance (about 10 millions have been identified so far), dense distribution (average 1 SNP per 332 basepair (bp)) and the ability to be genotyped in high throughput fashion render them indispensable as molecular markers in association studies [199-200]. In addition to serving as genetic markers, the fact that they occur at the genomic DNA level makes these markers plausible elements in disease pathogenesis [184,201]. For example, an insertion of a nucleotide at nucleotide position 3020 of the *CARD15* gene leads to a frameshift and a premature stop in translation. Cells expressing the truncated proteins have been shown to elicit a reduced NFκB activation to lipopolysaccaride compared to those with the normal proteins [202] and several studies have demonstrated that individuals possessing the insertion alleles are more likely to develop Crohn's disease compared to those with the wild type alleles [202-203].

1.4.4 Linkage Disequilibrium Mapping

The average density of SNPs is estimated to be 1 SNP per 332 bp [198]. The closer the proximity between a pair of SNPs, the more likely that SNP alleles will occur together in population surveys than expected by chance. This phenomenon has been termed linkage disequilibrium (LD) [188,198-199]. Many

different statistics have been proposed to assess the strength of association between pairs of SNPs. They all measure the difference between the observed number of chromosomes with two specific alleles occurring together and the corresponding number expected by chance (i.e. linkage equilibrium); they differ in the way they are standardized [204,205]. One frequently used measures of LD is D' [204-206], it is relatively easy to compute and interpret. Its values range from 0 (equilibrium) to 1 (complete disequilibrium). D' is standardized by its theoretical maximum value and it is interpreted as evidence for historical recombination between 2 sites, i.e. D' near 1 indicates no / weak evidence of historical recombination, D' significantly < 1 and near 0 indicates strong evidence of recombination, and intermediate values (e.g. 0.3 – 0.7) are considered indeterminate [199,206]. Formally, $D' = D_{max}/D$ and $D_{max} = \min(p_1q_1, p_2q_2)$ with p_1q_1, and p_2q_2 being the allele frequencies of SNP1 and SNP2 [207].

The ability to identify genetic variants that cause disease in association studies depends on the correlation between the causative variants and the markers being tested. High correlation between variants, characterized by strong LD measures, likely allows a study to detect the genetic effect of a causative variant by examining its neighboring SNPs. High LD occurs when SNPs locate in proximity to each other and one SNP is young in age [208-209]. For example, consider 2 SNPs A and B. If at some point in time, a mutation occurred at position A resulting in two alleles A and a before another mutation occurred at position B (hence only allele B exists). On a strand of DNA, there are two possible haplotypes or combinations of alleles: A - B or a - B. If at a later time point, a mutation occurred at position B to form alleles B and b on haplotype background a - B, then 3 possible haplotypes now exist to be passed on to the next generations: A - B, a - B, a -b. If the two locations are close enough that the likelihood of recombination is small, then even after many generations alleles a and b are still likely to occur together on the chromosome, and allele b is said to be in complete LD with allele a. In other words, allele b always coexists with allele a. The frequency of haplotype a - b would be significantly different from the

product of allele frequencies of a and b, under the assumption that the two SNPs are not linked. Hence if allele b is causative, then even when only allele a is examined, the effect of allele b can be detected. This method to establish the LD pattern among a dense array of chromosomal markers is called LD mapping [198,206,209-210]. LD mapping can be used to narrow the location of disease risk variants [199,211].

Large population studies examining LD structures in various genomic regions revealed that LD varies along the chromosomes with regions of high LD interspersed with regions of low LD [199,210,212-214]. Regions of high LD can be conceptualized as blocks and many methods have been proposed and developed to define LD blocks and the corresponding haplotypes (i.e. allelic combinations) within the blocks [206,215]. One method to define haplotype blocks is to preset a threshold and then to define a block where all pairwise LD coefficients within a region exceed the preset threshold. For example, Gabriel *et al.* defined a block as sets of consecutive SNPs between which there is little or no evidence of historical recombination indicated by high LD as measured by of D' [212]. Specifically, for each pairwise D', the approach constructs a confidence interval (CI), and two or more SNPs are grouped together into a block if the outermost pair of SNPs is in strong LD (lower CI of D' >0.7, upper CI >0.98), and if, for all pairwise comparisons in the block, the number of pairs in strong LD is at least 95% greater than the number of pairs in weak LD (upper CI < 0.9).

The extent of LD has been shown to vary dramatically among populations [216-217]. On average, blocks are shorter in populations of African ancestry.[209,212,218]. This finding coincides with the 'out of Africa' theory of human history where the African ancestral genome had the longest time interval to mutate and recombine, resulting in shorter regions of high LD [206,219].

Conceptually, by knowing in advance the LD patterns of regions of interest, the minimal number of SNPs that capture the genetic diversity within the

region can be tested to maximize the probability of detecting associations while minimizing costs [198]. The extent of marker coverage for association studies may differ among chromosomal regions; in areas where LD is low, denser distribution of markers are needed to detect associations.

1.5 Genetics of TB

1.5.1 Establishment of genetic component in TB susceptibility in humans

Twin studies conducted to determine if heredity of TB susceptibility exists showed that TB rates among monozygotic twins are significantly higher than those among dizygotic twins (at least 2 fold), indicating that susceptibility to TB is at least partially influenced by one's genetic makeup [220,221].

In addition to twin studies, evidence of genetic susceptibility to TB came from epidemiological observations of outbreaks. The survival of 173 babies in the tragic accident in Lübeck, Germany, in 1926 when 249 babies were vaccinated with live doses of virulent *M. tuberculosis,* instead of *BCG*, clearly demonstrated the presence of innate resistance to *M. tuberculosis* infection [222]. High ethnic-specific TB incidence rates during outbreaks also suggest a genetic component in TB susceptibility. Indigenous populations have been shown to have higher risks of developing TB during outbreaks than their white counterparts[223,224-226].

Although the presence of a genetic component in TB susceptibility is well established, its genetic model is not. Only one segregation study has been done to describe the genetic model of TB, and concluded that TB susceptibility was under the control of two major loci [227]. Failure to identify loci exerting major effects in TB susceptibility questions the likelihood of a 2-loci model mode of inheritance.

1.5.2 Linkage studies

Genome-wide linkage studies allow for screening of genes exerting a strong effect on susceptibility to multifactorial diseases. For TB susceptibility, a genome-wide scan was carried out in a cohort of 173 sib-pairs from the Gambia and South Africa [228]. Two regions on chromosome 15q11 - q13 (LOD score = 2) and Xq26 (LOD score = 1.77) showed suggestive evidence of linkage to TB susceptibility. A total of 15 polymorphisms located in chromosome region 15q11 - q13 were tested for association and 1 variant (7bp deletion) that located to the 5' end of *UBE3A* demonstrated non-random allele transmission to affected offspring ($p = 0.002$) [229]. No gene in the Xq26 region has been identified as being associated with TB susceptibility.

In addition to the comprehensive approach of genome-wide linkage analysis, selective regions have been investigated for non-random segregation with TB in multiplex families. Often, regions of interest are derived from results obtained in animal models. In mice, positional cloning had identified *Nramp1* on chromosome 1 as a host resistance to mycobacteria locus [230-231]. In humans, the syntenic region of mouse chromosome 1q39 locates to region 2q35, and has been shown to non-randomly segregate with pulmonary TB in a large pedigree of aboriginal Canadians [232] and in Brazilian families [227]. Also in the Brazilian families, chromosomal region 17q was shown to be linked to pulmonary TB. Candidate genes in these linked regions include *NRAMP1, IL8RB, NOS2A* and *SCYA2* [227]. The limited findings from linkage studies implied that in humans, genetic effects in TB susceptibility are likely not from genetic factors exerting large population - wide effects. Alternatively, as suggested by the analysis of the TB outbreak in the Canadian family, it is possible that modulating gene – environment interactions (e.g. exposure intensity to mycobacteria) is critical to reveal strong genetic effects [232].

1.5.3 Association studies

The human leukocyte associated antigens (*HLA*) locus, located on chromosomal region 6p21, has been studied extensively for TB susceptibility. The majority of genes located in the *HLA* region have immune functions e.g. they are involved in antigen presentation to T cells and in inflammatory responses [233-237]. In addition to the *HLA* genes, other non – *HLA* genes have been identified to influence TB susceptibility.

1.5.3.1 Natural Resistance Associated Macrophage Protein 1 *(NRAMP1)*

The human *NRAMP1* gene became a plausible candidate gene for TB susceptibility when the mouse *Nramp1* gene was identified as the first mycobacterial susceptibility gene. In mice, *Nramp1* was identified by positional cloning before its function was known. Subsequent sequencing and characterization of the gene in resistant and susceptible mice revealed a glycine to aspartic acid change in amino acid 169 (G169D) to be responsible for conveying susceptibility to a number of pathogens including *Salmonella typhimurium* [238], *Leishmani donovani* [239], *M. lapraemurium* [240], *M. intracellulare* [241], *M. avium* [242] and the vaccine form of *M. bovis* (Bacillus Calmette-Guerin (*BCG*)) [243]. The exchange of glycine for the negatively charged aspartic acid occurs within the putative membrane spanning domain number 4 and has been shown *in vitro* to cause incomplete glycosylation, localized misfolding and delivery failure of the Nramp1 protein to the late endosomes, lysosomes and phagolysosomes of macrophages, with the end result being an absence of the protein [244]. The association of allele G169 with resistance to BCG triggered interest to investigate human *NRAMP1* in TB susceptibility because 1) *M. bovis* is the bovine equivalent of *M. tuberculosis* and 2) *BCG* has been used as a vaccine for TB prevention in humans [226,245]. Hence, if in mice resistance to *M. bovis* infection is determined by the presence of allele G169 of *Nramp1*, then in

humans, resistance to *M. tuberculosis* infection may also be determined / influenced by genetic variants of *NRAMP1*.

To identify the genetic variants of *NRAMP1* responsible for TB susceptibility, many association studies were conducted. A human homologue of the murine variant G169D has not been found, and likely does not exist, yet over 10 other variants have been identified and validated [246-248]. In a population based association study done in the Gambia, 4 polymorphisms (5'$(CA)_n$, 469+14G>C, D543N and 1729+55del4) displayed association with TB [249]. For each of these polymorphisms, the rare alleles increased the risk of developing TB (P-value $\leq$ 0.008), with odd ratios of 1.13-1.85. Further analyses on the Gambian TB patients showed that alleles 1 and 2 of the promoter 5'$(CA)_n$ variant conferred risk to TB while allele 3 offered protection [250]. The 2 polymorphisms at the 5' end of the gene (5'$(CA)_n$ and 469+14G>C) are in strong LD, as are the 2 polymorphisms (D543N and 1729+55del4) at the 3'end of *NRAMP1*. However, LD between the 5' and 3' ends of *NRAMP1* was limited, implying that the 5'and 3' polymorphisms are independently associated with TB susceptibility, and the risk to develop TB increased dramatically for individuals who carried the risk alleles of both the 5'and 3'polymorphisms (OR = 4.07; 95% CI 1.86 - 9.12) [249].

Following the Gambian study, *NRAMP1* polymorphisms have been investigated by various groups in other populations. Confirmations and contradictions exist. The association of the 5' $(CA)_n$ variant with TB has been confirmed in Japanese, Cambodian and South African populations [251-253] A family based association study done in Guinea-Conakry confirmed the association of the 469+14G>C polymorphism with TB [254] and a case control South African study reproduced the finding that the deletion allele of variant 1729+55del4 was associated with TB risk [252]. However, a study conducted among Caucasian TB patients in Denmark failed to detect an association of *NRAMP1* polymorphisms with TB susceptibility, but it observed increased frequencies of the rare alleles of the 5' *NRAMP1* polymorphisms in patients with

microscopy - positive TB as compared to microscopy - negative cases [255]. It is not clear if this finding was confounded by the unbalanced distribution of patients belonging to different racial groups among microscopy - positive and – negative TB cases. The 3' *NRAMP1* polymorphisms associations with TB have been confirmed in Korean and Japanese TB populations; however, studies conducted in Guinea-Conakry, Denmark and Taiwan did not detect any 3'polymorphism association with TB [254-255;256] A study of Cambodian patients detected a protective effect of the rare allele of the D543N polymorphism, which is contrary to the findings in Gambian, Japanese and Korean studies.[249,253,257] Additional findings came from a study of Caucasian TB patients in Houston, US, that observed an excess of the 5'$(CA)_n$ allele 3 in non TB controls compared to patients with pulmonary TB, extrapulmonary TB and HIV / TB ($p = 0.004$) [258]. Within the different clinical forms of TB, the Houston study observed a significant increase of the 5'$(CA)_n$ allele 2 in extrapulmonary as compared to pulmonary cases. This observation raised the enticing possibility that an effect of *NRAMP1* on TB susceptibility may be more pronounced among extrapulmonary cases of TB.

The notion that *NRAMP1* variants are genetic risk factor in TB susceptibility is mostly agreed upon. It is difficult to envision that *NRAMP1* polymorphisms are in LD across racial boundaries with unknown disease causing polymorphisms in a gene other than *NRAMP1*. The only gene that one could suspect of being in LD with *NRAMP1* is *NLI-IF* [259] and a contribution of this gene to TB susceptibility has been ruled out [259]. What is not clear is the specificity of the causal relationship between these *NRAMP1* risk factors and TB disease. Are the genetic effects of *NRAMP1* more pronounced for specific forms of TB? What gene – environment interactions underlie disease pathogenesis? For example, the findings that *NRAMP1* variants are associated with microscopy - positive TB and extrapulmonary TB suggest that *NRAMP1* is involved in the control of bacillary growth and possibly dissemination rather than susceptibility to TB *per se* or susceptibility to infection with *M. tuberculosis* [258]. By modeling the mode of

inheritance with exposure intensity to *BCG* and / or *M. tuberculosis* in a linkage study of a large Aboriginal Canadian family, the finding suggests that *NRAMP1* may act as a progression gene; that it modulates the rate of developing TB symptoms from infection [232].

The exact function of the human NRAMP1 protein is unknown; however, the high degree of similarity of the human protein to its mouse homologue suggests that NRAMP1 may function as a divalent cation transporter [260]. In mice, the gene encodes a transmembrane protein that is found at the late endosomal / lysosomal compartment of macrophages [261-262]. Kinetic studies have shown that Nramp1 transports cations out of the phagosomes, and consequently mediates depletion of nutrients potentially essential for survival of pathogens in the host cell phagosomes [263-264]. The depletion of divalent cations from the phagosomes has also been shown to up - regulate various macrophage functions designed to combat an infection, such as regulation of IL-1β, nitric oxide and MHC class II antigen expression [265]. Furthermore, macrophages carrying the D169 allele demonstrate suboptimal antigen processing and presentation, resulting in a shift of the immune system towards a Th2 response when infected [266-269]. However, a similar effect on the Th1 / Th2 paradigm has yet to be been shown in humans.

1.5.3.2 Vitamin D Receptor *(VDR)*

Active 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), upon binding to vitamin D receptor (VDR) can modulate the neural, immune, and endocrine systems. Activities modulated by $1,25[OH]_2D_3$ include calcium and phosphorous homeostasis, apoptosis, and cell differentiation [270-271]. The immunoregulatory function of $1,25[OH]_2D_3$ is of particular relevance to the pathogenesis of infectious and immune – mediated diseases. $1,25[OH]_2D_3$ has been shown to suppress T cell proliferation [272], inhibit IFN - γ and IL – 2 production [273], and enhance the generation of Th2 cells [274]. The pleiotropic roles of $1,25[OH]_2D_3$ rendered its receptor to be the target for association studies of numerous diseases in humans.

There are 4 variants which have been widely studied for disease associations and are given the common aliases of *Fok*I, *Bsm*I, *Apa*I and *Taq*I. These genetic variants have been found to be associated with serum osteocalcin levels [275], bone mineral density [276-279], prostate cancer [280], hyperparathyroidism [281], insulin-dependent diabetes mellitus [282-283], Crohn's disease [284], leprosy [285], immunodeficiency syndrome [286] and asthma/atopy [287-288].

In particular, associations of *VDR* genetic variants and TB susceptibility have been explored in various epidemiological settings [289-294]. Of the 7 cohorts reported in 6 TB susceptibility studies, 5 concluded associations with *VDR* variants, but no agreement on the location and identity of the risk variant(s) emerged from these studies. At the translation start site, variant *Fok*I is a thymidine to cytosine nucleotide change resulted in the elimination of a methionine start site, and the encoded protein is shortened by 3 amino acids [295-296]. The *Fok*I variant has been tested in 5 cohorts for TB susceptibility, and 3 detected associations. However, the risk genotype and phenotype associated differed. For example, in a Chinese study the *Fok*I "TT" genotype was over represented in pulmonary TB patients [293], whereas in a West African study, it was the *Fok*I"C" allele that was found to be associated with risk, but only in the presence of the *Apa*I "T" allelic variant [292].

Association discrepancies are also observed for variants at the 3' end of *VDR*. Genotype *Taq*I "CC" was found to be associated with TB protection in a Gambian study [291], while in another West African study, no such association was observed for *Taq*I alone, but global association was detected in combination with other variants [292]. In a small Indian study, a different genotype, *Taq*I "TT", was found to be associated with TB protection in female subjects [290]. In a Gujurati Asian study, in the presence of 25-hydroxycholecalciferol deficiency, genotypes *Taq*I "TT" and *Taq*I "TC" were found to be associated with TB susceptibility [289].

One possible explanation for the inconsistent results across studies is

that these markers are not causative themselves, but in LD with the biological causative variants. High-resolution analysis of LD in the *VDR* gene demonstrated that the gene can be partitioned into 3 blocks of high LD [297]. The *Fok*I variant does not belong to any block, hence if the causal variant(s) resides upstream or downstream of the translation start site, no association would be detected with *Fok*I. While the other 3 widely studied variants at the 3'end belong to one block, it is noteworthy that they do not capture all the information of other common SNPs in the block. In other words, if the causative variant(s) resides at the 3'end, analysis of the variants *Bsm*I, *Apa*I and *Taq*I may not be sufficient to detect associations.

1.6 Genetics of asthma

1.6.1 Establishment of a genetic component in the development of asthma

Incidence of asthma was shown to be aggregated in families where the probability of an individual developing asthma is at least 3-fold higher if a sibling or a parent has asthma [298-299]. Further supporting heredity for asthma, twin studies showed that monozygotic twins are more likely to develop asthma (2-3 fold) if the other twin is asthmatic, compared to dizygotic twins, and segregation analyses estimated that the heritability for asthma is between 40% to 80% for Caucasians [167,300-308]. These segregation analyses have come to varying conclusions regarding the pattern of inheritance: mixed models, co-dominant inheritance, single locus contribution, multiple loci contribution and multifactorial (gene-environment) interactions.

1.6.2 Genome wide linkage and candidate gene association studies

Seven genome screens using asthma as a dichotomous trait have been conducted [310-318]. A total of 19 chromosomes have been found to be linked to asthma and 5 regions (1p, 5q, 6p, 8p and 12q) have been identified in more than

one study. In addition to testing linkage with asthma as a dichotomous trait, several other genome wide screens have located other regions linked to asthma-related phenotypes such as serum IgE level, bronchial hyper responsiveness and atopy. Due to the large number of linkage regions detected in genome screens, and the complexity of immunological pathways involved in asthma, more than 500 loci have been tested in genetic association studies [319-320]. Over 20 genes have been found associated with asthma – traits in two or more studies. Assuming that the hygiene hypothesis is valid, known asthma genes with immunoregulatory functions are strong candidates for TB risk factors.

1.6.2.1 Interleukin 4 (*IL–4*) and IL-4 Receptor (*IL4R)*

The 5q31 chromosomal region harbours a cluster of cytokine genes (IL-4, IL-5, IL-9 and IL-13), and has been shown to be linked to asthma in US and Japanese genome wide screens [311,315]. IL-4 has generated great interest in asthma / atopy pathogenesis since it has been shown to induce immature T effectors cells (Th0) to express Th2 phenotypes while reducing Th1 cell signals [24], and to trigger B cells to produce IgE and IgG4 [321-323]. Sensitized mice deficient in IL-4 showed no allergic symptoms when challenged with allergen [324]. However, when CD4+ T cells were transferred to these IL-4 deficient mice, they developed allergic phenotypes when challenged [325]. In addition, mice that normally develop allergic inflammation upon exposure to allergen failed to do so when their IL-4 cytokines were neutralized [326]. Hence, *IL-4* is a strong candidate gene for asthma / atopy genetic studies.

In humans, two *IL-4* promoter polymorphisms (-590C>T and -33C>T) have been studied extensively for associations with asthma and related phenotypes [327-333]. Variant –590C>T (i.e. a C to T base change at position –590 in the promoter region) has been shown to influence IL4 transcriptional activity by creating an extra binding site for the nuclear factor for activated T cells (NFAT) [327,334]. A German study observed that variant –590C>T was associated with asthma in school children ($p = 0.003$) [335]. Serum IgE level was also found to be

associated with this polymorphism (p = 0.01) in children with positive skin prick test responses.

IL4 exerts its effect through binding to its receptor IL4R, which consists of 2 subunits (IL4Rα and common (c) γ). Genetic variants of *IL4RA* have been shown to be associated with asthma and related phenotypes. A base change (A>G) at nucleotide position 1902 changes the amino acid from glutamine to arginine at codon 576 of *IL4RA* (alias Q576R). This variant has been found to be associated with atopic asthma in a British Caucasian cohort with the common allele, Q576, being the risk allele (p = 0.018) [336]. The rare allele R576 has been found to be associated with lower IgE level in a German study (p = 0.002) [337]. However, in a US cohort, it was allele R576 that was associated with atopy [338]. Furthermore, no such association was observed in either Japanese, Italian or 2 other German study populations [339-342]. There were other studies which failed to identify Q576R as a risk factor for atopy, but continued to detect association with haplotypes including this variants [343-344]. Taken together, in addition to Q576R, there are likely other causative variants residing in the region.

1.6.2.2 *HLA*

The chromosomal region of 6p21 has been found to be linked to asthma in at least 4 genome screens, making it the most replicated region. *TNFA* presents itself as a strong candidate in asthma development by being involved in the proinflammatory response [345-346] and by being found abundantly in bronchoalveolar lavage fluid, serum and bronchial submucosa of asthmatics [347-348]. A promoter variant at position –308 relative to the transcription start site has been found to be associated with asthma. Three of the 4 studies reporting association of the *TNFA*-308G>A polymorphism with asthma observed the rare allele (–308A) [349-351] to be the risk allele for asthma, whereas one Australian study observed the opposite allele –308G as the risk allele in children (p = 0.004) [352]. Yet, in other populations (Chinese, Belgian Caucasian, Czech and Italian),

no association with the –308G>A variant was detected [353-357]. In 13 functional studies, 4 reported an increase of LPS stimulated TNFα production in individuals with the –308A allele [358-360], while the remaining studies found no correlation [361-366]. Several studies have focused on detecting transcriptional differences between the –308 alleles. One study showed no difference in transcriptional activity, regardless whether the 3'UTR was present in the constructs or not [367] while another study showed that allele A drives a higher transcription than allele G, but only in the presence of the 3'UTR in the constructs [368-369]

Also known as the tumour necrosis factor β gene (*TNFβ*), the lymphotoxin α gene (*LTA*) locates immediately upstream and telomeric of *TNFα*. Immunologically, LTA is involved in early immune and inflammatory responses [370]. An intronic G>A SNP, located at nucleotide position 252 relative to the transcription start site (+252G>A), was found to be associated with asthma. In two Australian cohorts, the +252AA genotype increased the odds of asthma 5 folds in children ($p = 0.019$) in one cohort [352], while the opposite allele (252G) was found to be associated with asthma risk ($p = 0.005$) in the second cohort [349]. No association was observed in a Japanese children study [371] and in an multi-ethnic American cohort [351]. The functional significance of this polymorphism is controversial; some studies showed that the haplotype bearing the 252G allele induced greater LTA protein production [372-374], while no such increase was observed in other studies [375-376].

1.7 *M. tuberculosis* or asthma

As developing countries continue to thrive for economic and social progress, they will face the health burden of immune – mediated disorders, as experienced by their developed counterparts. The hygiene hypothesis connects the reverse trends of prevalence between infectious and immune – mediated diseases, and proposes that the absence of microbial exposure infection in

developed countries is responsible for the rise of immune – mediated diseases. Assuming the hygiene hypothesis is valid, the search for such infective agents becomes a crucial step to combat this class of chronic diseases including asthma. Asthma already affects 150 million people worldwide and resulting in 180 000 annually. To take advantage of the availabilities of a vast number of genetic markers and high throughput genotyping technologies, we hope to identify *M. tuberculosis* as one such infective agent by evaluating its possible relationship with asthma at the molecular level.

Chapter 2

Association of Vitamin D Receptor Genetic Variants with Susceptibility to Asthma and Atopy

In the comparative genetic study of TB and asthma susceptibility, the first TB susceptibility gene to be investigated in detail for its role in asthma susceptibility was *VDR*.

Association of Vitamin D Receptor Genetic Variants with Susceptibility to Asthma and Atopy

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ABSTRACT

Genome scans for asthma have identified suggestive or significant linkages on 17 different chromosomes, including chromosome 12, region q13-23, housing the vitamin D receptor gene. Through interaction with vitamin D receptor, 1,25-dihydroxyvitamin D_3 mediates numerous biological activities; such as regulation of helper T cell development and subsequent cytokine secretion profiles. Variants of the vitamin D receptor have been found to be associated with immune-mediated diseases that are characterized by an imbalance in helper T cell development, such as Crohn's disease and tuberculosis. The vitamin D receptor, hence, is a good candidate to be investigated for association with asthma, which is characterized by enhanced helper T cell type 2 activity. Here, we examined vitamin D receptor genetic variants in an asthma family-based cohort from Quebec. We report six variants to be strongly associated with asthma and four with atopy ($0.0005 \leq p \leq 0.05$). Analysis of the linkage disequilibrium pattern and haplotypes also revealed significant association with both phenotypes ($0.0004 \leq p \leq 0.01$). The findings have been replicated in a second, but not in a third cohort, by another research team. These results identify vitamin D receptor variants as genetic risk factors for asthma/atopy and implicate a non human leukocyte antigen immunoregulatory molecule in the pathogenesis of asthma and atopy.

Number of words: 199

Key words: VDR, polymorphism, genetic predisposition

INTRODUCTION

The interaction of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) with the vitamin D receptor (VDR) modulates many biological activities of the neural, immune, and endocrine systems, including calcium and phosphorous homeostasis, apoptosis and cell differentiation (reviewed by [1;2]). Once bound to $1,25(OH)_2D_3$, VDR binds to specific DNA sequence elements in vitamin D responsive genes, termed vitamin D receptor response elements (VDRE), to influence the rate of RNA polymerase II-mediated transcription [3-5]. Vitamin D-dependent rickets and osteomalacia are classical manifestations of vitamin D deficiency (OMIM 601769).

Abnormalities related to the pleiotrophic functions of VDR underlie the pathogenesis of several diseases. Table E.1 in the online supplement summarizes results of association studies of four *VDR* variants (*Fok*I, *Bsm*I, *Apa*I and *Taq*I) with serum osteocalcin levels, bone mineral density, prostate cancer, hyperparathyroidism, insulin-dependent diabetes mellitus, Crohn's disease, leprosy, tuberculosis (TB) and acquired immunodeficiency syndrome [6;7-13]. Among the biological effects of ligand-bound VDR, its influence on helper T (Th) cell development [14] is of particular interest for diseases involving the immune system. In the mouse, $1,25(OH)_2D_3$ has been shown to inhibit Th1 development and interferon-gamma production and to stimulate Th2 cell development and the production of interleukin 4 and interleukin 10 [15-17]. In humans, although vitamin D has been shown to inhibit Th1 responses, Th2 enhancement has not been demonstrated [18]. However, several of the associated phenotypes in Table E.1 are characterized by an imbalance in Th1/Th2 cell activity, e.g. acquired immunodeficiency syndrome progression [19;20], tuberculoid leprosy [12], lepromatous leprosy [12], Crohn's disease [11] and tuberculosis [13;21-23]. Owing to the well known immunoregulatory role of VDR and its known association with several immune-mediated diseases, *VDR* presents itself to be a candidate gene for asthma susceptibility. Since asthma is characterized by a shift of Th cell

responses towards type 2, we hypothesized that VDR may function as a regulator of asthma and atopy susceptibility. We investigated this hypothesis through the characterization of genetic variants of *VDR* in an asthma family-based cohort from northeastern Quebec.

Some of the results of this study have been previously reported in the form of an abstract (Poon A, et al., Comparative Genetic Study of Tuberculosis and Asthma Susceptibilities [abstract]. Am J Hum Genet 2003; 73:S385.) [24]

METHODS

Cohort Description

Families are from the Saguenay-Lac-St-Jean region of northeastern Quebec, Canada. Probands were recruited if they fulfilled at least two of the following three criteria: 1) a minimum of three clinic visits for acute asthma within one year; 2) two or more asthma-related hospital admissions within one year; or 2) steroid dependency, as defined by either six months of oral, or one year of inhaled corticosteroid use. Families were included for study if at least one parent was available for phenotypic assessment, at least one parent was unaffected, and all four grandparents were of French Canadian origin. When possible, grandparents and other relatives were also recruited to the study.

After recruitment of probands and their family members, the affection status of all study participants was determined by clinical evaluation and the completion of a standard respiratory questionnaire that was modified to include questions about asthma and atopy severity, family history of asthma and/or atopy, age-of-onset and the presence of other respiratory failure diagnoses[25]. In 41 cases, the age-of-onset described by parents was below 2 years; because of the uncertainty of this information, we used a default class of 2 years. We defined participants as asthmatics if (1) a reported history of asthma (questionnaire- based) and a history of physician-diagnosed asthma (past/current) were available, or (2) confirmation by a positive methacholine provocation test done only on subjects older than 12 years of age. See online supplementary methods section for description of clinical tests performed. Subjects were deemed atopic if they had at least one positive response (wheal diameter $\geq$ 3 mm at 10 min) to skin-prick tests. The family participation rate was about 60% and all subjects gave informed consent. A total of 223 independent families (1139 individuals) with family size ranging from 3 to 17 and the number of affected family members (including probands) ranging from 1 to 10 were analyzed.

Single Nucleotide Polymorphism (SNP) Selection and Genotyping

We investigated 93 kilobases (kb) of genomic DNA harboring *VDR*, spanning from chromosome 12 position 46586093 to 46492363 on build 34 hg16 genome assembly released by the National Center for Biotechnology Information (NCBI). An initial panel of 20 SNPs was selected from public databases (NCBI and The SNP Consortium) based on 1) location in the gene, 2) relative distances to each other, 3) compatibility with the genotyping methods employed, which is dependent on types of base change and flanking sequences, and 4) the known association with diseases. A final collection of 12 SNPs was tested for association. The remaining 8 SNPs were discarded due to low information content or unreliable genotyping data. SNPs described in this report are cited using their reference SNP identifier (rs#) from the NCBI database, except for *VDR* SNPs having commonly used aliases (*Apa* I, *Bsm*I, *Fok*I, and *Taq*I).

SNP genotyping was performed using SNPstream® UHT (Orchid Biosciences, Princeton, NJ) [26]. All protocols, and reaction conditions are available at this journal's online repository. See Table E.3 in the online data supplement for oligonucleotides used in genotyping and Table E.4 for those used in sequencing.

Statistical Analysis

Hardy-Weinberg equilibrium was tested in a subset of DNA samples using MERLIN [27]. These samples correspond to parents of probands whose DNA are available. These samples are independent, so that unbiased estimates of Hardy-Weinberg equilibrium for the variants can be obtained.

Allele distribution patterns were assessed by the family based association test (FBAT version 1.4) [28;29]. This software uses an empirical variance – covariance estimator to account for the possibility of non-independent allelic transmission to affected sibs [30]. Asthma and atopy phenotypes were tested separately under an additive genetic model.

Associations between *VDR* variants were assessed. Strength of linkage disequilibrium (LD) between pairs of SNPs was measured as D prime (D') [31], using HAPLOVIEW (http://www.broad.mit.edu/personal/jcbarret/haplo/documentation.php). Regions of strongly associated markers (LD blocks) were inferred and modified from the definition proposed by Gabriel et al. [32] as implemented in HAPLOVIEW. Specifically, the parameter confidence interval minima for strong LD was relaxed from the Gabriel et al. definition and upper confidence level was set from 0.98 to 0.90.

Haplotype-specific associations were investigated using the 'hbat' command implemented in FBAT (version 1.4) [28;29]. An empirical variance estimator was used [30]. Asthma and atopy phenotypes were tested separately under an additive genetic model.

VDR Resequencing

The promoter region and all exons were sequenced to detect novel coding SNPs using ABI PRISM BigDye Terminator (version 2) kit on an ABI 3700 DNA sequencer (Applied BiosSystems, Foster City, CA) as described elsewhere [33]. The protocols, reaction conditions and primers used are described in this issue's online data supplement and at http://www.genomequebec.mcgill.ca/VDR.

RESULTS

Patient Characteristics

Clinical characteristics were obtained for 1139 individuals (Table 1). At the time of recruitment, study participants were aged 3 to 88 years. There were 570 asthmatics of which 223 are probands. The median age of onset for cases and their affected siblings is 5 years (2 – 46 years). We note that 419 (74%) of the 570 asthmatic and 218 (38%) of the 569 non-asthmatic subjects were atopic. The sex ratio (male:female) for probands is 1:1.2, for affected family members is 1:1.4 and for unaffected family members is 1:1.2. Compared to their affected family members, the asthmatic probands had higher immunoglobulin E levels and co-existence of atopy.

Genetic Analysis of the *VDR* Locus

A final panel of 12 SNPs was used from an initial collection of 20 obtained from public databases for *VDR* characterization and association testing (see table E.2 for SNPs characteristics). Of the 8 discarded SNPs, 7 are uninformative, with minor allele frequencies <0.025, and 1 SNP failed the genotyping assay. Among the 12-SNP panel are four widely studied variants: *FokI* C>T (rs2228570) [13;22;34], *ApaI* A>C (rs7975232) [35;36], *Bsm*I G>A (rs1544410) [7;37-40], and *TaqI* C>T (rs731236) [13;21;23]. Of the 12 SNPs, *Fok*I and *Taq*I reside in the coding region while the remaining 10 SNPs locate in non-coding regions; between 4.8 kb upstream of the translation start site and 32 kb downstream of exon 9 (Figure 1A).

Of the 12 SNPs, only rs2239182 gave a significant deviation from Hardy-Weinberg equilibrium ($p = 0.039$); given the number of SNPs tested, this deviation can be expected to occur by chance alone.

Table 1 : Clinical Characteristics of Subjects

	Families Probands (n=223)	Members affected (n=347)	Members unaffected (n=569)
Male:Female Ratio	1: 1.2	1: 1.4	1 : 1.2
Mean age in years (range)	18 (3-46)	40 (2-83)	48 (3-96)
Median age in years	16	41	48
Mean age of onset in years (range)	7.8 (2-46)	7.5 (2-75)	Not applicable
Median age of onset in years	5	5	Not applicable
Smoking Status			
Never	186 (83.4%)	176 (50.7%)	243 (42.7%)
Ex-smoker	12 (5.4%)	105(30.3%)	199 (40%)
Smoker	25 (11.2%)	66 (19%)	127 (22.3%)
FEV_1 as % predicted (SD)*	92.2 (16.3)	88.9 (22.7)	98.9 (17.01)
PC_{20} in mg/ml (SD) †	2.66 (3.33)	3.36 (4.53)	26.91 (3.04)
Serum IgE in mg/l (SD) †	229.09 (4.61)	157.4 (4.55)	80.9 (3.72)
Number of persons with Subphenotypes			
Asthma	223 (100%)	347 (100%) ‡	not available
Atopy	182 (82%)	237 (68.3%)	218 (38.3%)
AHR §	169 (90%)	217 (82.2%)	64 (11.2%)
IgE >100mg/l	141 (63%)	185 (53.3%)	155 (27.2%)
IgE >280mg/l	95 (43%)	106 (30.5%)	70 (12.3%)

* FEV_1 = Forced expiratory volume in one second

† Geometric mean; PC_{20} = provocative concentration of methacholine inducing a 20% fall in FEV_1

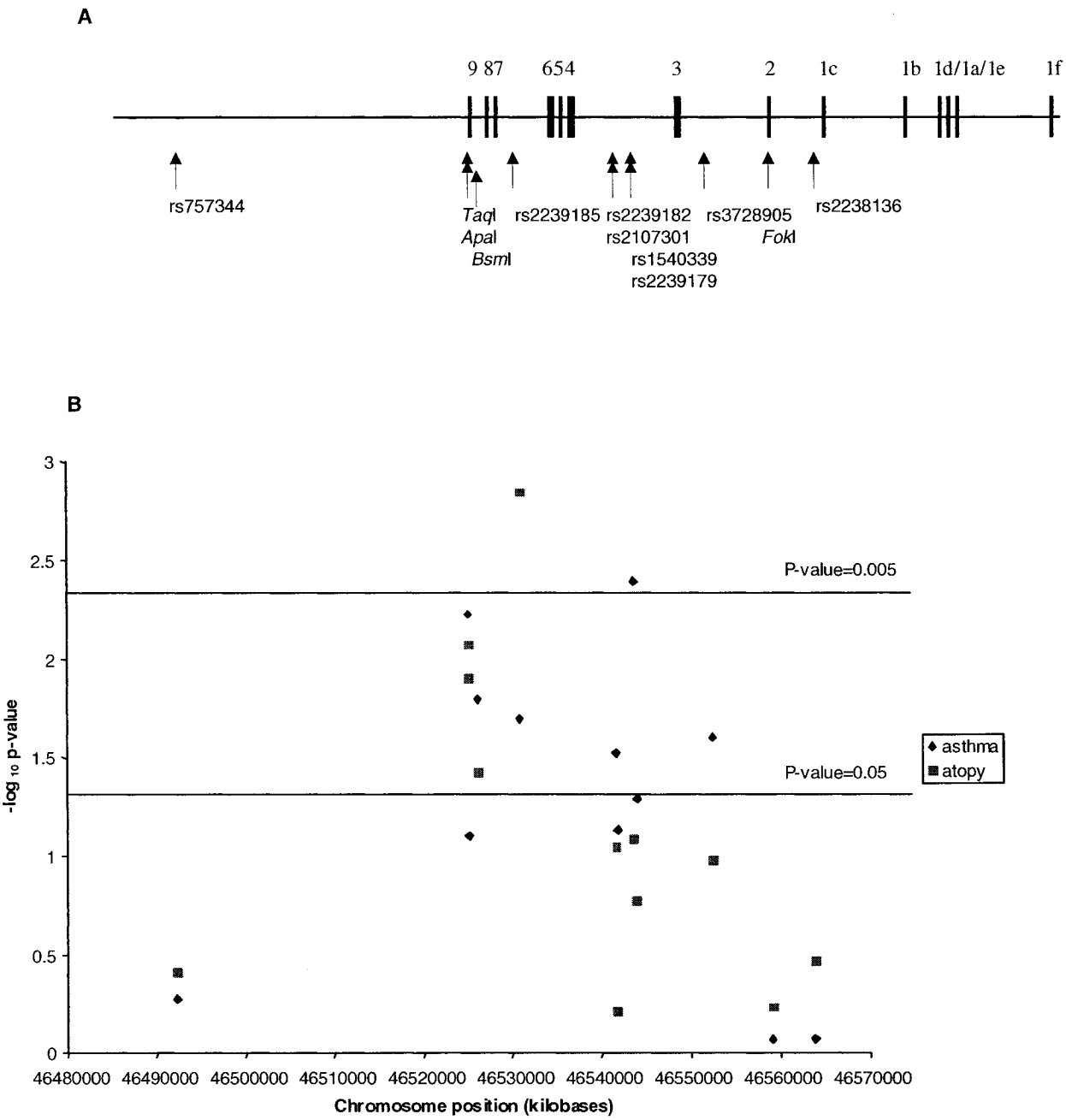
‡ Present asthma or past documented clinical history of asthma

§ AHR = Airway hyperreactivity according to American Thoracic Society criteria

Evaluated for 188 probands, 486 unaffected family members and for 264 affected family members.

Figure 1. A) Genomic organization of *VDR* and B) association plot between *VDR* variants and the two phenotypes: asthma and atopy. Exons are represented by black boxes connected by a straight line representing introns and 3'and 5' noncoding regions. Locations of exons 1a-1f are described elsewhere[54]. Positions and names of the twelve SNPs analyzed are represented by arrows below the gene structure. B) Significance of association given as $-\log_{10}$ (p-value) is plotted against relative SNP position given in kilobases. Three levels of significance (p=0.05 and 0.005) are indicated by straight lines drawn across the plot. Association with asthma is depicted by blue diamonds, and atopy by pink squares. Chromosome positions are based on the July 2003 freeze of the University of California Santa Cruz genome browser (http://genome.ucsc.edu/).

Figure 1


A
9 87
654
3
2
1c
1b
1d/1a/1e
1f
rs757344
TaqI
ApaI
BsmI
rs2239185
rs2239182
rs2107301
rs1540339
rs2239179
rs3728905
FokI
rs2238136
B
3
2.5
2
1.5
1
0.5
0
-log 10 p-value
P-value=0.005
P-value=0.05
asthma
atopy
46480000
46490000
46500000
46510000
46520000
46530000
46540000
46550000
46560000
46570000
Chromosome position (kilobases)

Family-based Association Analysis of *VDR* with Asthma and Atopy

The 12 SNPs were tested individually for association with asthma and atopy (Figure 1B). In the absence of *a priori* evidence for transmission models at this locus, we tested allelic associations under an additive genetic model. Six alleles (rs3782905C, rs1540339A, rs2239182A, rs2239185C, *Bsm*IG, *Taq*IT) and four alleles (rs2239185C, *Bsm*IG, *Apa*IC and *Taq*IT) were significantly overtransmitted to asthmatic and atopic offspring ($p<0.05$), respectively.

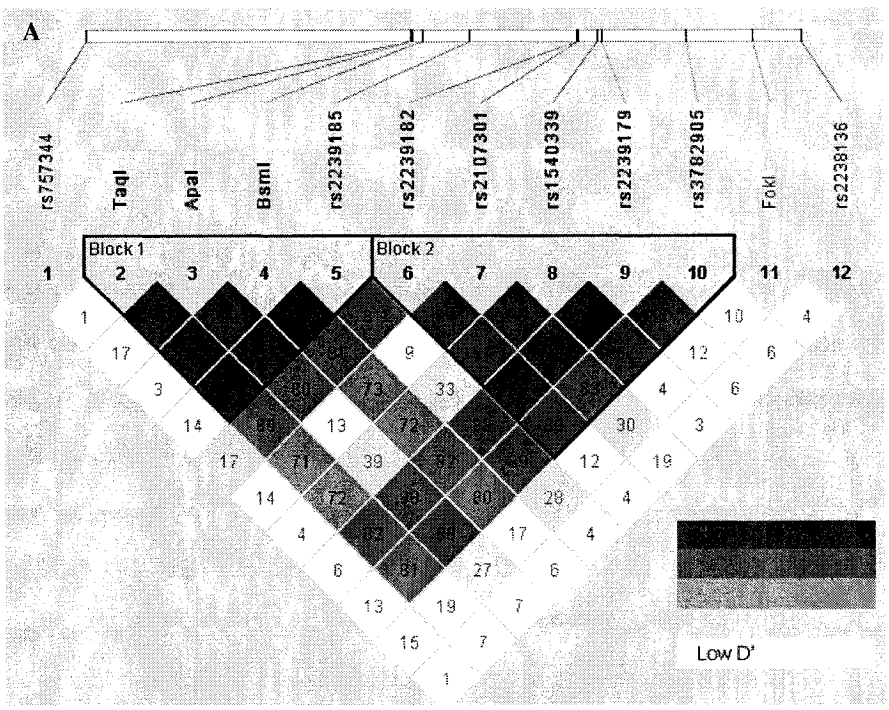
IgE data were collected and analyzed both as quantitative and dichotomous traits, independent of atopy status. In a dichotomous trait model, subjects can be classified as either high or low responders according to their IgE levels. In this cohort, log (IgE) values were normally distributed and a cut-off point of 100mg/L divided the subjects into low (2/3 of participants) and high (1/3) responders. Under an additive model, 3 alleles (rs2239185C , *Apa*IC and *Taq*IT) are associated with being a high responder at $0.006<p<0.02$. The alleles associated with high IgE are the ones also seen associated with asthma and atopy. As a quantitative trait, the phenotype investigated is high IgE responder. When regressed on age and sex, 2 alleles (rs2239185C and *Taq*IT) are associated at $p<0.005$.

Intragenic LD Structure of *VDR*

Associations among the 12 SNPs were assessed by measuring pairwise LD using D'(Figure 2A). The two flanking upstream SNPs (rs2238136, *Fok*I) and the most downstream SNP (rs757344) are not in significant LD ($D'\leq 0.30$) with any other marker tested. Thus, the LD of the 2 central *VDR* blocks is unlikely to have extended to these SNPs and they are likely to be outside the core *VDR* blocks. The remaining 9 SNPs are distributed within a 28kb region, between intron 2 and

Figure 2. A) Pairwise linkage disequilibrium pattern of *VDR* measured by D' and B) the common haplotypes of the 28 kilobases linkage disequilibrium region. The location of each tested SNP along the chromosome is indicated on top. The number in each diamond indicates the magnitude of LD (D'x 10^{-2}) between respective pairs of SNPs. For example, the pairwise D' for SNPs rs1540339 and rs2107301 is 0.96. Squares without D' written on them represent perfect LD (D'= 1.0). Strength of LD is depicted by progression of color, for all D' with LOD >2, the color moves from red to light blue as D' runs from 1 to 0; for D' with LOD <2, it is represented by white. B) Common haplotypes of the two blocks are listed with their frequencies within parentheses. Thick lines joining haplotypes from each block represent combined haplotypes with frequency > 0.1 and thin lines for frequency < 0.01.

Figure 2



B

Block 1	Block 2
T C G C (0.448)	A T A A C (0.265)
C A A T (0.390)	G C G G G (0.287)
T A G T (0.140)	A C G A C (0.163)
	A C A A C (0.111)
	G C G G C (0.096)
	G C G A C (0.035)
	G C A A C (0.017)
	A T A A G (0.012)

Frequency > 0.10
Frequency < 0.01

exon 9 of *VDR*, and are in strong LD (D'≥0.8) with at least one additional SNP (80% of the pairwise LDs are D'≥0.80). Of the 36 pairwise LDs calculated between these 9 SNPs, 8 D's are low (0.09≤D'≤0.73), and they further separate the region into 2 blocks of tightly associated SNPs. We relaxed Gabriel's criteria for haplotype block definition: the outer-most marker pair was required to be in LD with an upper confidence limit that exceeds 0.90, and a lower confidence limit that exceeds 0.7. Block 1 locates towards the 3'end of *VDR* and consists of SNPs *Taq*I, *Apa*I, *Bsm*I, and rs2239185, while block 2 locates towards the 5' end of *VDR* and comprises SNPs rs2239182, rs2107301, rs1540339, rs2239179 and rs3782905. Block 1 spans about 5.8 kb and block 2 spans roughly 8.4 kb. The 2 blocks which are separated by 10.8kb, show moderate LD between blocks (D'=0.77, data not shown). Three common haplotypes (frequency>0.1) are observed within block 1: haplotypes TCGC (frequency = 0.45), CAAT (0.39) and TAGT (0.15) and within block 2, four common haplotypes are observed: haplotypes GCGGG (0.29), ATAAC (0.27), ACGAC (0.16) and ACAAC (0.11). Three common haplotypes of the 9 SNPs that extended across the 2 blocks were observed: (3'to 5' SNPs) CAATGCGGG, TCGCATAAC and TCGCACGAC (Figure 2B).

Haplotype-Specific Association Analysis

FBAT results and LD patterns indicate that associations between *VDR* variants and asthma/atopy occur across the two blocks within a 28kb region. To characterize haplotype transmission in this region, 10 common haplotypes previously inferred using HAPLOVIEW were assessed for non-random transmissions using FBATv1.4 [28;29] (Figure 3). For asthma, 5 of the 10 common haplotypes show non-random distribution of haplotypes. In block 1, the non-randomly transmitted haplotypes were TCGC (overtransmitted, p = 0.007) and CAAT (p = 0.02, undertransmitted). In

Figure 3. Haplotype transmission patterns for (A) asthma and (B) atopy. E(S) = expected FBAT statistic; S = FBAT statistic; Z = Z score

A

Taq I	*Apa* I	*Bsm* I	rs2239185	rs2239182	rs2107301	rs1540339	rs2239179	rs3782905	S*	E(S)†	Z‡	p-value
				G	C	G	G	G	98.30	109.50	-1.69	NS§
				T	T	T	T	T	120.93	100.13	2.89	0.004
				A	C	G	A	C	56.63	66.00	-1.50	NS
				A	C	A	A	C	38.00	31.70	1.63	NS
C	A	A	T						89.49	107.20	-2.40	0.02
T	A	G	T						56.50	50.02	1.16	NS
T	T	T	T						145.85	125.86	2.71	0.007
C	A	A	T	G	C	G	G	G	68.27	83.46	-2.65	0.008
T	T	T	T	T	T	T	T	T	59.82	44.08	2.83	0.005
T	C	G	C	A	C	G	A	C	40.96	44.97	-0.83	NS

B

Taq I	*Apa* I	*Bsm* I	rs2239185	rs2239182	rs2107301	rs1540339	rs2239179	rs3782905	S	E(S)	Z	p-value
				G	C	G	G	G	90.33	98.31	-1.24	NS
				A	T	A	A	C	115.96	104.60	1.57	NS
				A	C	G	A	C	61.43	66.99	-0.84	NS
				A	C	A	A	C	41.20	34.93	1.40	NS
C	A	A	T						86.48	99.64	-1.67	NS
T	A	G	T						53.52	60.03	-1.00	NS
T	T	T	T						172.85	144.44	3.56	0.0004
C	A	A	T	G	C	G	G	G	61.23	72.62	-2.20	0.03
T	T	T	T	T	T	T	T	T	58.88	44.35	2.53	0.01
T	C	G	C	A	C	G	A	C	48.96	49.95	-0.18	NS

Overtransmitted Haplotypes (p-value <0.05)
Undertransmitted Haplotypes (p-value <0.05)

S* = FBAT statistic

E(S)† = Expected S

Z‡ = Z - score

block 2, haplotype ATAAC was overtransmitted (p = 0.004). In the combined block, haplotype TCGCATAAC was overtranmitted (p = 0.005) and haplotype CAATGCGGG was undertransmitted (p = 0.008).

For atopy, 3 of the 10 common haplotypes show non-random distribution of haplotypes. In block 1, haplotype TCGC was overtransmitted (p = 0.0004). In the combined block, haplotype TCGCATAAC was overtransmitted (p = 0.01) and haplotype CAATGCGGG was undertransmitted (p = 0.03).

VDR sequencing

In order to exclude the presence of a coding *VDR* polymorphism that was not seen in previous analyses of *VDR*, we sequenced the proximal promoter region housing the 6 alternatively spliced exons 1 (1a-1f), exons 2-9 and intron/exon boundaries of *VDR* locus from genomic DNA obtained from 24 cases; the selection was based on their haplotype diversity. A total of 15 SNPs were identified, including *Fok*I, *Apa*I, and *Taq*I. The remaining 12 SNPs were only seen in non coding regions; these SNPs are listed in Supplementary Table E.5.

DISCUSSION

Using a family-based cohort, we observed association between common allelic variants of *VDR* and phenotypes of asthma and atopy in a French-Canadian founder population. Six SNPs, in introns 2, 3, 6, 8 and exon 9 of *VDR* spanning about 28kb of genomic sequence, were associated with asthma. These markers fall into 2 haplotype blocks, with very substantial LD among markers of both blocks. With our present LD pattern obtained from 12 SNPs, the precise boundaries of the 2 blocks are unknown, and additional SNPs might be needed to better represent the LD pattern at this locus. Nevertheless, significant non-random segregation of marker haplotypes spanning this 28kb region of *VDR* further confirmed association of this region with asthma and atopy.

Interestingly, the same 28kb haplotypes spanning both core *VDR* LD blocks are associated with asthma and atopy. If considering the blocks individually, the same haplotypes show the same direction of over or undertransmission, but some of these haplotypes do not reach statistical significance for association with one of the phenotypes. At this point, the current level of information is not sufficient for us to assign a greater likelihood of association for any of the 2 haplotype blocks to either of the phenotyes. Given the high clinical correlation between both phenotypes and the similarity of their haplotype associations, we believe it likely that there is one or more functional variants at the 3'end of the *VDR* locus responsible for susceptibility to both asthma and atopy.

In a separate association study of *VDR* variants described in a companion article (Raby and colleagues) [41], association with asthma was evaluated in two study populations: a family-based cohort and a case control cohort. The family cohort is part of the Childhood Asthma Management Program (CAMP) study [42;43], with families being recruited from 8 centres across North America

(www.jhucct.com/camp/open/sites.htm). The case control cohort is part of the Nurses' Health Study [44] and only included women.

In the Nurses' Health Study cohort, 4 of the 5 SNPs tested are associated with asthma (Table 3). Of these 4 SNPs, 3 are with the same alleles found to be associated in the Quebec cohort. For the remaining associated SNP *Apa*I, the C allele was overtransmitted in both the Quebec and the Nurses' Health Study cohorts, but only reaches 95% significance level in the Nurses' Health Study cohort. In the Quebec cohort, SNP *Apa*I showed significant association with the atopy phenotype. SNP *Apa*I is the only tested SNP being associated with asthma in the CAMP cohort, but the distribution pattern of its alleles was different from both the Quebec and the Nurses' Health Study cohorts: allele A was overtransmitted in the CAMP cohort, and it was undertransmitted in the other two cohorts. Hence, the same *VDR* alleles were observed to be associated with asthma in 2 out of 3 study populations.

We note that the CAMP study recruited patients with median age-of-onset of 3 (boys) and 4 (girls) (ranging from 2 to 6 years), comparable to that of the Quebec probands (5 years, with a wide range of 2 to 46 years). However, the median age of recruitment of the Quebec probands was older (16 years), which correlates with a longer duration of disease and/or recurrence in adulthood. The NHS cohort includes female nurses with asthma present in adulthood [44]. The lack of *VDR* associations in the CAMP study may be explained by differences in phenotypes related to age or duration of disease and/or sample size issues [45,46].

By sequencing the promoter, exons and their surrounding regions, we excluded novel missense polymorphisms that could have been responsible for the observed associations. Of the SNPs associated with asthma/atopy, only *Taq*I resides in the coding region (exon 9), and the polymorphism does

Table 3: Comparison of associations observed in the Quebec, Childhood Asthma Management Program and Nurses' Health Study cohorts

Variant	Quebec	Childhood Asthma Management Program	Nurses' Health Study
rs3782905	S	NS	S*
rs1540339	S	NS	NS
rs2239182	S	NT	NT
rs2239185	S	NS	S*
*Bsm*I	S	NS	NT
*Apa*I	NS (S for atopy)	S†	S
*Taq*I	S	NS	S*

S = statistically significant association ($p<0.05$)

NS = not significant

NT = not typed

* same allele of association as the Quebec cohort

† different allele of association from both the Quebec and the Nurses' Health Study cohorts

not result in an amino acid change. *Taq*I has been shown to be associated with many metabolic and immune-mediated diseases. The T allele, which is associated with asthma and atopy in our cohort, is also associated with tuberculosis[13;21-23], chronic hepatitis B infection[23], lepromatous leprosy[12] and type 1 diabetes in Eastern Europeans[47] and South Indians[39]. The C allele, on the other hand, is associated with Crohn's disease[11], tuberculoid leprosy[12] and type 1 diabetes in Germans[48]. The functional consequence of this polymorphism is not fully understood. In lymphocytes, the *Taq*IC allele is 30% less abundant [48], while in pituitary adenomas and transfected green monkey kidney (COS-7) cells, the same allele is associated with higher mRNA levels [7;37].

Similar to variant *Taq*I, SNPs *Apa*I and *Bsm*I have been widely studied and shown to be associated with many diseases. Alleles *Bsm*IG and *Apa*IC are associated with asthma/atopy in this cohort, the same alleles are also associated with high bone mass density[7] and sporadic hyperparathyroidism in females[37]; while allele *Bsm*IA is associated with fast acquired immunodeficiency syndrome progression[6] and type 1 diabetes risk *per se* and acute-onset type 1 diabetes[40].

Given that association of asthma with *VDR* involves variants from intron 2 to exon 9, spanning approximately 28kb, and that no SNPs giving rise to an amino aid change were found, it is possible that the functional variant that confers susceptibility to asthma is a regulatory SNP [50], located in a *VDR* intron. Since VDR is a known immunoregulatory switch molecule, and many of the associated phenotypes have the characteristics of Th1/Th2 imbalance, the mechanism of VDR in immune-mediated diseases may involve varying levels of VDR in immune cells upon stimuli.

In summary, we identified a strong association between genetic variants at the *VDR* locus and asthma/atopy in a Quebec cohort. Along with other known asthma risk genes identified such as *ADAM*33[51], *TNFA*[52], *RANTES*[53], and

GPRA[54], the addition of *VDR* involvement in the understanding of asthma/atopy pathogenesis will shed light for better control and treatment.

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DATA SUPPLEMENTARY

Patients Recruitment

Families from the Saguenay-Lac-St-Jean region of Northeastern Quebec, Canada, were recruited through media advertisement and from Chicoutimi Hospital. This population is inhabited by 287,000 individuals who descend predominantly from approximately 2500 founders originating from France that settled in Quebec in the 17th century [55]. The population grew at a high rate with little admixture over 12 to 14 generations, and is an example of a young founder population [56].

Each study participant completed a standard respiratory questionnaire that was modified to include questions about asthma and atopy severity, family history of asthma and/or atopy, and the presence of other respiratory failure diagnoses[25]. Clinical evaluation consisted of skin prick tests, serum eosinophil counts and serum IgE levels measurement. In most subjects greater than 11 years of age, spirometry (with pre- and post- bronchodilator response) and methacholine challenge were performed. Measurements of expiratory flows and bronchodilator response were performed according to American Thoracic Society recommendations[57]. Methacholine bronchoprovocation tests were performed according to the method described by Juniper and colleagues[58]. We defined participants as asthmatics if (1) a reported history of asthma (questionnaire-based) and a history of physician-diagnosed asthma (past/current) were available, or (2) confirmation by a positive methacholine provocation test.

Subjects were excluded from methacholine tests for the following reasons: forced expiratory volume over 1 second to be less than 1.5L or 60% of the predicted value, 20% fall in forced expiratory volume over 1 second with saline, recent myocardial infarctus, history of coronary diseases, pregnancy, unstable

asthma, and use of short or long-term bronchodilator medication in the 8 or 24 hours prior to spirometry. The dose of methacholine that resulted in a 20% fall in forced expiratory volume was recorded as the PC_{20}. Skin-prick tests were performed for 26 inhalant allergens[59]. Serum immunoglobulin E level was measured with enzyme immunofluorometry (Quanticlone Total IgE kit #839). Subjects with unconfirmed asthma status are categorized as uncertain affection status in the analysis. Subjects were deemed atopic if they had at least one positive response (wheal diameter $\geq$ 3 mm at 10 min) to skin-prick tests.

Genotyping

SNP genotyping was performed using SNPstream® UHT (Orchid Biosciences)[26]. To confirm the accuracy of the genotypes determined by SNPstream®, selected SNPs (rs1540339, rs3782905, rs2239179, rs2239185 and *TaqI*) were repeat genotyped by HEFP™ (Molecular Devices)[60]. To further assess the reliability of genotypes determined by SNPstream® UHT, discordant rates were calculated from genotypes determined by SNPstream® UHT and HEFP™, followed by mendelian error check implemented in the family based association test (FBAT v.1.4)[28]. Genotypes determined by SNPstream® were considered reliable for association testing; five SNPs were genotyped using two methods, which allowed us to measure discordancy rates of 0.7-1.6% (data not shown). FBAT detected one mendelian error in the entire cohort, and the genotypes of that family were reset to zero in the analysis.

For the UHT Orchid platform genotyping, SNP flanking sequences were tested for the presence of repeats or duplicated regions using the BLAT program (http://www.genome.ucsc.edu). PCR and SBE primers were designed using the Autoprimer program (http://www.autoprimer.com). The program selects PCR primers that will generate products ranging between 90 and 180 basepair and an optimized single base-pair extension primer 5' to the SNP site[26]. See table E1

for oligonucleotides used for genotyping in this study. The program also assembles SNPs into groups of twelve by SNP extension type (e.g. T/C, A/T, etc.) and appends a unique DNA tag to the 5' ends of the extension primers. Twelve-plex PCR reactions were performed in 384-well plates (MJS BioLynx) in a 5μL volume using 6ng of DNA, 75μM dNTPs, 0.5 Unit of AmpliTaq Gold (Perkin-Elmer), and the 24 PCR primers at a concentration of 50nM each in 1 X PCR buffer. Thermal cycling was performed in GeneAmp PCR system 9700 thermal cyclers (Applied Biosystems) using the following program: initial denaturation at 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 50-55°C for 55 seconds, 72°C for 30 seconds. After the last cycle, the reaction was held at 72°C for 7 minutes. Following PCR, plates were centrifuged briefly and 3μL of a mixture containing 0.67 Unit Exonuclease I (Amersham Pharmacia) and 0.33 Unit Shrimp Alkaline Phosphatase (Amersham Pharmacia) were added to each well. The plates were sealed and incubated for 30 minutes at 37°C and at 95°C for 10 minutes. Extension reactions and hybridizations to the Orchid UHT microarray plates were carried out as described in[26], using the SNPware UHT reagent kit and the appropriate extension mix kit containing two dideoxynuclotides labeled with either Bodipy-Fluorescein or TAMRA dye (Beckman Coulter). Finally, the plates were read using the SNPstream Array Imager (Beckman Coulter) and fluorescence intensities were measured with help of the UHTImage software (Beckman Coulter). Intensities were plotted and genotypes were called by the UHTGetGenos software (Beckman Coulter). After visual inspection of the clusters, manual adjustments were made for some of the assays.

For the HEFP platform, PCR primers were designed using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCR reaction were carried out as follows: 6.0 ng of genomic DNA were added to an 8.0μL reaction mixture containing 2.5mM of $MgCl_2$, 25mM of dNTPs, 0.2 Unit of HotstartTaq DNA polymerase (Qiagen) and 100nM of each primer. PCR was initiated by denaturing the samples at 94°C for 15 minutes followed by 45 cycles

of denaturation at 94°C for 30 seconds, annealing at between 56°C -57°C (primers specific) for 30 seconds and extension at 72°C for 30 seconds. Final extension was done at 72°C for 6 minutes. PCR products were treated with Exonuclease I and Shrimp Alkaline Phosphatase as recommended by the manufacturer (AcycloPrime-FP SNP Detection Kit, Perkin Elmer, Wellesley, MA). Single-base extension (SBE) detection primers of minimum 16 bp length and melting temperature above 55°C were designed in one or both orientations for each SNP using Primer3. See table E.3 for oligonucleotides used in the HEFP platform. FP-SBE reactions were performed in one or both orientations as suggested by the manufacturer (AcycloPrime-FP SNP Detection Kit, Perkin Elmer, Wellesley, MA). If necessary, we optimised the FP-SBE reaction by varying the annealing temperature and $MgCl_2$ concentration. After addition of reading buffer, we read the plates using an Analyst HT reader (Molecular Devices, Sunnyvale, CA) as described previously[61;62].

SNP Discoveries

Exons and surrounding regions were first amplified using the following 12 sets of primer. See table E.4 for oligonucleotides, annealing temperature and $MgCl_2$ concentration used in the sequencing reaction. PCR reaction was carried out with 20μmole of genomic DNA, 0.1μl of Hot Star TAQ Polymerase (Qiagen), 2.5μl of 10X buffer (Qiagen), 0-2.5μl of 25μM of $MgCl_2$ depending on the primers, 0.50μl of 20μM primers and 0.5μl of 10mM dNTPs (Qiagen) to a final volume of 25μl. Cycling conditions were: 96°C for 15 minutes, 35 cycles of 96°C for 30 seconds, 56°C - 60°C depending on the primers for 1 min and final extension at 72°C for 10 minutes.

Purification was done using the Millipore Montage PCR 96 Cleanup kit (Millipore Corp). Briefly, 100μl of sterile water and PCR products were transferred to the purification plates and vaccum pumped for 10 minutes. After adding 25μl of water, the plate was vortex and shaken for 5 minutes and stored

in –20°C. PCR products were sequenced using the appropriate ABI PRISM BigDye Terminator kit on an ABI 3700 DNA sequencer (Applied BioSystems) as described elsewhere[33].) Data analysis was done using Sequencing Analysis software (version 3.6) and Autoassembler 2.1 (Applied BioSystems).

Twelve novel SNPs have been identified by direct sequencing of 24 individuals. See table E.5 for their base change, allele frequency, chromosome location and genomic characteristics.

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Table E.1: Examples of genetic association study of *VDR* variants and disease susceptibility

Authors / Year	Study Site	Phenotype	SNPs Tested	Association
Bellamy R. et al., 1999[13]	The Gambia	Pulmonary TB, chronic hepatitis B	*Taq*I	*Taq*I CC genotype is the protective factor for TB (p-value=0.01) and chronic hepatitis B infection (p-value=0.01)
Wilkinson R.J. et al., 2000[14]	United Kingdom (Gujarati Asians)	TB	*Taq*I, *Bsm*I and *Fok*I	*Taq*I TT/TC genotypes + vitamin D3 (VD) deficiency are risk factors for TB (OR=2.8, 95% CI=1.2-6.5). *Fok*I TC+VD deficiency are risk factors for TB (OR=5.1, 95% CI=1.4-18.4)).
Selvaraj P. et al., 2000[15]	India	Pulmonary TB	*Taq*I	1. *Taq*I TT genotype is the protective factor and CC genotype is the risk factor for TB in female subjects (p<0.02 for both). 2. *Taq*I CC genotype is the risk factor in female subjects.
Liu W. et al., 2003[16]	People Republic of China	Pulmonary TB	*Fok*I	*Fok*I TT genotype was associated with pulmonary TB in men (OR=3.036, 95%CI=1.117-8.253)
Barber Y et al., 2001[17]	Spain (European origins)	Human immunodeficiency virus / acquired immunodeficiency syndrome progression	*Bsm*I	*Bsm*I AA genotype is associated with fast disease progression (OR=2.4, 95%CI=1.3-4.7).
Roy S et a., 1999[18]	India	Tuberculoid leprosy, lepromatous leprosy	*Taq*I	*Taq*I CC genotype is associated with tuberculoid leprosy (OR=3.22, 95%CI=1.47-7.13), and *Taq*I TT genotype is associated with lepromatous leprosy (OR=1.67, 95%CI=1.02-2.75).
Simmons J.D. et al., 2000[19]	United Kingdom (European Caucasoids)	Inflammatory bowel disease (Crohn's disease, ulcerative colitis)	*Taq*I, *Apa*I, *Fok*I	*Taq*I CC is a risk genotype for Crohn's disease (OR=1.99, 95%CI=1.14-3.47)
McDermott M.F. et al., 1997[20]	South India	Insulin dependent diabetes mellitus	*Taq*I, *Apa*I, *Bsm*I, D12S85	*Bsm*I *G* is a risk allele for insulin dependent diabetes mellitus (p-value = 0.016)
Chang T.L. et al., 2000[21]	Taiwan	Insulin dependent diabetes mellitus	*Bsm*I, *Apa*I,*Taq*I	*Bsm*I A allele is a risk allele for insulin dependent diabetes mellitus
Motohashi Y et al., 2003[22]	Japan	Onset pattern of type 1 diabetes	*Bsm*I	*Bsm*I A allele is a risk allele for type 1 diabetes *per se* (p-value = 0.001), and a risk allele in acute-onset type 1 diabetics (p-value = 0.0002).
Guy M et al., 2003[23]	United Kingdom (Caucasians)	Breast cancer	*Bsm*I, *Fok*I	*Bsm*I GG genotype is associated with breast cancer risk (OR=1.79, p-value = 0.02).
Sillanpaa P et al., 2004[24]	Finland	Breast cancer	*Apa*I	*Apa*I C allele is associated with breast cancer protection (OR=0.73, 95% CI=0.54-0.98).
Houston L.A. et al., 1996[25]	United Kingdom	BMD	*Bsm*I	*Bsm*I AA genotype had a higher femoral neck bone density than those with GG (p-value<0.02)
Kiel D.P. et al., 1997[26]	United States (Caucasians)	BMD	*Bsm*I	*Bsm*I GG genotype is associated with 1) higher BMD to calcium intake (p-value<0.05), 2) higher BMD compared to AG/AA location specific in high calcium intake group (p-value<0.05).
Carling T. et al., 1997[27]	Sweden	development of sporadic primary hyperparathyroidism, hyperparathyroidism of multiple endocrine neoplasia type 1 (MEN1) and hyperparathyroidism of uremia	*Apa*I, *Bsm*I, *Taq*I	*Bsm*I GG, *Apa*IGG and *Taq*ITT genotypes are associatd with sporadic hyperparathyrodism in female subjects (OR=2.6 to 3.4)

Table E.2: *VDR* Polymorphisms characteristics

SNP ID#	Base change	*MAF	†Chromosome location	Genomic features
rs2238136	G>A	0.36	46563980	intron 1
*Fok*I	C>T	0.36	46559162	nonsynonymous, translation start site, met --> Thr
rs3782905	C>G	0.31	46552434	intron 3
rs2239179	A>G	0.39	46544033	intron 3
rs1540339	C>T	0.4	46543593	intron 3
rs2107301	C>T	0.27	46541837	intron 3
rs2239182	A>G	0.45	46541678	intron 3
rs2239185	A>G	0.44	46530826	intron 6
*Bsm*I	C>T	0.4	46526102	intron 8
*Apa*I	A>C	0.45	46525104	intron 8
*Taq*I	T>C	0.4	46525024	synonymous, exon 9
rs757344	A>G	0.36	46492363	*HDAC7A*

*MAF=minor allele frequency

† chromosome position is based on the July 2003 freeze of the University of California Santa Cruz genome browser http://genomeluscsc.edu/

Table E.3: Oligonucleotides used in genotyping assays

Method	SNP ID	Oligonucleotide Name	Sequence (5' to 3')	Base Change
SNPstream□ UHT	rs2238136	VDRrs2238136U3CTu	AGGGAGCATCTGAGGAAAA	G>A
		VDRrs2238136U3CTl	AGCAGACACCTCCCACTG	
		VDRrs2238136U3CTsu	CGTGCCGCTCGTGATAGAATACTA-GTGGACAATGAGCCAAGATAA	
SNPstream□ UHT	*Fok*I	VDRFok1U7CTu	ACTGACTCTGGCTCTGACCG	C>T
		VDRFok1U7CTl	TCAAAGTCTCCAGGGTCAG	
		VDRFok1U7CTsu	AGGGTCTCTACGCTGACGATGGC-CTGCTTGCTGTTCTTACAGGGA	
SNPstream□ UHT	rs3782905	VDRrs3782905U8CGu	CATGCTAGGCACCCAGAG	C>G
		VDRrs3782905U8CGl	AGAAGAGAAACTAGCAGAAAGA	
		VDRrs3782905U8CGsu	GTGATTCTGTACGTGTCGCCACTTTA-AAATCTACTTTCACCCACT	
HEFP□	rs3782905	rs3782905incgF	ACTTTAAAATCTACTTTCACCCACT	C>G
		rs3782905incgR	TGGGAGGGAGTGCTGA	
		rs3782905outF	GTCTGCTTCAGGGGTCTCAA	
		rs3782905outR	CAGGACCTGGACAAAGGAAA	
SNPstream□ UHT	rs2239179	VDRrs2239179U4CTu	TATCCTCTGTCCCTGACACA	A>G
		VDRrs2239179U4CTl	ATGCGGACCCTCCTGGCTAT	
		VDRrs2239179U4CTsu	AGCGATCTGCGAGACCGTATCTTCC-TGTTACCTGACCTCTCCCCA	
HEFP□	rs2239179	rs2239179inctF	GTTACCTGACCTCTCCCCA	A>G
		rs2239179inctR	GTTTGGAGTGGTTGGGG	
		rs2239179outF	TCTGTCCCTGACACATCTTCC	
		rs2239179outR	TCCTAGCTGTGGGTCTGAGG	
SNPstream□ UHT	rs1540339	VDRrs1540339U4CTu	GGAGATGGGACTGTGCTG	G>A
		VDRrs1540339U4CTl	TTAAGAGGCTTCACACACATTCT	
		VDRrs1540339U4CTsu	AGCGATCTGCGAGACCGTATCACA-CCTTGTTGGTGCCCACCCTAA	
HEFP□	rs1540339	rs1540339inctF	GTTGGTGCCCACCCTAA	
		rs1540339inctR	CTGCTCAGGGTGAGGC	G>A
		rs1540339outF	GCTTTAGAAACCGGACTCCC	
		rs1540339outR	CACACACATTCTCAGTGGGC	
SNPstream□ UHT	rs2107301	VDRrs2107301U4CTu	TTGGCTTCGTTAAGGAGAG	C>T
		VDRrs2107301U4CTl	TTGACTTCATTTTAAGCTCCTTG	

		VDRrs2107301U4CTsu	AGCGATCTGCGAGACCGTATACAT-GTCTTGCATGGGAATAACTTG	
SNPstream□ UHT	rs2239182	VDRrs2239182U1CTu	TTTTTCAATGGATTGAACCTAAG	A>G
		VDRrs2239182U1CTl	ATCACCAGACAGCCCAAC	
		VDRrs2239182U1CTsu	ACGCACGTCCACGGTGATTTGAT-ATATGAAGCCATTGACCTAGAA	
SNPstream□ UHT	rs2239185	VDRrs2239185U12GAu	AAACAGCAACACAATTCCAGT	T>C
		VDRrs2239185U12GAl	CCTCCCTCACCTGTGTGA	
		VDRrs2239185U12GAsl	CGACTGTAGGTGCGTAACTCCAT-TTACACCCTCCTCTGTCTTCAC	
HEFP□	rs2239185	rs2239185inctF	CAGAAGCGGCTGCAGG	T>C
		rs2239185inctR	TACACCCTCCTCTGTCTTCAC	
		rs2239185outFNEW	GGGAAAGACGAAACAGCAAC	
		rs2239185outR	TTGTTCCACGATGATAGGCA	
SNPstream□ UHT	*Bsm*I	VDRrs1544410U10CTu	AAAGTTTTGTACCCTGCCC	G>A
		VDRrs1544410U10CTl	ATTCTGAGGAACTAGATAAGCAGG	
		VDRrs1544410U10CTsu	AGATAGAGTCGATGCCAGCTGAG-CAGAGCCTGAGTATTGGGAATG	
SNPstream□ UHT	*Apa*I	VDRApaIU6CAu	ATCTGTGGGCACGGGGATA	A>C
		VDRApaIU6CAl	TTGAGTGTCTGTGTGGGTG	
		VDRApaIU6CAsu	GGCTATGATTCGCAATGCTTAAG-GCACAGGAGCTCTCAGCTGGGC	
SNPstream□ UHT	*Taq*I	VDRrs731236U12CTu	TTCTTCTCTATCCCCGTGC	T>C
		VDRrs731236U12CTl	ATGTACGTCTGCAGTGTGTTG	
		VDRrs731236U12CTsu	CGACTGTAGGTGCGTAACTCCTG-GGGTGCAGGACGCCGCGCTGAT	
HEFP□	*Taq*I	rs731236inctF	GGACGCCGCGCTGAT	T>C
		rs731236inctR	GGTCCTGGATGGCCTC	
		rs731236outF	TGAGAGCTCCTGTGCCTTCT	
		rs731236outR	ACGTCTGCAGTGTGTTGGAC	
SNPstream□ UHT	rs757344	VDRrs757344U8GAu	TGTTCCAGGCCGCGATGG	A>G
		VDRrs757344U8GAl	ACGAAGAACAGAAACTGGGC	
		VDRrs757344U8GAsl	GTGATTCTGTACGTGTCGCCCTAG-CCCCAGTTCACAGAAAAAACC	

Table E.4: Oligonucleotides used in sequencing

Exon and surrounding region	Primer Name	Sequence (5'-3')	Annealing temperature (°C)	MgCl2 concentration (mM)
exon 1a	exon1aForward	ATTAACACAGGCTGAAGCGG	56	0
	exon1aReverse	AGACTCTAATGCTCGCAGCC		
exon 1b	exon1bForward	GATCTTCATGCCTCTCTGGC	57	2.5
	exon1bReverse	GCTGGCAAGCCGTGTAAAG		
exon 1c	exon1cForward	GAGGCTGTTTCCTTCTGCCT	57	0
	exon1cReverse	CCCACACACTCATGCATCTC		
exon 1d	exon1dForward	GAACAGCTTGTCCACCCG	57	0
	exon1dReverse	AATTACTTAAAAGACCCAACTCCA		
exon 1e	exon1eForward	CCAAACGCAGAACCTCTGA	60	0
	exon1eReverse	CAACCACCAATACCTTGGGA		
exon 1f	exon1fForward	ATGGCCAGAGTTCATGGAAA	57	0
	exon1FReverse	CGCATACCCGACACTTGTT		
exon 2	exon 2Forward	AATCATGTATGAGGGCTCCG	60	0
	exon2Reverse	CATCTGGAGCTGAGAGGAGG		
exon 3	exon3Forward	TTGGAGAAATGGAGACCAGG	57	0
	exon3Reverse	CAGCTACAGAGGAAGGGCAG		
exon 4 and 5	exon4&5Forward	ATCTTGGACCTTTACCCCCA	60	0
	exon4&5Reverse	CTGTTGTGAAGACGCTGCAT		
exon 6	exon6Forward	AGAGAGTCCCAGAGGGAAGC	60	0
	exon6Reverse	AGGACTCTGACTCTGTTCCCC		
exon 7 and 8	exon7&8Forward	CTCCTCGATGAAAGACCCAG	57	2.5
	exon7&8Reverse	TACGTCTCCCTTCAGGTTGC		
exon 9	exon9Forward	CTGCCGTTGAGTGTCTGTGT	57	2.5
	exoon9Reverse	GTGAGGAGGGCTGCTGAGTA		

Table E.5: Characteristics of novel SNPs discovered

SNP ID#	Base change	*MAF	†Chromosome location	Genomic features
VDR0001	insA	0.13	46587093	5'flanking
VDR0002	T>G	0.05	46586800	5'flanking
VDR0003	C>T	0.03	46586785	5' flanking
VDR0004	C>A	0.24	46579983	noncoding, 5103bp downstream of transcription start site
VDR0005	C>T	0.02	46579937	noncoding, 5149bp downstream of transcription start site
VDR0006	G>C	0.24	46579872	noncoding, 5214bp downstream of transcription start site
VDR0007	T>C	0.02	46562699	noncoding, 3537bp upstream of translation start site
VDR0008	G>A	0.05	46559260	noncoding, 98bp upstream of translation start site
VDR0009	C>T	0.025	46559010	noncoding, 152bp downstream of translation start site
VDR0010	A>G	0.5	46537800	noncoding
VDR0011	G>A	0.07	46537343	noncoding
VDR0012	insG	0.02	46526633	noncoding

*MAF=minor allele frequency

† chromosome position is based on the July 2003 freeze of the University of California Santa Cruz genome browser http://genomeluscsc.edu/

Chapter 3

NRAMP1 is not associated with asthma, atopy, and serum immunoglobulin E level in the French Canadian population

After the successful detection of associations between *VDR* genetic variants and asthma / atopy, a second TB susceptibility gene was tested in the French Canadian asthma study.

NRAMP1 is not associated with asthma, atopy, and serum immunoglobulin E level in the French Canadian population

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ABSTRACT

Reduced infection by mycobacteria, including *Mycobacterium tuberculosis*, may be partly responsible for increased prevalence of allergic and autoimmune diseases in developed countries. In a murine model of innate resistance to mycobacteria, the *Nramp1* gene has been shown to affect asthma susceptibility. From this observation it was proposed that human NRAMP1 may be a modulator of asthma risk in human populations. To experimentally test the candidacy of NRAMP1 in asthma susceptibility, we characterized five genetic variants of *NRAMP1* (5'CA_n, 274C>T, 469+14G>C, D543N and 1729+del4) in an asthma family-based cohort from northeastern Quebec. We did not observe any significant association between *NRAMP1* variants (either allele or haplotype specific) with asthma, atopy or serum immunoglobulin E levels. These results demonstrate that in spite of direct involvement of Nramp1 in a murine asthma model, in human populations NRAMP1 is not likely to be a major contributor to the genetic etiology of asthma and asthma related phenotypes.

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INTRODUCTION

In 1989, David Strachan reported the findings of a prospective study of 17414 British children from birth to 23 years of age. [1] The study found a significant inverse correlation between the number of older siblings in the household and the prevalence of hay fever at age 11 and 23 years. A similar correlation was also observed for eczema in the first year of life. Subsequent epidemiologic studies confirmed the inverse association between family size and atopic markers such as skin prick positivity and specific immunoglobulin (Ig) E titers. [2] To explain the inverse correlation between family size and atopy, it was suggested that declining family size, improved household amenities and higher standards of personal cleanliness, all associated with improved hygiene, had reduced the opportunities of cross-infection, and this may have increased the prevalence of atopic diseases. This explanation is now known as the hygiene hypothesis and it has been extended to include autoimmune diseases in general.

One proposed mechanism underlying the hygiene hypothesis is that microbial exposures to viruses, bacteria and parasites, elicit an immune response towards the maturation of T helper type 1 cells (Th1), and the subsequent production of cytokines such as interleukin (IL)-2 and interferon-γ. This predominating Th1 immune response suppresses the production of immunoglobulin (Ig) E and IgG1 as well as cytokines IL4, IL5, IL9 and IL13, characteristic of type 2 (Th2) responses. The possibility of a Th1/Th2 imbalance and an association between *Mycobacterium tuberculosis* infection and atopy expression was first shown by a study of Japanese school children. [3] The study reported observed an inverse association between delayed hypersensitivity to tuberculin, a broad measure of exposure to mycobacteria (Th1 response), and serum IgE levels (Th2 response), and concluded that exposure to *M. tuberculosis* may inhibit the development of atopic diseases. In humans, *M. tuberculosis* is the cause of tuberculosis

(TB). However, not all individuals exposed to *M. tuberculosis* will become infected, and of those infected only a small proportion will develop clinical disease. [4, 5] It is now well established that both genetic and environmental factors determine the progression from exposure to infection and from infection to disease. If *M. tuberculosis* infection protects against atopy, then genetic risk factors for TB susceptibility may be protective for asthma and vice versa. A well established TB susceptibility gene is the Natural Resistance Associated Macrophage Protein 1 gene (*NRAMP1*). In addition to its known role in TB susceptibility, its associations with numerous immune – mediated disorders such as rheumatoid arthritis, type 1 diabetes and multiple sclerosis are making *NRAMP1* a prime candidate to test the hypothesis of inverse genetic control of asthma and TB susceptibility. [6, 7]

Data from the mouse support the candidacy of *NRAMP1* as an asthma susceptibility gene. It has been shown that *NRAMP1* resistant (*NRAMP1*^{r}) mice have a lower IgE and IL-4 response compared to *NRAMP1* susceptible (*NRAMP1*^{s}) mice after infection with an attenuated strain of *Salmonella typhimurium*, [8] demonstrating that *NRAMP1* can modulate Th1/Th2 host responsiveness. Moreover, in *M. vaccae* and allergen sensitized mice, subsequent allergen challenge triggered higher levels of Th2 cytokines (IL-4, IL-5, IL-13) and IgE in *NRAMP1*^{s} as compared to *NRAMP1*^{r} mice. [9] This finding implies that the ability to develop atopy associated Th2 responses is dependent on resistance to infection, dictated in this model by *NRAMP1*. Finally, *M. vaccae* is more efficient in lowering allergic and asthmatic symptoms in allergen challenged *NRAMP1*^{s} than in *NRAMP1*^{r} mice, [10] directly demonstrating that *NRAMP1* can modify immune responses following mycobacterial infection. Taken together these observations provide a direct experimental link between genetically controlled resistance to infection and altered asthma susceptibility and constitute the main motivating force for the present study.

RESULTS

Patient Characteristics

Clinical characteristics of the study participants have been reported previously. [11] Briefly, 1139 individuals between ages 3 years and 88 years were recruited. The median age of onset for index cases and their affected siblings is 5 years (2-46 years). Of the 570 subjects with asthma and 569 without asthma, 419 (74%) and 218 (38%) were atopic, respectively. The male to female ratios in probands, affected and unaffected family members are 1:1.2, 1:1.4 and 1:1.2, respectively. Index cases have higher IgE levels and coexistence of atopy compared to other affected family members.

Genetic Variants of the *NRAMP1* locus

We characterized five polymorphisms of the *NRAMP1* gene in the family-based cohort (Figure 1). Variant 5'$(CA)_n$ is a promoter dinucleotide repeat polymorphism; variant rs2276631 (reference SNP identifier from the National Center for Biotechnology Information database) is a synonymous C>T polymorphism in exon 3 (common alias 274 C>T); rs3731865 involves a G>C base change in intron 4 (common alias 469+14G>C); variant D543N is a G>A substitution resulting in an aspartic acid to asparagine amino acid change in exon 15, and variant 1729+55del4 is a TGTG tetranucleotide deletion polymorphism in the 3' untranslated region (Table 1). All variants have previously been described. [32] *NRAMP1* variants were selected due to their known associations with susceptibility to infectious and autoimmune diseases. In the French-Canadian families, variants in the 5' *NRAMP1* region (5'$(CA)_n$, 274C>T and 469+14G>C) are polymorphic with minor allele frequencies of 0.31, 0.28, 0.31, respectively, and all variants are in Hardy-Weinberg equilibrium. Variants 1729+del4 and D543N were uninformative

Figure 1: Chromosomal location of *NRAMP1* polymorphisms associated with common diseases. In the schematic presentation of the *NRAMP1* genomic organization, exons are depicted as black boxes with the corresponding exon numbers on top. Introns are depicted as lines between exon boxes. Genomic distances between exons are indicated by the scale in kilobases (kb) above the panel with 0 kb being the transcription start site of exon 1 and 13.6 kb indicating the end of exon 15. The white box 4a represents the alternatively spliced exon 4A. [10] Positions of variants are indicated by arrows. Names or identification numbers of variants are given underneath the arrows.

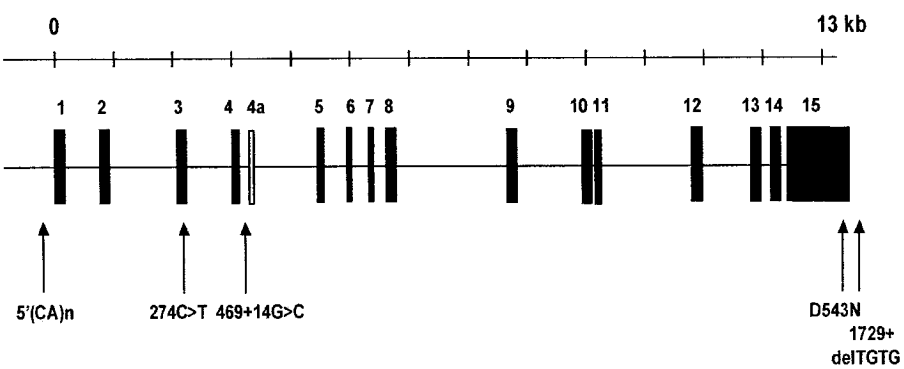

0
13 kb
1
2
3
4
4a
5
6
7
8
9
10
11
12
13
14
15
5'(CA)n
274C>T
469+14G>C
D543N
1729+
delTGTG

Table 1. *NRAMP1* polymorphisms and disease associations

Variant	Chrosome Position[c]	Allele	Genomic Characteristics	Human Disease Associated	References
5'$(CA)_n$	219072130	2[d,e] t$(gt)_5$ac$(gt)_5$ac$(gt)_{10}$ggcaga$(g)_6$	promoter	TB	12, 13, 14
		3 t$(gt)_5$ac$(gt)_5$ac$(gt)_9$ggcaga$(g)_6$		HIV infection	15
				Kawasaki	16
				rheumatoid arthritis (RA)	17, 18
				inflammatory bowel disease	19
				sarcoidoisis	20
				type 1 diabetes	21, 22
				multiple sclerosis	23
				primary biliary cirrhosis	24
rs2276631[a], common alias: 274C>T[b]	219074518	G[e]	exon 3, synonymous	HIV infection	15
		A			
rs3731865, common alias: 469+14G>C	219075508	G	intron 4	TB	12, 25, 26
		C		HIV infection	15
common alias: D543N	219085237	G[e]	exon 15, aspartic acid to asparagine	TB	12, 14, 27, 28
		A		RA	29, 30
				sarcodosis	20
common alias: 1729+delTGTG	219085319	TGTG[e]	3' untranslated region	TB	12
		-TGTG		leprosy	31
				RA	29, 30

[a] rs numbers are the reference SNP identifiers from the NCBI database.

[b] common alias perviously described. [32]

[c] chrosome positions are based on July 2003 freeze of the University of California Santa Cruz genome browser (http://genome.ucsc.edu).

[d] at least 9 alleles have been identified in five studies, in all studied populations, allele 3 is the most common allele, followed by allele 2, the remaining 7 alleles are all rare variants. [6,7]

[e] base changes indicated are on the negative strand of July 2003 freeze of the University of California Santa Cruz genome browser.

(minor allele frequency = 0.012 and 0.017, respectively). Hence, these two 3' end variants were not further analyzed.

Family-based Association Analysis of *NRAMP1* with Asthma, Atopy and IgE Level

The three informative *NRAMP1* polymorphisms located in the 5' region of the gene were tested individually for association with asthma, atopy and IgE level (Table 2). IgE serum levels were analyzed as dichotomous trait, independently of atopy status. Subjects were classified either as high or low responders according to their IgE levels. Based on the normal distribution of the log (IgE) values, a cut-off point of 100 mg/L divided the subjects into low (two-thirds of participants) and high (one-third) responders. We tested allelic associations under additive and dominant genetic models. No allele was significantly ($p < 0.05$) transmitted nonrandomly to offspring with asthma or atopy, and to high IgE responders. The 469+14G allele, under an additive model, was non-significantly overtransmitted to asthmatic offspring ($p=0.08$).

Haplotype – Specific Association Analysis

Association among the 3 variants was assessed by measuring pairwise linkage disequilibrium (LD) using D'. The 3 variants are strongly associated as evidenced by a high degree of LD (D' > 0.90) among them (data not shown).

Alleles of the 3 variants are likely to be transmitted together as groups, or haplotypes. Within this region of high LD, 2 haplotypes with frequency > 0.1 are observed: haplotype (5'$(CA)_n$) allele 3-(274)C-(469+14)G (frequency = 0.683) and haplotype allele 2-T-C (0.258). Other less frequently occurring

Table 2. Allele transmission pattern for asthma and related phenotypes

Phenotype/model	Variant	Allele (frequency)	S[c]	E(S)[d]	Z[e]	P-value
asthma/additive	5'$(CA)_n$	3[a] (0.687)	270	263.33	0.95	NS[f]
		2[b] (0.313)	112	118.67	-0.95	NS
	274C>T	C (0.72)	242	234.41	1.11	NS
		T (0.28)	92	99.58	-1.11	NS
	469+14G>C	G (0.69)	267	254.17	1.74	0.08
		C (0.31)	101	113.83	-1.74	0.08
asthma/dominant	5'$(CA)_n$	3	61	58.91	0.65	NS
		2	101	105.58	-0.74	NS
	274C>T	C	49	46.16	0.89	NS
		T	80	84.74	-0.80	NS
	469+14G>C	G	66	63.41	0.79	NS
		C	90	100.25	-1.58	NS
atopy/additive	5'$(CA)_n$	3	286	283.58	0.34	NS
		2	128	130.42	-0.34	NS
	274C>T	C	252	248.50	0.51	NS
		T	104	107.50	-0.51	NS
	469+14G>C	G	298	294.00	0.54	NS
		C	128	132.00	-0.54	NS
atopy/dominant	5'$(CA)_n$	3	60	58.99	0.31	NS
		2	109	110.41	-0.23	NS
	274C>T	C	52	48.49	1.11	NS
		T	93	92.99	0.002	NS
	469+14G>C	G	66	64.49	0.47	NS
		C	113	115.49	-0.39	NS
IgE/additive	5'$(CA)_n$	3	165	165.50	-0.09	NS
		2	71	70.50	0.09	NS
	274C>T	C	150	154.42	-0.85	NS
		T	70	65.58	0.85	NS
	469+14G>C	G	161	164.42	-0.62	NS
		C	73	69.58	0.62	NS
IgE/dominant	5'$(CA)_n$	3	35	34.16	0.36	NS
		2	62	60.66	0.28	NS
	274C>T	C	26	25.91	0.04	NS
		T	58	53.50	0.96	NS
	469+14G>C	G	34	34.41	-0.17	NS
		C	64	61.00	0.62	NS

[a] allele 3: $t(gt)_5ac(gt)_5ac(gt)_9ggcaga(g)_6$, [b] allele 2: $t(gt)_5ac(gt)_5ac(gt)_{10}ggcaga(g)_6$, S[c]=FBAT statistic, E(S)[d]=Expected FBAT statistic, Z[e] = Z-score, NS[f]=not significant

haplotypes are allele 2-C-G (0.021), allele 2-C-C (0.015) and allele 3-C-C (0.010). All haplotypes were assessed for nonrandom transmissions in the asthma families using Family Based Association Testing software (FBAT), version 1.5 [33, 34] (Table 3). Under additive and dominant genetic models, all haplotypes were randomly transmitted to offspring with either asthma, atopy, or high IgE responsiveness.

Power Calculations

We examined the possibility that the failure to detect association of *NRAMP1* variants with asthma and related phenotypes was due to insufficient power of the study sample. Power was calculated for 60 sets of parameters defined by susceptibility allele frequency (q) and genetic attributable fraction (GAF) for an additive disease model with disease prevalence set at 0.10 (Figure 2). The result shows that with the present cohort of 1139 individuals there is excellent power (> 80%) to detect variants across a wide allele frequency range (0.20 to 0.50) for a heterozygotes odds ratio (HET OR) > 1.6. For example, for q = 0.20, power > 80% is achieved with GAF ≥ 0.20, corresponding to a HET OR > 1.6 (Figure 2). For the low allele frequencies of 0.05 and 0.10, power > 80% is achieved for a HET OR > 1.8. For the high allele frequency of 0.70, power > 80% is achieved for a HET OR > 1.7 (data not shown). By contrast, power to detect variants with a HET OR < 1.4 is poor (< 60%) for the allele frequency range of 0.10 to 0.70, although such small risk modifiers may still account for a substantial proportion of cases, especially if the risk variant is present at high

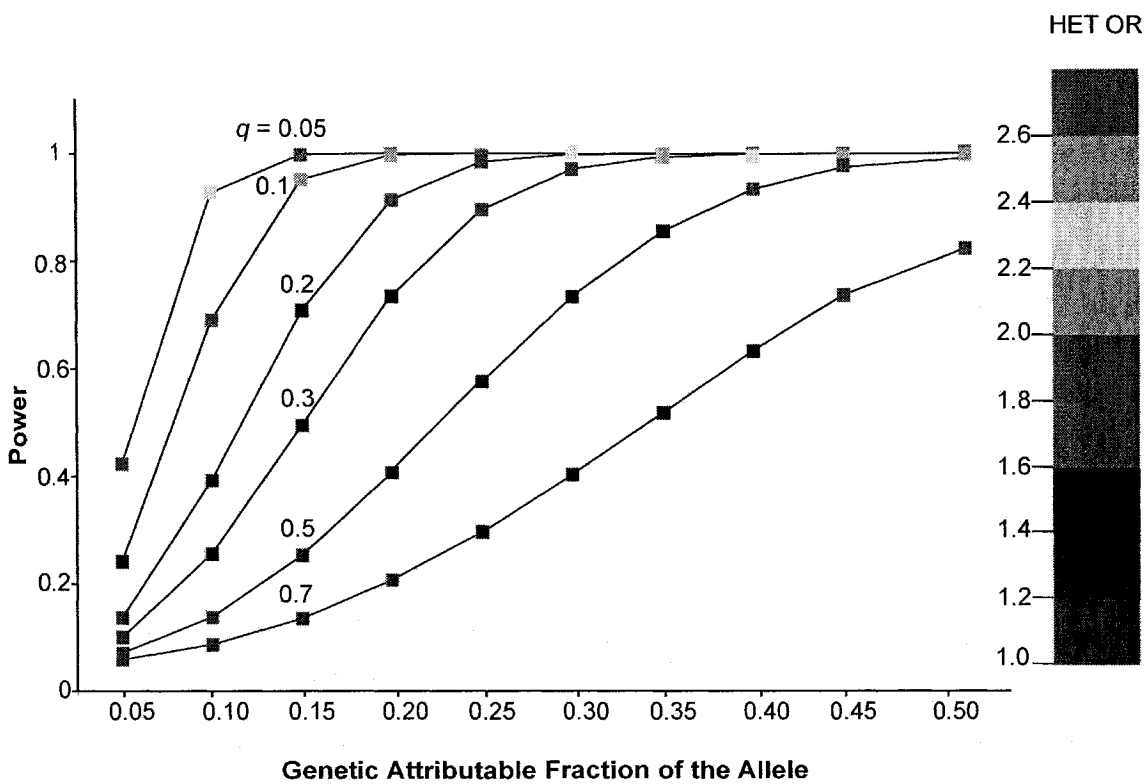
Table 3. Haplotype transmission pattern of *NRAMP1*

Asthma

5'$(CA)_n$	274C>T	469+14G>C	Frequency	$S^{a,b}$	S^{d}	$E(S)^{a,c}$	$E(S)^{d}$	$Z^{a,e}$	Z^{d}	p-value[a]	p-value[d]
3	C	G	0.683	221.995	146.995	214.023	145.334	1.242	0.607	0.214	0.544
2	T	C	0.258	101.995	82.995	106.671	85.515	-0.721	-0.462	0.471	0.644
2	C	G	0.021	9.005	9.005	8.01	8.01	0.575	0.575	0.565	0.565
2	C	C	0.015	****	****	****	****	****	****	****	****
3	C	C	0.010	****	****	****	****	****	****	****	****
Atopy											
3	C	G	0.683	241.99	161.99	237.41	160.69	0.729	0.453	0.466	0.650
2	T	C	0.258	109.99	91.99	111.78	91.647	-0.294	0.066	0.769	0.947
2	C	G	0.021	8.01	8.01	7.51	7.51	0.243	0.243	0.808	0.808
2	C	C	0.015	****	****	****	****	****	****	****	****
3	C	C	0.010	****	****	****	****	****	****	****	****
IgE											
3	C	G	0.683	122.99	83.99	124.6	83.918	-0.362	0.033	0.717	0.973
2	T	C	0.258	66.99	54.99	63.887	51.813	0.647	0.768	0.517	0.442
2	C	G	0.021	****	****	****	****	****	****	****	****
2	C	C	0.015	****	****	****	****	****	****	****	****
3	C	C	0.010	****	****	****	****	****	****	****	****

[a] additive model, [b] FBAT statistic; [c] expected FBAT statistic; [d] dominant model, [e] Z score

Figure 2: Power estimates. The genetic attributable fraction (GAF) of a susceptibility allele is plotted on the x-axis against power to detect association on the y-axis. Susceptibility allele frequencies (q) of 0.05, 0.1, 0.2, 0.3, 0.5 and 0.7 are tested. Each line of squares corresponds to the power calculated under an additive disease model with disease prevalence of 0.10, for a specific q across a range of GAF. The colour of the squares represents the associated odds ratios of the heterozygotes (HET OR), according to the colour gradient on the right of the graph. A heterozygote odds ratio of 2 indicates that an individual with 1 copy of the susceptibility allele has twice the risk to develop the disease as does a person with no copy of the susceptibility allele. For example, if the susceptible allele of frequency 0.30 attributes to 25% of the cases (GAF = 0.25), and exerts a HET OR between 1.6 and 1.8 (light blue), then the present cohort has power > 0.80 (80%) to detect the association between allele and disease.


HET OR
q = 0.05
0.1
0.2
0.3
0.5
0.7
Power
0
0.2
0.4
0.6
0.8
1
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
Genetic Attributable Fraction of the Allele

allele frequencies. For low allele frequencies < 0.05, power is poor even for a HET OR between 1.6 to 1.8. Overall, the results of the power calculation argue against *NRAMP1* alleles being asthma susceptibility factors with relative risk > 1.8 in the French Canadian population.

DISCUSSION

In light of the hygiene hypothesis and the Th1/Th2 paradigm in TB and asthma pathogeneses, the established TB susceptibility gene, *NRAMP1*, is a strong candidate gene for asthma susceptibility. In mice, the *NRAMP1* gene encodes a 90 to 100 kD transmembrane protein and its mRNA is expressed in primary macrophages and granulocytes. The *NRAMP1* protein is found at the late endosomal / lysosomal compartment of macrophages. [35, 36] *NRAMP1* is a major determinant of innate host resistance to infection. The gene affects intracellular replication of a wide range of pathogens including *Salmonella typhimurium*, [37] *Leishmania donovani*, [38] *M. lepraemurium*, [39] *M. intracellulare*, [40] *M. avium*, [41] and the TB vaccine strain *M. bovis* – Bacillus Calmette-Guérin (*BCG*). [42] Specifically, a glycine to aspartic acid change at amino acid 169 (G169D), located in predicted transmembrane domain number 4 (TM 4), has removed the host's ability to inhibit pathogen growth. [43, 44] Comparative sequence analysis of the Nramp gene family suggested that *NRAMP1* functions as a divalent cation transporter. [45] Kinetic studies demonstrated that *NRAMP1* transports cations out of the phagosomes, and consequently, mediates depletion of nutrients potentially essential for survival of pathogens in the host cell phagosome. [46, 47] In addition, *NRAMP1* is critical to overcome pathogen-triggered blockages of intracellular vesicle trafficking. [48] The exact function of human *NRAMP1* is not known. However, due to its high sequence homology with mouse *NRAMP1* (93% overall sequence similarity and 88% sequence identity), the *NRAMP1* protein is likely to be a divalent cation transporter [49, 50, 51, 52, 53]. The amino acid sequences of *NRAMP1* and *NRAMP1* in predicted TM 4 are identical, but the homologous G169D variant in TM 4 has not been found in human *NRAMP1.* [49] However, genetic variants at the 5' and 3' ends of the human gene are associated with infectious and autoimmune disease susceptibility. [6, 7]

Based on the previous findings in a murine model of asthma demonstrating that the propensity to mount an atopic immune response is dependent on *NRAMP1*, we carried out a family-based association study to test if in humans, *NRAMP1* genetic variants are associated with asthma and related phenotypes such as, atopy and IgE levels. We selected five *NRAMP1* variants that had previously been shown to be associated with susceptibility to a variety of immune disorders and infectious diseases (Table 1). Specifically, polymorphisms in the 5' region of *NRAMP1* have been found to be risk modifiers for TB in multiple populations. This suggests that a variant located in the 5' *NRAMP1* region impacts on *NRAMP1* function. The 5'$(CA)_n$ promoter variant has been linked to variable *NRAMP1* mRNA expression. [54, 55] Promoter allele 3 drives a higher mRNA expression compared to other alleles such as allele 2 (Table 1) in the absence of any stimulant. When stimulated with interferon-γ, alleles 2, 3 and other rare alleles demonstrate enhanced mRNA expression. When co-stimulated with interferon-□ and bacterial antigen lipopolysaccharide, expression by allele 2 is reduced while that of allele 3 is further enhanced. [54] Interestingly, allele 3 has been found to be associated with TB protection, and with risk for type I diabetes, an autoimmune disorder. [6]

Despite supportive evidence from a mouse model of atopic disease, we failed to detect an impact of human *NRAMP1* on asthma and related phenotypes. Our findings suggest that *NRAMP1* genetic variants do not play a major role in human atopic disease. Detailed power calculation indicated that our cohort is of sufficient size (power > 80%) to detect associations of allelic variants with weak impact on disease risk (HET OR > 1.6). However, cohort size is insufficient (power < 60%) to detect variants with very low impact on disease risk (HET OR < 1.4). The inability to detect genetic risk factors with such low impact on disease risk is not unique to the present

study. Due to unfavourable cost-benefit ratios, genetic studies are rarely powered to detect risk variants with OR < 2.

The reason why we could not replicate the mouse findings in a human population is unknown. However, it is becoming increasingly clear that the majority of common human diseases are multifactorial and complex, and that animal systems might not be able to accurately model all aspects of human diseases. In the case of *NRAMP1*, differences between the mouse model of innate resistance/susceptibility to mycobacteria and human mycobacterial diseases are well known. In mice, *NRAMP1* controls intracellular replication of several atypical mycobacteria and BCG, but does not seem to affect resistance to *M. tuberculosis*. [56, 57]. In humans, *NRAMP1* has been shown in multiple studies to be a risk modifier of TB. This species-dependent permissiveness in mycobacterial replication may be due to dosage and route of pathogen administration in mice that do not accurately mimic the natural infection in humans. Likewise, if resistance to *M. tuberculosis* is under different genetic controls in mice and humans, it is possible that asthma susceptibility may also be under different control in mice and humans. Another major difference between mouse *NRAMP1* and human *NRAMP1* is the tissue-specific gene expression. In humans, *NRAMP1* mRNA expression is more pronounced in the lung than in the spleen and liver, [49] whereas in mice, *NRAMP1* mRNA is expressed strongly in the spleen and liver, with almost no detectable expression in the lung. [46, 58-60] Since *M. tuberculosis* infection in the mouse manifests itself as progressive lung disease, low or absent *NRAMP1* expression in the lung of mice coincides with their inability to control *M. tuberculosis* infection. It is possible that similar, but presently unknown differences, in tissue expression impact on asthma susceptibility in mice and humans. Finally, the mouse findings that provided the rationale for our study used *M. vaccae* as the mycobacterial stimulant. At present, the effect of *NRAMP1* on *M. vaccae* susceptibility in

humans is unknown and it is possible that, like *M. tuberculosis*, *M. vaccae* is under different genetic controls in mice and humans.

In conclusion, the results of our study have two implications. First, even in well-developed animal models of complex human diseases such as atopy, the genetic control elements may differ between humans and the model system. Second, it seems unlikely that a single inverse relationship exists between variants that predispose to asthma / atopy and those that predispose to TB. Hence, reduced *M. tuberculosis* infection may not be the driving force behind increased asthma / atopy prevalence in developed countries, and if it is, the genetic mechanism is not likely to have included *NRAMP1*.

PATIENTS AND METHODS

Populations

Families are from the Saguenay-Lac-St-Jean region of northeastern Quebec, Canada. The recruitment scheme has been described previously. [11] Briefly, probands were recruited if they fulfilled at least two of the following three criteria: 1) a minimum of three clinic visits for acute asthma within one year; 2) two or more asthma-related hospital admissions within one year; or 3) steroid dependency, as defined by either six months of oral, or one year of inhaled corticosteroid use. Families were included for study if at least one parent was available for phenotypic assessment, at least one parent was unaffected, and all four grandparents were of French Canadian origin. When possible, grandparents and other relatives were also recruited to the study.

The affection status of all study participants was determined by clinical evaluation and the completion of a standard respiratory questionnaire that was modified to include questions about asthma and atopy severity, family history of asthma and / or atopy, age-of-onset and the presence of other respiratory failure diagnoses. [61] We defined participants as asthmatics if (1) a reported history of asthma (questionnaire- based) and a history of physician-diagnosed asthma (past/current) were available, or (2) confirmation of diagnosis by a positive methacholine provocation test was obtained (only on subjects older than 12 years of age). Subjects were deemed atopic if they had at least one positive response (wheal diameter ≥ 3 mm at 10 minutes) to skin-prick tests. The family participation rate was approximately 60% and all subjects gave informed consent. A total of 223 independent families (1139 individuals) with family size ranging from 3 to 17 and number of affected family members (including probands) ranging from 1 to 10 were analyzed.

Polymorphism Selection and Genotyping

Five polymorphisms of the *NRAMP1* gene were selected based on their known association with disease. Two of the variants have reference SNP identifiers (rs#) from the NCBI database. In this report, we referred to the common aliases of the variants: 5'$(CA)_n$, 274C>T, 469+14G>C, D543N and 1729+del4. [32] Variant 5'$(CA)_n$ was genotyped by length polymorphism analysis using ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA). Primers for amplifying polymerase chain reaction (PCR) products were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCR products of sizes ranging from 168 basepairs (bp) to 182 bp were amplified using primer pair: 5'-AACGAGGGGTCTTGGAACTC-3' and 5'-GCCTCCCAAGTTAGCTCTGA-3'. PCR reactions were carried out in PTC-100® Peltier thermal cyclers (MJ Research, MA) under the following condition: 10 ng of genomic DNA were added to 20 µl reaction mixture containing 1X PCR buffer, 2.5 mM of $MgCl_2$, 0.5 Unit of Platinum Taq polymerase (Qiagen, CA), 0.50 mM of dinucleotides and 0.30 µM of primers. PCR was initiated by denaturing the samples at 96°C for 10 minutes followed by 30 cycles of denaturation at 96°C for 25 seconds, annealing at 67°C for 1 minute. Final extension was done at 72°C for 5 minutes. Finally, 1 µl of PCR product was mixed with 0.30 µl of formamide and 10 µl of GeneScan™ – 500 Liz™ Size Standard (Applied Biosystems, CA) before being denatured at 95°C for 5 minutes and injected into ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA). Fluorescence signal was read and analyzed using ABI PRISM® GeneMapper™ Software version 3.5 (Applied Biosystems, CA).

Variants 274C>T and 469+14G>C were genotyped using HEFP™ (Molecular Devices), [62] a single-base extension (SBE) fluorescence polarization platform, as previously described. [11] Briefly, PCR and SBE

primers were designed using the Primer3 software. PCR reactions were carried out using primer set 1 (274C>T): 5'-GCCAGCCTGAAGATCTGACT-3', 5'-GGACCCCCTCACTCTACTCC-3'and set 2 (469+14G>C): 5'-ATCGTGGAAGCTGAAAATGG-3', 5'-GCGAGGTCTGCCATCTCTAC-3'. A total of 6 ng of genomic DNA was added to 8 µl reaction mixture containing 2.5 mM of $MgCl_2$, 25 mM of dinucleotides, 0.2 Unit of HotstartTaq DNA polymerase (Qiagen) and 100 nM of primers. PCR was initiated by denaturing the samples at 94°C for 15 minutes followed by 45 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C (primers specific) for 30 seconds and extension at 72°C for 30 seconds. Final extension was done at 72°C for 6 minutes. PCR products were treated with Exonuclease I and Shrimp Alkaline Phosphatase as recommended by the manufacturer (AcycloPrime-FP SNP Detection Kit, Perkin Elmer, Wellesley, MA). Single-base extension (SBE) detection primers used were (274C>T): (sense) 5'-GGAAAGCAATGCTCATGAG-3', (anti-sense) 5'-TTCACGGGGCCTGGCTT-3', (469+14G>C): (sense) 5'-TGGTTCTCCCTGTCCAGG-3' and (anti-sense) 5'-TAAGGTGAGCTTGGGGG-3'. FP-SBE reactions were performed in one or both orientations as suggested by the manufacturer (AcycloPrime-FP SNP Detection Kit, Perkin Elmer, Wellesley, MA). After the addition of reading buffer, the plates were read using the Analyst HT® reader (Molecular Devices, CA) as described previously. [63]

Variants D543N and 1729+55del4 were genotyped by TaqMan assays. [64] Each variant was analysed using 2 sets of oligonucleotides (external primers and internal probes) designed using the Primer3 software. The internal probes were labelled with fluorescent dyes; TAMRA (6-Carboxytetramethyl-rhodamine) at 3' ends, FAM (6-Carboxy-fluorescein) and TET (6-Carboxy-4,7,2',7'-Tetrachlorofluoroscein) (one per oligonucleotide) at 5' ends. For variant D543N, primers set 5'-CCACCACCACTTCCTGTATG -3', 5'-CACGTCATACATGCCACTCC -3', and probes set 5'-FAM-

CCCTTTCTGGTCCTCTTCAAGGA-TAMRA - 3' and 5'-TET-CCCTTTCTGGTTCTCTTCAAGGAGC-TAMRA were used. For variant 1729+55del4, primers set 5'-GGGAGTGGCATGTATGACG-3', 5'-TCTATCCTGCTGCCTGCAC-3', and probes set 5'-FAM-TGGCCTGCTGGATGTGGAG-TAMRA and 5'-TET-TGACTGGCCTGCTGGAGAGG-TAMRA were used. For both variants, 10 ng of genomic DNA were added to a 20 µl reaction mixture containing 1X PCR buffer, 5.0 mM of $MgCl_2$, 0.2 mM of each nucleotide, 0.5 Unit of HotstartTaq DNA polymerase (Qiagen), 0.03 µM of probes and 0.30 mM of primers. PCR was initiated by denaturing the samples at 96°C for 10 minutes followed by 40 cycles of denaturation at 96°C for 30 seconds, annealing and extending at 60°C for 1 minute. Final extension was done at 72°C for 5 minutes. PCR endpoint fluorescence reading was done using ABI PRISM® 7700 Sequence Detector System (Applied Biosystems, CA). The fluorescence intensity was adjusted and recorded using Sequence Detector Software version 1.7 (Applied Systems, CA).

Statistical Analysis

Hardy-Weinberg equilibrium was tested in a subset of independent DNA samples (parents of probands) using HAPLOVIEW. [65] Allele distribution patterns were assessed by the family-based association test (FBAT, version 1.5). [33, 34] This software uses an empirical variance-covariance estimator to account for the possibility of nonindependent allelic transmission to affected sibs. [66] Asthma, atopy and IgE level phenotypes were tested separately under additive and dominant genetic models.

Associations between variants were assessed by calculating D', a measurement of the LD strength [67] using HAPLOVIEW. [65] Based on the LD strength of variants, haplotypes were inferred and assessed for nonrandom transmission using the "hbat"command of FBAT version 1.5. [33, 34] An

empirical variance estimator was used. [66] Asthma, atopy and IgE level phenotypes were tested separately under additive and dominant genetic models.

To assess whether the cohort size has sufficient power to detect association between variants and phenotypes, we used Power calculation of the Family Based Association Tests (PBAT). [68, 69] The family design was based on the observed count of nuclear families, according to the number of affected sibs, unaffected sibs and missing parents. The genetic models considered assumed a constant population prevalence of 0.10, varying susceptibility allele frequencies (*q*) and varying genetic attributable fraction of the allele (GAF), under an additive model for risk. GAF corresponds to a reduction of incidence in the study families when the risk variant is removed, i.e. a GAF of 5% indicates that removal of the risk variant would reduce incidence by 5% in the study families. We used values for q ranging from 0.05 to 0.70 and values for GAF ranging from 0.05 to 0.50. For an additive disease model with prevalence at 0.10, the odds ratio for the heterozygotes was calculated for each q and GAF parameter set. The level of significance was set at 0.05.

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Chapter 4

Association Studies of Vitamin D Receptor Genetic Variants with Tuberculosis

The identification of *VDR* as a susceptibility gene to both TB and asthma / susceptibility gene underlies its importance in validating the hygiene hypothesis. *VDR* genetic variants had previously been found to be associated with TB susceptibility, however, with inconsistent results. To better characterize the nature of the *VDR* – TB association i.e. to define stages of pathogenesis that are associated with specific *VDR* regions, 14 SNPs of the *VDR* gene were investigated in a Mexican TB case-control study.

Association Studies of Vitamin D Receptor Genetic Variants with Tuberculosis

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ABSTRACT

OBJECTIVE: To define genetic risk factors for tuberculosis of the vitamin D receptor (*VDR*) gene in a case - control population from Orizaba, Mexico, and pediatric TB families from Houston, Texas, USA.

RESULTS: Fourteen single nucleotide polymorphisms (SNPs) spanning the *VDR* gene were studied in both samples. In the case - control study, the *Fok*I polymorphism was associated with TB susceptibility (allele 'T', p = 0.01; genotype 'TT', p = 0.04) and susceptibility to *Mycobacterium tuberculosis* infection (allele 'T', p = 0.02, genotype 'TT', p = 0.024), and SNP rs4760655 (allele 'G', p = 0.03) was associated with *M. tuberculosis* infection. In addition, *Fok*I (allele 'T', p = 0.02, genotype 'TT', p = 0.03) and *Bsm*I (allele 'G', p = 0.03 and genotype 'GG', p = 0.05) variants were associated with progression from infection to disease. Based on linkage disequilibrium (LD) pattern of the *VDR* genomic region, haplotypes of 3 intronic SNPs located in the middle part of the *VDR* gene were associated with disease progression (p = 0.005). In the pediatric cohort, variants rs4760655 (allele 'A', p = 0.01) and *Fok*I (allele 'C', p = 0.02) were associated with TB susceptibility. Haplotype analysis detected under - transmission of a promoter region haplotype (p = 0.009) that had not been observed in the case-control study.

CONCLUSION: Our results are consistent with the suggestion of *VDR* variants as genetic risk factors for TB. However, the causative variants have not been identified and the mechanisms on how *VDR* alleles impact on VDR function are unknown but will likely need to be interpreted in the context of specific gene – environment interactions.

INTRODUCTION

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), can modulate the neural, immune, and endocrine systems following binding to the vitamin D receptor (VDR) (reviewed in [1-2]). Due to the pleiotropic effects of $1,25[OH]_2D_3$ on cell and physiological functions, its receptor has been the target for several genetic association studies with human disease. Four genetic variants have been widely studied (*Fok*I T>C, *Bsm*I G>A, *Apa*I C>A and *Taq*I C>T), and observed to be associated with serum osteocalcin levels [3], bone mineral density [4-7], prostate cancer [8], hyperparathyroidism [9], insulin-dependent diabetes mellitus [10-11], Crohn's disease [12], leprosy [13], immunodeficiency syndrome [14] and asthma / atopy [15-16]. In particular, associations of *VDR* genetic variants and TB susceptibility have been explored in various epidemiological settings [17-23] (Table 1). In 5 out of 7 populations studied, significant associations of *VDR* variants with TB susceptibility were detected. However, there was little consistency with respect to the identity of the risk variant(s).

At the translation start site, variant *Fok*I corresponds a thymidine to cytosine nucleotide change resulting in the elimination of the methionine start site, and truncation of the encoded protein by 3 amino acids [24-26]. The *Fok*I variant has been tested for TB susceptibility in 5 cohorts and 3 significant associations were detected. Yet, the risk genotype and the specific TB susceptibility phenotypes found to be associated differed among the studies. For example, in a Chinese study, the *Fok*I 'TT' genotype was found over - represented in pulmonary TB patients [21], whereas in a West African study, it was the *Fok*I 'C' allele that was significantly associated with pulmonary TB, and only in the presence of another allelic variant *Apa*I 'T' [20]. The *Taq*I 'CC' genotype at the 3'end of the *VDR* gene was found to be associated with TB protection in a Gambia study [19]. In another West African study no such association was observed for variant *Taq*I alone, while a global association

was detected in combination with other variants [20]. In a small Indian study, a different genotype, *Taq*I 'TT', was found to be associated with TB protection in female subjects [18]. In Gujurati Asians, no association was detected with variant *Taq*I alone, but genotypes *Taq*I 'TT', *Taq*I 'TC' and the presence of 25-hydroxycholecalciferol deficiency were associated with TB susceptibility [17]. One possible explanation for the inconsistent results across studies is that these markers are not causative, but in LD with the causative variants. However, detailed investigation of *VDR* gene LD pattern in TB susceptibility studies has not been conducted.

We undertook an association study between *VDR* genetic variants and TB susceptibility in a Mexican case control population. To allow the analysis of LD pattern, we genotyped 14 genetic variants (including *Fok*I, *Bsm*I and *Taq*I) across a 94 kb region on chromosome 12 harbouring the *VDR* gene. The recruitment scheme of the Mexican TB population allowed for the study of 2 other TB susceptibility phenotypes: infection to *M. tuberculosis* and disease progression from infection. To validate the findings for disease progression, a replication cohort of pediatric TB families was used.

Table 1: Genetic association studies of VDR and TB related phenotypes

Authors	Ethnic Group	Study Site	Sample Size	TB related Phenotype	SNPs Tested	Association
Bellamy R. *et al*., 1999 reference (ref.) 19	Africans	Gambia	408 pulmonary TB (PTB) cases 414 controls	PTB	*Taq*I T>C	*Taq*I 'CC' genotype is the protective factor for TB (p=0.01)
Wilkinson R. *et al*., 2000 (ref. 17)	Gujarati Asians	West London	126 tuberculosis (TB) patients 116 controls	TB	*Taq*I T>C, *Bsm*I G>A and *Fok*1 C>T	*Taq*I 'TT', 'TC' genotypes and 25-hydroxycholecalciferol deficiency were associated with TB (OR=2.8, 95%CI= 1.2-6.5). *Fok*I 'TT' genotype or 25-hydroxycholecalciferol deficiency were associated with TB (OR=5.1, 95%CI=1.4-18.4).
Selvaraj P. *et al*., 2000 (ref. 18)	Southern Indians	India	202 cases 109 controls (47 female cases) (33 female controls)	PTB	*Taq*I T>C	*Taq*I 'TT' genotype is the protective factor and 'CC' genotype is the risk factor for TB in female subjects (p<0.02 for both).
Delgado J *etal.,* 2002 (ref.23)	Cambodians	Cambodia	358 PTB cases 106 controls	PTB	*Fok*I C>T *Taq*I C>T	F*ok*I variant was not informative. *Taq*I variant was not associated with PTB.
Liu W. *et al*., 2004 (ref. 21)	Chinese	China	120 PTB cases 240 controls	PTB	*Fok*I C>T, *Taq*I C>T	*Fok*I 'TT' genotype was associated with pulmonary TB (p=0.002).
Roth D *et al*., 2004 (ref. 22)	Peruvians	Peru	103 PTB caess, 206 healthy control, 78 treated PTB cases	PTB, treatment to PTB (fast sputum mycobacterial culture and auramine stain conversions)	*Fok*I C>T, *Taq*I C>T	No association between *Fok*I, *Taq*I and PTB. *Fok*I 'CC' was associated with faster sputum mycobacterial culture and auramine stain conversions (p=0.03 and 0.025, respectively). *Taq*I 'TC'genotype was associated with faster sputum mycobacterial culture conversion (p=0.012).
Selvaraj P. *et al*., 2004 (ref. 46)	Southern Indians	India	46 PTB cases and 64 normal healthy controls	phagocytic potential of macrophages, lymphocyte response to *M. tuberculosis*	*Fok*I C>T, *Bsm*I G>A, *Apa*I A>T, *Taq*I T>C,	*Bsm*I 'AA', *Apa*I 'AA', *Taq*I *'CC'* and *Fok*I 'CC' genotypes were associated with increased phagocytic potential in normal individuals (p=0.017, 0.016, 0.034 and 0.013; respectively). *Bsm*I 'TT', *Taq*I 'CC' and *Fok*I 'CC' genotypes were associated with decreased lymphocyte response in normal individuals (p=0.0009, 0;.016 & 0.008; respectively).
Bornman L. *et al*., 2004 (ref. 20)	Africans	The Gambian, Guinea and Guinea-Bissau	417 PTB cases, 722 healthy controls, 436 TB families	PTB	*Fok*I C>T, *Bsm*I G>A, *Apa*I A>T, *Taq*I T>C	A global haplotype containing *Fok*I 'C' and *Apa*I 'A' was associated with PTB (p=0.0063).

STUDY POPULATIONS AND METHODS

Patients and controls

A detailed description of patients and controls for the case-control cohort has been given previously [27]. Briefly, patients were recruited following 2 recruitment schemes: (1) health promoters visited households, shelters, jails, orphanages, and self support groups for alcoholics, diabetics and drug users to identify individuals with cough persisting for more than 2 weeks, and (2) recruiters reviewed the TB register of the local Tuberculosis Prevention and Control Program to identify TB patients. Coughers recruited by scheme 1 were asked to produce 3 sputum samples for Acid Fast Bacilli (AFB) staining. AFB smear-positive individuals were offered to participate. Recruited TB patients underwent physical examination, human immunodeficiency virus testing, chest radiography and a standardized interview focusing on clinical history and social behavior. A total of 218 individuals with pulmonary TB participated in the study as patients (P). Two groups of controls were recruited; (1) unrelated individuals who lived in the same household as the patients and suffered a persistent cough but were AFB smear - negative were recruited as household controls (HC) (n = 215); and (2) non coughers who lived in the same neighborhood as the patients were recruited as community controls (CC) (n = 214). All participants are HIV negative and gave informed consent. A family-based association cohort of pediatric TB was recruited from Harris county, Texas. The diagnosis of TB was by culture confirmation or by fulfilling clear clinical criteria of disease [28, 29]. Informed consent was obtained from all study participants. The study received ethical clearance from the Institutional Review Board at Baylor College of Medicine, Houston, Texas, USA, Instituto Nacional de Salud Pública, Cuernavaca, Mexico and the Ethics Committee at the Research Institute of the McGill University Health Centre, Montreal, Québec, Canada.

DNA extraction

DNA samples of the Mexican cohort was extracted from blood. Briefly, an equal volume of lysis buffer 2x and proteinase K was added to blood to a final concentration of 100 µg/ml and incubated overnight at 37 °C. An equal volume of phenol was added to the lyzed blood. The phenol containing tubes were then rotated at room temperature for 30 min, and centrifuged at 2000 rpm for 30 minutes at room temperature. The phenolic phase was discarded, and an equal volume of new phenol was added. The tubes were then rotated and centrifuged in the same fashion as previously and the phenolic phase was discarded. An equal volume of chloroform was added, and tubes were rotated and centrifuged as above. The chloroform phase was discarded and three times volume of ethanol was added until DNA precipitation. DNA samples of the pediatric cohort was extracted from blood using the Nucleon extraction kit (Pharmacia – Amersham).

Genotyping

We investigated 93.73 kb of genomic DNA harboring the *VDR* gene, extending from chromosome 12 position 46,614,960 to position 46,492,363 on the July 2003 freeze of the University of California Santa Cruz genome browser http://genome.ucsc.edu. A panel of 14 SNPs were selected from public databases (National Center for Biotechnology Information (NCBI) and the SNP Consortium) based on (1) the location in the gene, (2) relative distances between SNPs and (3) known associations with diseases. SNPs described in this report are cited using their reference SNP identifier from the NCBI database, except for those that have commonly used aliases (*Fok*I, *Bsm*I and *Taq*I).

SNP genotyping was performed using SNPstream Ultra High Throughput System (Orchid biosciences, Princeton, NJ) [30]. Protocols and reaction conditions have previously been described [31]. See Table 2 for primer sequences used in genotyping.

Statistical Analysis

All variants were tested for Hardy-Weinberg equilibrium using HAPLOVIEW in the case – control study (P, HC and CC groups individually) and the pediatric TB cohort (parents of probands) [32].

Differences in allele and genotype distributions for the adult TB cohort were assessed by χ^2 tests, and Fisher's exact test was used where appropriate. Three phenotypes were tested for association with *VDR* alleles: (1) for susceptibility to TB, community controls and patients were compared using a 2x2 χ^2 test with 1 degree of freedom for alleles and 3x2 χ^2 test with 2 degrees of freedom for genotypes; (2) for susceptibility to *M. tuberculosis* infection, community controls were compared to combined household controls and patients; (3) for disease progression, household controls and patients were compared.

Allelic transmission patterns for the pediatric cohort were assessed by the family-based association test (FBAT, version 1.5.5) [33-34]. This software uses an empirical variance-covariance estimator to account for the possibility of nonindependent allelic transmission to affected sibs [35]. TB susceptibility was tested under an additive model.

Associations between *VDR* variants were assessed in the case-control (the combined controls group) and the pediatric cohorts using HAPLOVIEW [32]. Strength of LD between pairs of SNPs was measured as D' [36]. Regions of strongly associated markers (LD blocks) were inferred as proposed by

Gabriel and colleagues [37] and implemented in HAPLOVIEW. However, the upper confidence limit of D' for the outermost SNP pair of a block was set from 0.98 to 0.90 in both cohorts.

Haplotypes within LD blocks were inferred and associations were tested using PHASE version 2.1.1 [38-39] for the adult cohort and FBAT (version 1.5.5) [33-34] for the pediatric cohort. Haploview derived LD blocks were tested by permutation for evidence of association with TB phenotypes in the case – control study. The PHASE software version 2.1.2 tests for significant differences in haplotype frequencies in different groups and the null hypothesis that the haplotypes in the different groups are a random sample from a single set of haplotype frequencies [38-39].

Table 2: Oligonucleotides used in genotyping assays

SNP ID	Oligonucleotide Name	Sequence (5' to 3')
rs4516035	VDRrs4516035U11CTu	ATAATCTTCTGGAATAGAAATGCTCA
	VDRrs4516035U11CTl	ACCCTTTCACCTTGTCCCT
	VDRrs4516035U11CTsu	AGAGCGAGTGACGCATACTAGATGACCTCCTTTAGCCAGGGAAGA
rs4760655	VDRrs4760655U11GAu	TGTTCTCAGGTCAACTTGTTCA
	VDRrs4760655U11GAl	TTAACCTGCATGGAACTCTCC
	VDRrs4760655U11GAsu	AGAGCGAGTGACGCATACTACTCTCAGCTCATAAACATTTACTGC
rs2238136	VDRrs2238136U3CTu	AGGGAGCATCTGAGGAAAA
	VDRrs2238136U3CTl	AGCAGACACCTCCCACTG
	VDRrs2238136U3CTsu	CGTGCCGCTCGTGATAGAATACTA-GTGGACAATGAGCCAAGATAA
rs2408876	VDRrs2408876U8CTu	TACCAAGACAATCAACATTTCAAG
	VDRrs2408876U8CTl	TAGATGATAATGCCTGTTTAGAAAAA
	VDRrs2408876U8CTsu	GTGATTCTGTACGTGTCGCCTATTTCCCCCCCCTTTTTTTGGCAA
*Fok*I	VDRFok1U7CTu	ACTGACTCTGGCTCTGACCG
	VDRFok1U7CTl	TCAAAGTCTCCAGGGTCAG
	VDRFok1U7CTsu	AGGGTCTCTACGCTGACGATGGC-CTGCTTGCTGTTCTTACAGGGA
rs2239179	VDRrs2239179U4CTu	TATCCTCTGTCCCTGACACA
	VDRrs2239179U4CTl	ATGCGGACCCTCCTGGCTAT
	VDRrs2239179U4CTsu	AGCGATCTGCGAGACCGTATCTTCC-TGTTACCTGACCTCTCCCCA
rs1540339	VDRrs1540339U4CTu	GGAGATGGGACTGTGCTG
	VDRrs1540339U4CTl	TTAAGAGGCTTCACACACATTCT
	VDRrs1540339U4CTsu	AGCGATCTGCGAGACCGTATCACA-CCTTGTTGGTGCCCACCCTAA
rs2107301	VDRrs2107301U4CTu	TTGGCTTCGTTAAGGAGAG
	VDRrs2107301U4CTl	TTGACTTCATTTTAAGCTCCTTG
	VDRrs2107301U4CTsu	AGCGATCTGCGAGACCGTATACAT-GTCTTGCATGGGAATAACTTG
rs2239182	VDRrs2239182U1CTu	TTTTTCAATGGATTGAACCTAAG
	VDRrs2239182U1CTl	ATCACCAGACAGCCCAAC
	VDRrs2239182U1CTsu	ACGCACGTCCACGGTGATTTGAT-ATATGAAGCCATTGACCTAGAA
rs2239185	VDRrs2239185U12GAu	AAACAGCAACACAATTCCAGT
	VDRrs2239185U12GAl	CCTCCCTCACCTGTGTGA
	VDRrs2239185U12GAsl	CGACTGTAGGTGCGTAACTCCAT-TTACACCCTCCTCTGTCTTCAC
*Bsm*I	VDRrs1544410U10CTu	AAAGTTTTGTACCCTGCCC
	VDRrs1544410U10CTl	ATTCTGAGGAACTAGATAAGCAGG
	VDRrs1544410U10CTsu	AGATAGAGTCGATGCCAGCTGAG-CAGAGCCTGAGTATTGGGAATG
*Taq*I	VDRrs731236U12CTu	TTCTTCTCTATCCCCGTGC
	VDRrs731236U12CTl	ATGTACGTCTGCAGTGTGTTG
	VDRrs731236U12CTsu	CGACTGTAGGTGCGTAACTCCTG-GGGTGCAGGACGCCGCGCTGAT
rs2853563	VDRrs2853563U8CTu	TTACATTTACAAATGTCTATTTCACACTC
	VDRrs2853563U8CTl	TAGGAGCTGGGAGGAAAAG
	rs2853563CTU9	GACCTGGGTGTCGATACCTACATTCTAAAGTAGAATCGATGATGA
rs757344	VDRrs757344U8GAu	TGTTCCAGGCCGCGATGG
	VDRrs757344U8GAl	ACGAAGAACAGAAACTGGGC
	VDRrs757344U8GAsl	GTGATTCTGTACGTGTCGCCCTAG-CCCCAGTTCACAGAAAAAACC

RESULTS

Characteristics of patients and controls

The case - control population consisted of 218 smear – positive TB patients and 429 controls recruited from the Orizaba Health Region in the province of Vera Cruz located in South – Eastern Mexico. Among the controls were 2 subgroups. The first subgroup (n = 215) termed "household controls", consisted of biologically – unrelated individuals living in the same household as the patients. These individuals suffered from persistent cough but were AFB smear negative, hence they were presumed to be latently infected with *M. tuberculosis.* The second subgroup (n = 214), termed "community controls", consisted of healthy individuals living in the same neighborhood as the patients. They are presumed to be uninfected with *M. tuberculosis.* Of the 647 individuals, 34 had no data on ethnicity (n = 8 [3.7%], 13 [6%] and 15 [14.7%] for patients, household controls and community controls, respectively). The majority of patients, household and community controls described themselves as having mixed Mexican heritage (n=180 [86%], 180 [89%] and 181 [95%]; respectively). A total of 21 (10%) patients, 17 (8.4%) household controls and 17 (8.5%) community controls belonged to the indigenous Náhuatl population. Only 1 patient and 1 household control belonged to the indigenous Totonaco population. The remaining individuals described themselves as belonging to a "other" catergory. The male: female ratios are 1:1 for patients, 0.3:1 for household controls and 1:1 for community controls.

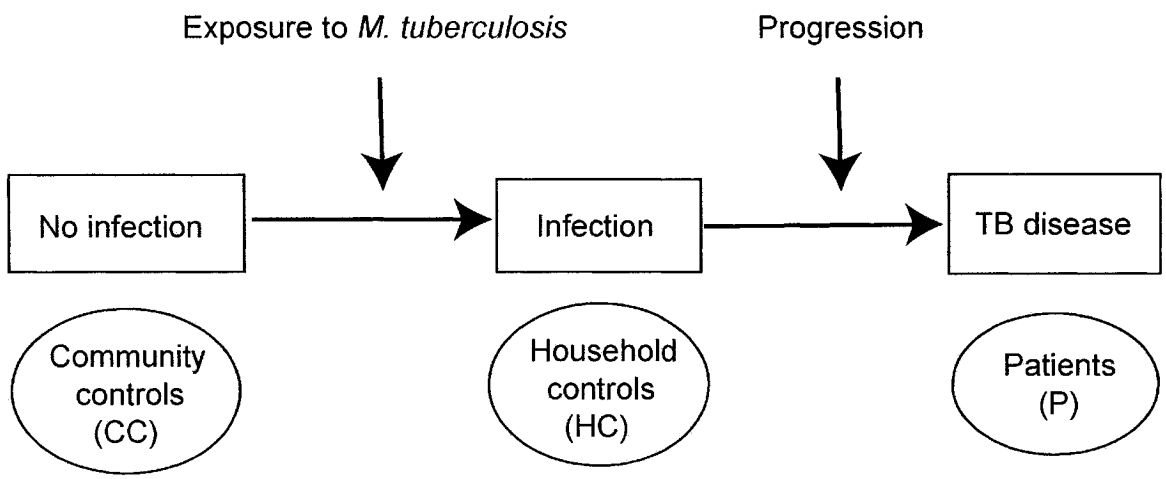
Patients and control groups were compared differently in the analyses to study specific phenotypes of TB susceptibility. For the phenotype TB susceptibility patients (individuals with the disease) were compared to community controls (healthy uninfected individuals). For the phenotype

susceptibility to *M. tuberculosis* infection patients and household controls (individuals who are infected) were combined and compared to community controls. For the phenotype disease progression patients were compared to household controls (Figure 1).

Association Analysis of *VDR* with Susceptibility to TB and related phenotypes

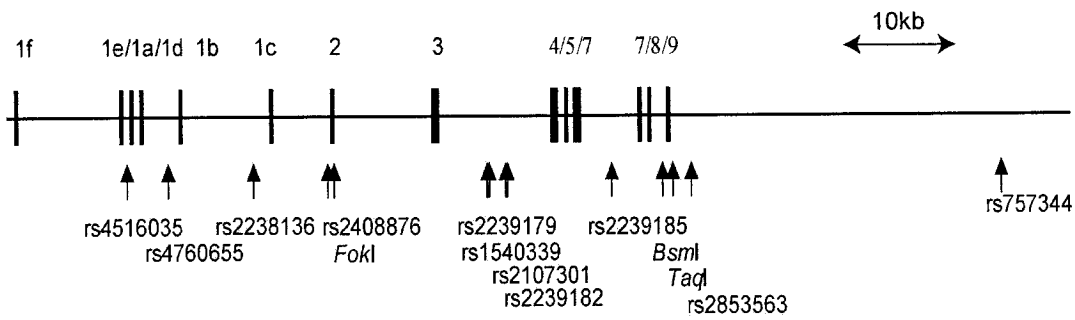
Fourteen single nucleotide polymorphisms (SNPs) were genotyped in the case - control TB population (see Figure 2). Among these SNPs are the three widely studied variants: *Fok*I T>C (rs2228570) [17,20-21,31,40-41], *Bsm*I G>A (rs1544410) [4,9,11,31,42-43] and *Taq*I T>C (rs731236) [17,19-20,31,41]. The T>C change for variant *Fok*I eliminates the translation start codon methionine, resulting in a protein that is shortened by the 3 most N – terminal amino acids. Variant *Taq*I corresponds to a synonymous isoleucine > isoleucine amino acid change in exon 9. The remaining 12 SNPs locate to noncoding regions, between 26.9kb upstream of the translation start site at exon 2 and 32kb downstream of exon 9 (Figure 2). All variants are in Hardy-Weinberg equilibrium. Of the 14 SNPs, variants rs2238138 and rs2853563 have minor allele frequency <0.05.

Figure 1: Study design and subjects classification scheme. Operationally, tuberculosis pathogenesis can be divided into 3 distinct stages that are shown in boxes: uninfected with *M. tuberculosis,* infected with *M. tuberculosis* but no clinical disease, and patients displaying clinical symptoms of the disease. The 3 groups of subjects community controls, household controls and patients corresponding to the three stages of tuberculosis pathogenesis are indicated in circles. Group comparisons that were used to study *VDR* polymorphisms impacting on different stages of tuberculosis are given below the pathogenesis scheme.



Phenotypes	Comparison groups
(case - control study)	
Susceptibility to disease	CC versus P
Susceptibility to *M. tuberculosis* infection	CC versus (HC + P)
Disease progression	HC versus P
(replication study)	
Disease progression	Not Applicable

Figure 2: Schematic representation of the genomic organization of *VDR* and location of studied SNPs. Exons are represented by black boxes connected by a straight line representing introns and 3'and 5' noncoding regions. Locations of exons 1a-1f are described elsewhere[48]. Positions and names of the 14 SNPs analyzed are represented by arrows below the gene structure. Distances are drawn to scale (a distance of 10kb is scaled by the length of the double arrow line). Chromosome position is based on the July 2003 freeze of the University of California Santa Cruz genome browser http://genomeluscsc.edu/. SNPs rs4516035, rs2408876, *Fok*I (rs2228570) and *Taq*I (rs731236) are T>C nucleotide changes. *Fok*I corresponds to methionine > threonine amino acid change. SNPs rs4760655, rs2239179, rs2239182 and rs757344 are A>G nucleotide changes. SNPs rs2238136, rs154-339, *Bsm*I (rs1544410) and rs2853563 are G>A nucleotide changes. SNPs rs2107301 and rs2239185 are C>T nucleotide changes.


10kb
1f
1e/1a/1d
1b
1c
2
3
4/5/7
7/8/9
rs4516035
rs4760655
rs2238136
rs2408876
FokI
rs2239179
rs1540339
rs2107301
rs2239182
rs2239185
BsmI
TaqI
rs2853563
rs757344

All *VDR* polymorphisms were tested individually for allelic and genotypic associations with three phenotypes: (1) susceptibility to TB, (2) susceptibility to *M. tuberculosis* infection and (3) disease progression (Tables 3 and 4). Variants *Fok*I and *Bsm*I were associated with susceptibility to TB. Specifically, allele *Fok*I 'T' was over represented in patients compared to community controls (allele 'T': p = 0.01, Odds Ratio (OR)=1.43 (95% CI=1.09, 1.90). In addition, genotypes *Fok*I 'TT' and *Bsm*I 'AA' were more frequent among patients than among community controls (p=0.04; OR= 1.5 (95% CI=0.99, 2.34), and p=0.02, OR=5.13 (95% CI=1.09, 24.15), respectively). Allele rs4760655 'G' was over-represented in the combined patients and household controls compared to community controls (p=0.03, OR=1.33 (95%CI=1.03-1.71)), identifying this SNP as risk factor for infection with *M. tuberculosis.* Finally alleles *Fok*I 'T' and *Bsm*I 'A' were over-represented in patients compared to household controls (p=0.02, OR=1.38 (95%CI=1.06-1.82); p=0.03, OR=1.60 (95% CI=1.06-2.41), respectively), and their homozygous genotypes *Fok*I 'TT' and *Bsm*I 'AA' were also significantly more frequent in the patients than in the household controls (p=0.024, OR=1.77 (1.15, 2.72); p=0.02, OR=5.3 (95% CI=1.12, 24.92)). These results suggest VDR as genetic modulator of speed of progression from infection to disease.

Haplotype – specific Associations

Associations among the 14 SNPs were assessed by measuring pair-wise LD using D'. We defined LD blocks in the combined controls group using the definition proposed by Gabriel *et al.*[37]. Three blocks of LD were observed (Figure 3A). Block 1 is located toward the 5'end of *VDR*, contains variants rs4516035 and rs4760655 and spans roughly 5.7kb. Block 2 is located in intron 3, approximately 37kb downstream of block 1, contains variants

rs2239179, rs1540339 and rs2107301 and is approximately 2.2kb in length. Block 3, containing variants rs2239185, *Bsm*I and *Taq*I, is located near the 3'end of the gene and extends for 5.8 kb from intron 7 to exon 9. The association between the blocks, as measured by D', is greater for blocks 2 and 3 (D'=0.69) as compared to blocks 1 and 2 (D'=0.23). Within each block, 3 to 4 common haplotypes (frequency >0.1) were observed: (block 1) haplotypes TG (frequency =0.64), CA (0.26) and TA (0.10); (block 2) haplotypes AGC (0.37), GGC (0.31), AAT (0.16) and AAC (0.16); (block 3) haplotypes CGT (0.64), TGT (0.22) and TAC (0.13) (Figure 3A). Of these LD blocks, only block 2 was significantly associated with disease progression (p=0.005) (Table 5). Among the 4 common haplotypes within block 2, haplotype AAT gives the greatest difference in frequencies between patients (frequency =0.24) and household controls (frequency =0.20). No other haplotype in the 3 blocks was found to be associated with any TB related phenotypes.

Replication of *VDR* Association with TB in a Pediatric Family Based Study

The most consistent result of the case-control study was an association of *VDR* alleles, genotypes and haplotypes with progression from infection to TB disease. Rapid progression from infection to disease is a hallmark of primary TB. Pediatric TB disease is in its majority primary TB (Figure 1). Hence, we decided to replicate the findings from the case-control study in a panel of pediatric TB cases. To avoid potential confounding of association by improper selection of controls, we opted for a family-based design. In such a design, deviations from the 50:50 transmission ratio of biallelic marker alleles from informative parents to affected children are indicative for association of the marker with disease [44]. The same 14 *VDR*

Table 3 : Allelic frequencies and associations of *VDR* polymorphism with TB observed in Mexican TB cases and controls

Marker	Allele	Patients (frequency)	HC (frequency)	CC (frequency)	P-value*, OR (95% CI)	P-value†, OR (95% CI)	P-value‡, OR (95% CI)
rs4516035	T	288 (0.769)	293 (0.755)	276 (0.735)			
	C	86 (0.231)	95 (0.245)	100 (0.265)	NS	NS	NS
rs4760655	G	258 (0.669)	261 (0.668)	233 (0.606)		0.03, OR=1.33 (1.03-1.71)	
	A	128 (0.331)	125 (0.332)	151 (0.394)	NS		NS
rs2238136	G	300 (0.957)	298 (0.962)	301 (0.960)			
	A	94 (0.043)	94 (0.038)	95 (0.040)	NS	NS	NS
rs2408876	T	295 (0.767)	297 (0.758)	292 (0.750)			
	C	89 (0.233)	95 (0.242)	98 (0.250)	NS	NS	NS
*Fok*I	T	264 (0.611)	230 (0.544)	229 (0.540)	0.01, OR=1.43 (1.09-1.90)		0.02, OR=1.38 (1.05-1.82)
	C	158 (0.389)	190 (0.456)	197 (0.460)		NS	
rs2239179	A	286 (0.719)	280 (0.707)	262 (0.665)			
	G	114 (0.281)	116 (0.293)	130 (0.335)	NS	NS	NS
rs1540339	G	246 (0.650)	261 (0.659)	262 (0.690)			
	A	132 (0.350)	135 (0.341)	118 (0.310)	NS	NS	NS
rs2107301	C	300 (0.796)	320 (0.816)	322 (0.849)			
	T	76 (0.204)	72 (0.184)	58 (0.151)	NS	NS	NS
rs2239182	A	259 (0.690)	263 (0.674)	250 (0.656)			
	G	117 (0.310)	127 (0.326)	130 (0.344)	NS	NS	NS
rs2239185	C	255 (0.638)	262 (0.658)	253 (0.632)			
	T	147 (0.362)	136 (0.342)	145 (0.368)	NS	NS	NS
*Bsm*I	G	317 (0.835)	361 (0.889)	361 (0.867)			
	A	63 (0.165)	45 (0.111)	55 (0.133)	NS	NS	0.03, OR=1.60 (1.06, 2.41)
*Taq*I	T	338 (0.847)	348 (0.874)	345 (0.856)			
	C	62 (0.153)	50 (0.126)	57 (0.144)	NS	NS	NS
rs2853563	G	391 (0.982)	395 (0.992)	393 (0.987)			
	A	7 (0.018)	3 (0.008)	5 (0.013)	NS	NS	NS
rs757344	A	299 (0.762)	301 (0.764)	302 (0.772)			
	G	93 (0.238)	93 (0.236)	90 (0.228)	NS	NS	NS

* susceptibility to TB, †susceptibility to *M.tb* infection, ‡disease progression, §not significant

Table 4: Genotypic frequencies and associations of *VDR* polymorphism with TB observed in Mexican TB cases and controls

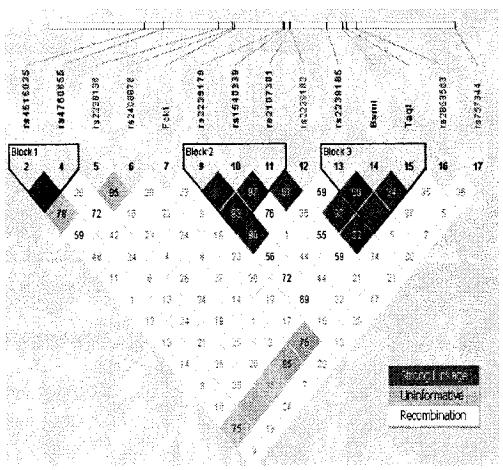
Marker	Genotype	Patients	HC	CC	P-value* (OR)	P-value† (OR)	P-value‡ (OR)
rs4516035	TT	111 (0.594)	106 (0.546)	96 (0.511)			
	TC	66 (0.353)	81 (0.418)	84 (0.447)	NS§	NS	NS
	CC	10 (0.053)	7 (0.036)	8 (0.043)	NS	NS	NS
rs4760655	GG	87 (0.451)	88 (0.456)	68 (0.354)			
	GA	84 (0.435)	85 (0.440)	97 (0.505)	NS	NS	NS
	AA	22 (0.144)	20 (0.104)	27 (0.141)	NS	NS	NS
rs2238136	GG	114 (0.579)	110 (0.561)	116 (0.586)			
	GA	72 (0.365)	78 (0.398)	69 (0.348)	NS	NS	NS
	AA	11 (0.056)	8 (0.041)	13 (0.066)	NS	NS	NS
rs2408876	TT	113 (0.587)	112 (0.571)	109 (0.559)			
	TC	69 (0.359)	73 (0.372)	74 (0.379)	NS	NS	NS
	CC	10 (0.052)	11 (0.056)	12 (0.062)	NS	NS	NS
*Fok*I	TT	87 (0.412)	60 (0.286)	64 (0.300)	0.04, OR*=1.5 (0.99, 2.34)		0.024, OR*=1.77 (1.15, 2.72)
	TC	90 (0.427)	110 (0.524)	101 (0.474)		NS	
	CC	34 (0.161)	40 (0.190)	48 (0.255)	NS	NS	NS
rs2239179	AA	102 (0.510)	94 (0.475)	90 (0.459)			
	AG	82 (0.410)	92 (0.465)	82 (0.418)	NS	NS	NS
	GG	16 (0.080)	12 (0.061)	24 (0.122)	NS	NS	NS
rs1540339	GG	82 (0.434)	87 (0.439)	88 (0.463)			
	GA	82 (0.434)	87 (0.439)	86 (0.453)	NS	NS	NS
	AA	25 (0.132)	24 (0.121)	16 (0.084)	NS	NS	NS
rs2107301	CC	120 (0.638)	134 (0.684)	138 (0.726)			
	CT	60 (0.319)	52 (0.265)	46 (0.242)	NS	NS	NS
	TT	8 (0.043)	10 (0.051)	6 (0.032)	NS	NS	NS
rs2239182	AA	93 (0.495)	85 (0.436)	86 (0.453)			
	AG	73 (0.388)	93 (0.477)	78 (0.411)	NS	NS	NS
	GG	22 (0.117)	17 (0.087)	26 (0.137)	NS	NS	NS
rs2239185	CC	86 (0.428)	81 (0.407)	87 (0.437)			
	CT	83 (0.413)	100 (0.503)	79 (0.397)	NS	NS	NS
	TT	32 (0.159)	18 (0.090)	33 (0.166)	NS	NS	NS
*Bsm*I	GG	136 (0.716)	160 (0.788)	155 (0.745)			
	GA	45 (0.237)	41 (0.202)	51 (0.245)	NS	NS	NS
	AA	9 (0.047)	2 (0.010)	2 (0.010)	0.02 OR=5.13 (1.09, 24.15)	NS	0.02, OR=5.29 (1.12, 24.92)
*Taq*I	TT	144 (0.720)	153 (0.769)	146 (0.726)			
	TC	50 (0.250)	42 (0.211)	53 (0.264)	NS	NS	NS
	CC	6 (0.030)	4 (0.042)	2 (0.010)	NS	NS	NS
rs2853563	GG	192 (0.965)	196 (0.985)	194 (0.975)			
	GA	7 (0.035)	3 (0.015)	5 (0.025)	NS	NS	NS
	AA	0	0	0	NS	NS	NS
rs757344	AA	120 (0.612)	115 (0.584)	120 (0.612)			
	AG	59 (0.301)	71 (0.360)	62 (0.316)	NS	NS	NS
	GG	17 (0.087)	11 (0.056)	14 (0.071)	NS	NS	NS

* susceptibility to TB, †susceptibility to *M.tb* infection, ‡disease progression, §not significant

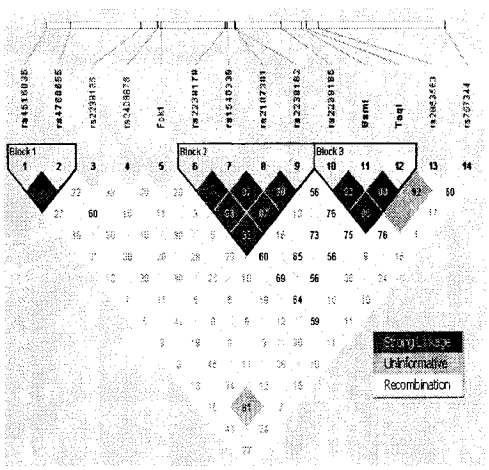
Figure 3: Pairwise LD pattern of *VDR* in a (A) Mexican (A) and a (B) Texan population measured by D'. The location of each tested SNP along the chromosome is indicated on top. The number in each diamond indicates the magnitude of LD (D'x 10^{-2}) between respective pairs of SNPs. For example, the pairwise D' for SNPs rs1540339 and rs2107301 is 0.97. Squares without D' written on them represent perfect LD (D'= 1.0). Strength of LD (or evidence of recombination) is depicted by shades (strong LD = black, weak LD = white, intermediate = grey). Below the D' matrixes the haplotypes of the three LD are depicted with their frequencies given within parentheses. Thick lines joining haplotypes from each block represent combined haplotypes with frequency > 0.1 while thin lines for frequency < 0.01. Strength of LD between block 1 and block 2 and block 2 and block 3 are given as D' below and between the corresponding blocks.

Figure 3

A



B



Block 1 Block 2 Block 3

T G(0.65) A G C (0.37) C G T (0.64)
C A(0.25) G G C (0.30) T G T (0.21)
T A(0.10) A A T (0.18) T A C (0.14)
A A C (0.16)

0.25 0.71

Block 1 Block 2 Block 3

T G(0.43) A G C A (0.29) C G T (0.54)
T A(0.38) G G C G (0.31) T A C (0.21)
C A(0.19) A A T A (0.20) T G T (0.19)

0.25 0.61

Table 5: Haplotype Distribution Pattern in the case - control study

Block	Haplotype	Patients Frequency	HC Frequency	CC Frequency	p-value*	p-value†	p-value‡
rs4516035-rs4760655	CA	0.239166	0.245228	0.271544	NS§	NS	NS
	CG	0.000026	0.000015	ND**			
	TA	0.091616	0.085814	0.112537			
	TG	0.669191	0.668943	0.615901			
rs2239179-rs1540339-rs2107301	AAC	0.141662	0.147111	0.161387	NS	NS	0.005
	AAT	0.236867	0.205384	0.146625			
	AGC	0.349289	0.361923	0.358361			
	GGC	0.269602	0.285361	0.327169			
rs2239185-*Bsm*I-*Taq*I	CGT	0.637847	0.655696	0.639906	NS	NS	NS
	TGT	0.195365	0.217429	0.217466			
	TAC	0.150492	0.113824	0.127441			

* susceptibility to TB, †susceptibility to *M.tb* infection, ‡disease progression, §not significant, **not detected

variants were genotyped and analyzed in all study families. All *VDR* variants were found to be in Hardy-Weinberg equilibrium among the parents.

When *VDR* alleles were analyzed individually under either an additive or a dominant genetic model, allele rs4760655 'A', located in the 5' proximal block 1, was over transmitted (p=0.01, additive model) and its 'G' allele was under transmitted (p = 0.01, additive model; p =0.007, dominant model) (Table 6). Likewise, the translation start site allele *Fok*I 'C' was over transmitted (p=0.02, additive model) and its T allele was undertransmitted (p = 0.02 additive; p = 0.03, dominant model). The *Fok*I polymorphism had also been found associated in the case – control study.

To evaluate haplotype associations in the pediatric TB disease families, the LD pattern was assessed using HAPLOVIEW[32] (Figure 3B). Overall, the LD block structure was very similar to the one observed in the Mexican population, and three LD blocks were observed. These three LD blocks are in low / moderate LD, i.e. D'=0.25 for blocks 1 and 2; and D'=0.60 for blocks 2 and 3. However, haplotype frequencies for each block were significantly different between the Mexican controls and the Texan parents (Figure 3B). When analyzed for association under either additive or dominant genetic models, haplotypes rs4516035C-rs4760655A of block 1 (p =0.05, additive) and rs2239179G-rs1540339G-rs2107301C-rs2239182G of block 2 (p=0.05, dominant) were over-transmitted, whereas block haplotype rs4516035T–rs4760655G was undertransmitted to affected offspring (data not shown).

Table 6: Transmission pattern of alleles in the pediatric TB cohort

Marker	Allele	MAF	S*[a]	S*[d]	E(S)[†a]	E(S)[†d]	Z[‡a]	Z[‡d]	p-value[a]	p-value[d]
rs4516035	T		77	39	84.233	36.017	-1.399	0.7	NS	NS
	C	0.202	47	11	39.767	15.25		-1.659		NS
rs4760655	A		85	34	70.583	30.443	2.548	1.136	0.01	NS
	G	0.405	49	22	63.417	32.86		-2.688		0.007
rs2238136	G		56	****	50.75	****	1.478	****	NS	****
	A	0.102	16	17	21.25	21.583		-1.408		NS
rs2408876	T		68	35	69.12	38.679	-0.233	-0.984	NS	NS
	C	0.316	50	21	48.79	25.889		-1.644		NS
*Fok*I	C		99	37	85.75	33.05	2.319	1.184	0.02	NS
	T	0.490	61	32	74.25	41.3		-2.23		0.026
rs2239179	A		91	20	95.393	18.914	-0.933	0.518	NS	NS
	G	0.305	55	44	50.607	38.521		1.33		NS
rs1540339	G		90	25	87.483	23.686	0.465	0.479	NS	NS
	A	0.310	56	35	58.517	36.203		-0.291		NS
rs2107301	C		84	11	83.85	10.611	0.031	0.239	NS	NS
	T	0.192	36	34	36.15	33.761		0.058		NS
rs2239182	A		67	27	69.417	26.19	-0.513	0.274	NS	NS
	G	0.403	51	38	48.583	34.773		0.924		NS
rs2239185	C		82	27	80.114	24.198	0.398	0.948	NS	NS
	T	0.449	68	31	69.886	30.083		0.259		NS
*Bsm*I	G		73	13	71.31	13.5	0.404	-0.234	NS	NS
	A	0.242	39	28	40.69	30.190		-0.647		NS
*Taq*I	T		60	12	62.917	14.139	-0.644	-0.922	NS	NS
	C	0.222	42	26	39.083	25.222		0.229		NS
rs2853563	G		41	****	42.786	****	-0.695	****	NS	NS
	A	0.065	17	17	15	15.298		0.682		NS
rs757344	A		86	24	79.183	22.858	1.293	0.482	NS	NS
	G	0.349	50	28	56.817	33.675		-1.383		NS

MAF =minor allele frequency, S* =FBAT statistic, [a] =additive genetic mdel, [d] =dominant genetic model, E(S)[†] =Expected FBAT statistic, Z[‡] =Z-score, NS[§] =not significant

DISCUSSION

For the present investigation, we have designed a case-control study that in addition to tuberculosis susceptibility allows analysis of susceptibility to infection with *M. tuberculosis* and progression from infection to tuberculosis disease, two decisive steps in tuberculosis pathogenesis (Figure 1). For this purpose, we have identified smear-positive pulmonary tuberculosis patients and two groups of controls: community controls, enrolled from the neighbourhood of patients and unrelated household contacts characterized by strong exposure force and persistent cough. Community controls are considered uninfected and given the incidence of tuberculosis in the study area (42/100,000) [27] it seems reasonable to assume that the majority of subjects in this group are indeed uninfected with *M. tuberculosis*. The group of household contacts presenting with a persistent cough represents individuals that are likely to be infected with *M. tuberculosis* without rapidly advancing to tuberculosis disease. We felt that selection of infected subjects based on epidemiologic (high exposure) and clinical parameters (persistent cough) would provide a better indication of infection than simple TST testing. In the resulting design indicated in Figure 1, a total of 3 *VDR* polymorphisms located in the 5' untranslated region, the translation start site, and the 3' region of *VDR* were found to be associated with *M. tuberculosis* infection, TB disease susceptibility, and progression from infection to disease.

Given the large number of tests conducted in the initial comparisons in the Mexican case-control study, replication of the detected associations in independent cohorts is critical for validation of the results. Of the 3 associations detected, only *Fok*I had previously been reported to be associated with tuberculosis disease susceptibility among male Chinese

tuberculosis patients [21]. Importantly, the direction of association, i.e. the *Fok*I "T" allele being a risk factor, was the same in both populations. The validity of the *Fok*I T allele association with tuberculosis susceptibility is also supported by the fact that the *Fok*I polymorphism is the only *VDR* genetic variant with a clear impact on VDR structure. The T>C base change eliminates the ATG translation start site resulting in the translation of a VDR protein that is truncated by its 3 N-terminal amino acids [24-26]. Moreover, the smaller protein has a higher transcriptional activation and mRNA expression *in vitro* consistent with the proposed protective effect of the shorter VDR variant [24-25,45]. A study in West-African tuberculosis patients failed to detect an impact of the *Fok*I polymorphism on tuberculosis risk [20]. However, the same study identified a *Fok*I C allele containing haplotype as risk factor for tuberculosis [20]. The significance of the latter observation is difficult to interpret since the evidence for global haplotypic association was weak and replication in the case-control study was not provided. However, it is possible that despite the biological support for a role of *Fok*I in tuberculosis susceptibility, *Fok*I may indeed not be the causal variant for altered tuberculosis risk but be in LD with the true causal polymorphism. Further support for the presence of an unknown causal variant is also provided by our observation that *Fok*I is located in a region of high recombinational activity since no extensive LD of *Fok*I with adjacent polymorphisms could be detected. To properly fine – map this region of low LD will require much higher marker density to more precisely delineate the physical borders of the causative polymorphism(s). Nevertheless, our own results and those obtained in the Chinese population argue for a role of a 5' *VDR* polymorphism in the vicinity of *Fok*I in tuberculosis susceptibility, possibly the *Fok*I C allele that gives rise to a truncated form of the receptor.

There are few studies in the literature that aim to specifically address the genetics of *VDR* in progression from infection to disease. One study of

smear-confirmed TB patients and skin test positive controls in Cambodia failed to detect association of *Fok*I or *Taq*I polymorphisms with progression to pulmonary TB [23]. A second study of Gujarati Asians provided suggestive evidence for association of the *Fok*I TT genotype with pulmonary tuberculosis disease [17]. Hence, to obtain independent validation of the *Fok*I and *Bsm*I genotypic associations with progression to disease, we decided to genotype a family panel of pediatric tuberculosis cases and to test all 14 *VDR* polymorphisms for association with pediatric tuberculosis. Pediatric cases in their majority represent a phenotype of fast progression from infection to disease and we reasoned that this was a close approximation to the phenotype tested in the comparison of infected household contacts with tuberculosis patients in the Mexican case-control population. We observed that the *Fok*I C allele was associated with pediatric tuberculosis disease, i.e. the direction of association was inverted from the one seen in the case-control study. Hence, the results obtained with the pediatric families are inconsistent with those obtained in the Mexican case-control study and the previous study in Gujarati Asians [17]. Why the same polymorphism would display different directions of association is not clear but could indicate that fundamentally different physiological processes underlie susceptibility to pediatric and adult tuberculosis disease.

There have been other studies demonstrating an effect of the *Fok*I C allele on tuberculosis related phenotypes. For example, a Peruvian study demonstrated faster sputum mycobacterial culture and auramine stain conversions during treatment in *Fok*I CC individuals [22], while a study in healthy Indians correlated the *Fok*I CC genotype with increased macrophage phagocytosis of *M. tuberculosis* and reduced lymphoproliferative responses to *M. tuberculosis* antigen [46]. How these disease-related phenotypes are connected to tuberculosis susceptibility as investigated in our study is unknown. Nevertheless, the results from these diverse investigations

confirm that 5' *VDR* polymorphism are significant risk factors for tuberculosis and related mechanistic phenotypes. However, given the divergent directions of association and widely differing phenotypes used, it is clear that other, presently unknown genetic, environmental or bacterial factors impact on pleiotropic VDR-mediated host responsiveness. Consequently, a simple linear relationship between *VDR* polymorphisms and tuberculosis is unlikely to exist.

Besides the *Fok*I polymorphism we also detected significant association of the *Bsm*I polymorphism with tuberculosis and progression to tuberculosis from the infected state. In previous studies, *Bsm*I was either not tested or did not show significant association with tuberculosis or progression. Likewise, in the pediatric patients no effect of *Bsm*I was observed (Table 6). However, it is noteworthy that *Bsm*I is closely linked to the *Taq*I polymorphism and both markers belong to haplotype block 3 (Figure 3). The *Taq*I polymorphism had been shown in a number of previous studies to be a risk factor for TB (Table 1). Combined, these results suggest that neither *Taq*I nor *Bsm*I are causally involved in TB risk. Rather, it seems likely that an unknown genetic variant in LD with both *Bsm*I and *Taq*I is the cause of altered TB susceptibility. Study group specific changes in LD patterns and their underlying allele frequencies may then result in either *Bsm*I or *Taq*I as being identified as TB risk factor.

We also detected significant association of a VDR haplotype in the case-control study (Table 5). This haplotype is different from the one identified in the West-African study [20]. Nevertheless, the same issues emerge: overall the evidence for global associations is weak ($p = 0.005$) and a replication is not available. This makes it likely that the observation is spurious resulting from the number of haplotype tests conducted. Moreover, when we directly tested the genotypic combinations found associated with

tuberculosis in West – African, we failed to detect evidence for association [20]. Finally, we detected association of 5' SNP rs4760655 with risk of infection ($p = 0.03$). This SNP is located in the 5' untranslated region of *VDR* where various exons 1 (1a to 1f) can combine differently, and result in different transcriptional activities [47]. The biological significance of a base change at this site is unknown. This SNP had not been studied in any of the previous genetic investigations of *VDR* polymorphisms with disease but was found associated with infection in the Mexican cohort as well with pediatric disease in the Texan families. It is plausible that a variant that predisposes to infection might be detected while studying disease in a family-based design. However, the fact that direction of association was opposite between the two cohorts raises doubts about the validity of the association.

Given the complex LD pattern within the *VDR* gene and considering the pleiotropic effects mediated by VDR, future studies will need to employ an even higher density grid of markers spanning the entire gene that will allow with high resolution to pinpoint a gene region(s) that is consistently associated with tuberculosis. Independently, it will be necessary to develop functional assay to quantify the effect of *VDR* polymorphism on VDR function. While the mechanistic basis of the VDR effect on tuberculosis susceptibility needs more detailed study, all genetic studies point to an important albeit complex contribution of VDR to tuberculosis susceptibility.

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Chapter 5

General Discussion and Conclusion

5.1 The search for TB and asthma susceptibility genes by candidate gene association studies

Since the proposal of the hygiene hypothesis in the early 1990s, the search for the early life exposure microbial agents that decrease the risk of developing allergic and autoimmune diseases has been ongoing. Murine models provided strong support for a protective role of mycobacteria in asthma / atopy [377-378]. In mice, mycobacteria such as *BCG* [377], *M. tuberculosis* [380] and *M. vaccae* [175,378-379]can prevent or downregulate the development of allergic reaction, airway hyperreactivity, airway inflammation and eosinophilia in lungs. Furthermore, the mycobacterial components responsible for reducing airway eosinophilia of sensitized mice are the mycobacterial cell wall lipoglycans lipoarabinomannan, phosphatidylinositol mannan and *M. tuberculosis* chaperonins [174,380]. In humans, however, the protective role of mycobacteria in asthma / atopy remains controversial, as reflected by the conflicting results in studies comparing mycobacterial exposures in asthmatic / atopic individuals and healthy control subjects [381-382]. In my thesis work I aimed to identify a causative effect of mycobacterial exposure on asthma using a genetic approach. If the hygiene hypothesis is valid and infection of *M. tuberculosis* confers protection against asthma / atopy, then genetic variants which confer susceptibility to TB may provide protection against asthma / atopy and vice versa. Hence various candidate genes that had previously been associated with altered risk of asthma or TB were simultaneously tested in populations of asthma and TB patients.

5.1.1 *VDR*

The findings that genetic variants of *VDR* are associated with both TB

and asthma / atopy risks are encouraging. The identification of a gene that affects pathogenetic processes in both diseases demonstrates a relationship between the two diseases at the molecular level. Ideally, if we had identified the same genetic variants to be inversely associated with TB and asthma / atopy, then we could have persuasively concluded that *M. tuberculosis* infection protects against asthma / atopy, or its absence predisposes to asthma / atopy development. However, in the three cohorts (1 adult TB, 1 pediatric TB and 1 childhood asthma) where we investigated the role of *VDR* in disease susceptibility, different variants of the same gene were associated with TB and asthma. The results obtained in my own experiments show that variants at the 5' end of the gene are associated with TB while those at the 3' end are associated with asthma / atopy. Due to the inconsistent findings and incomprehensive gene coverage of other *VDR* - TB susceptibility studies in the literature [289-293], Similar comparisons based on the data provided is difficult. Also inconclusive are the variants associated with asthma / atopy [287,288,383]. Since the publication of the *VDR* - asthma study in families from North – Eastern Québec, 3 replications have been attempted by others [287,384]. Although all replications have identified the 3' end of *VDR* to be relevant in asthma / atopy, the associated variants are different. In a women nurses cohort, 3 of the 5 SNPs to be associated with asthma risk are the same as those in the Québec asthma cohort [287], whereas in the Childhood Asthma Management Program cohort, only 1 SNP was associated with asthma and the risk variant was the opposite allele from the Québec and the nurses' cohorts [287]. More recently, a German study has detected a protective but not a risk effect of *VDR* alleles for asthma [383]. Several possibilities can be advanced to explain these inconsistent findings, e.g. varying patterns of LD [216-217,385], variable clinical phenotypes [386-387] or the impact of unmeasured gene – environment interactions [388], but all of these possibilities are highly speculative and no dedicated experimental effort to

elucidate the gene – epidemiologic basis of these inconsistencies has been forthcoming.

Since $1,25(OH)_2D_3$ is an immuno – modulating hormone [274,389-390], the mechanism of its interaction through VDR in TB and asthma / atopy pathogenesis could be simplistically speculated to be as follows: upon phagocytosis of *M. tuberculosis* by macrophages, $1,25(OH)_2D_3$ synthesis is enhanced to subsequently stimulate antimicrobial activity of macrophages [391], including production of reactive nitrogen and oxygen intermediates [392]. In addition, the $1,25(OH)_2D_3$ – VDR complex mediates the cytokine profile of T cells; IL2 [393-394] IL12 [395], IFN-γ [396] are downregulated while IL4 [274,397-398], IL1 [393] and IL10 [274] are upregulated. The protection against asthma / atopy would depend on the competition between the Th1 inducing effect of *M. tuberculosis* and the Th2 inducing effect of $1,25(OH)_2D_3$. The latter can be diminished in the presence of insufficient exposure or expression of (partly) incompetent VDR molecules. Hence, in light of the hygiene hypothesis, the absence of Th1 inducing pathogens would allow susceptible populations (i.e. individuals whose $1,25(OH)_2D_3$ – VDR interactions can promote strong Th2 effects) to develop asthma / atopy.

Inability to replicate genetic associations is a widespread phenomenon in common diseases [399]. Possible explanations for inconsistent findings include (1) genetic heterogeneity - the genetic effect may exist for certain populations but not others [400]; (2) allelic heterogeneity - different variants of the same gene contribute to disease risk in different populations [400]; (3) association by LD in populations with different LD patterns - the causative variant(s) have not been identified, and they are in LD with different marker alleles in different populations [401-402]; (4) gene environment interaction - genetic variants interact with environmental factors to contribute to disease risk, and such environmental factors may not be

present in all populations [185]; (5) broad phenotype definitions - true associations can be masked by testing cohorts consisting of other subphenotypes (i.e. childhood asthma and adult asthma) [387]; (6) different phenotype definitions among cohorts - studies may in fact be investigating different subphenotypes of complex traits [387]; (7) false positive - spurious associations [403]; and (8) false negative - insufficient sample size [403].

Despite some inconsistency, the associations observed between *VDR* genetic variants and asthma / atopy in the French Canadian cohort, and those with TB in the Mexican adult and Texan pediatric cohorts are unlikely to be spurious because (1) more than one *VDR* variant was associated with disease, and (2) the genetic effect was detected in two or more populations for each disease. However, the considerable inconsistency across studies as to the molecular identity and / or direction of association of *VDR* with TB [289-293] and asthma susceptibility [288,383-384] suggests that our understanding of the biological role(s) of *VDR* genetic variants is incomplete.

5.1.2 *NRAMP1*

Prior to our reporting of absence of associations between *NRAMP1* genetic variants and asthma / atopy, no studies that investigated the genetic effect of *NRAMP1* in asthma had been published. For atopy, one study reported association between a microsatellite marker located downstream of *NRAMP1* and atopy in *BCG* vaccinated Scandinavian children ($p = 0.01$), but no association was observed with the promoter variant 5'$(CA)_n$ regardless of the *BCG* status [405]. The authors suggested that the association was due to LD with the causative variant(s) likely to be located at the 3' end of *NRAMP1.* In our asthma / atopy study, we assessed the genetic effect of *NRAMP1* at both the 5' and 3' ends. Unlike the atopy

study in the Scandinavian children, we did not have data on exposure to mycobacteria, such as BCG vaccination, in our cohort. However, we suspect that even if we had the microbial exposure data, we would not have detected a genetic effect at the 3' end of the gene because the previously identified TB susceptibility alleles occurred infrequently in the French Canadian cohort (allele frequencies <3%). The absence of association between *NRAMP1* alleles in 2 cohorts argues against the hypothesis that a relationship exists between *M. tuberculosis* infection and asthma / atopy protection. However, genetic heterogeneity and gene – environment interaction may have prevented the detection of a genetic effect by *NRAMP1.* Since the correlations between *BCG* vaccination and protection against asthma / atopy are geography specific - observed in tropical but not in non – temperate regions [406], then perhaps the Scandinavians and French Canadians are not the ideal cohorts to detect genetic effect of *NRAMP1* in asthma / atopy. Populations from temperate or TB endemic regions may offer a greater chance to detect not only the genetic effect of *NRMP1*, but also the environmental conditions needed to exert such an effect. If the protective effect of *NRAMP1* parallels that of *TIM-1* - requiring the presence of HAV to exert a protective effect [37], then studies relying on cohorts from regions where exposures to *M. tuberculosis* or other mycobacteria are low would compromise the power to detect the genetic effect of *NRAMP1.*

5.1.3 Other genes

One might argue that the rejection of the hypothesis that a relationship exists between *M. tuberculosis* infection and protection against asthma / atopy (or the absence of the infection and asthma / atopy risk) is inappropriate since we have tested only two candidate genes in 1 to 3 populations. However, In addition to *VDR* and *NRAMP1*, we have tested 25

SNPs in 17 candidate genes (*IL1A, IL1B, IL3, IL4, IL4RA, IL6, IL10, IL12RB1, CARD15, CCR2, CD95, ELAM1, HSD11B1, LTA, MCP-1, TGFB* and *TNFA*), but in a less comprehensive fashion. In the Mexican TB cohort, none of the 25 SNPs was associated with either TB susceptibility or progression from infection. It is possible that we may have missed causal variants in the tested candidate genes since only a small number of SNPs per gene were tested. For example, we tested 2 SNPs in the *IL12RB1* gene (365 Met>Thr and 378 Gly>Arg) and did not detect any association with TB susceptibility or progression from infection, whereas a more comprehensive study of a Moroccan cohort assessing 13 SNPs across the *IL12RB1* gene did detect 2 SNPs (-111 A>T and –2 C>T, relative to the transcription start site) in the promoter region to be associated with pulmonary TB susceptibility ($p < 0.02$) [407]. Assuming that the same genetic variants contribute to risk of common human diseases [408], we feel that by being strategic in selecting variants to test (i.e. testing variants which have already been shown to be associated with other diseases), we had a greater probability to detect genetic risk factors by evaluating more candidate genes, although less comprehensively, than more comprehensively in fewer genes.

5.2 Challenges in the search of genetic risk variants for common diseases

Challenges in the search of genetic risk factors for common diseases range from conceptual to technical to analytical. Here I discuss some main issues that arise from the complexity of common traits as they became apparent from the genetic study of asthma and TB.

5.2.1 Gene - gene interactions

In the past, association studies involved analyzing few variants of the same gene in cohorts comprised of people with the disease and unrelated individuals without the disease in case-control cohorts, or relatives of the cases in family-based cohorts [186,188]. The low success rate of identifying genetic risk factors for common diseases illustrates the 'simple' fact that common human diseases are too complex to be detected by conventional 'single gene' association methods. Biochemical pathways indicate that proteins interact, and multiple changes in the characters of proteins (e.g. expression, affinity, function) brought on by nucleotide changes may combine together to exert an effect on a phenotype [409]. Interaction between genes is termed epitasis [410]. For example, in a Dutch case-control association study of asthma, variants of the *IL4RA* and *IL13* genes were assessed for their risk effect. Individually, variants of both genes are associated with asthma ($p < 0.02$, but the greatest effect was observed in individuals who possess the risk alleles at both genes (OR = 4.87; $p = 0.0004$) [411]. Since IL13 binds to IL4RA [412], changes in IL13 level may enhance the effect of IL4RA and the subsequent downstream signaling events. In the *NRAMP1* study of asthma, however, in the absence of *a priori* evidence for epistasis with specific genes, systematic screening of the entire genome or a large number of candidate genes poses the problems of false findings due to multiple testing [403], or the lack of power to detect associations if stringent corrections for multiple testing are introduced [413].

5.2.2 Gene – environment interactions

For common complex traits such as allergic and infectious diseases, genetic variants alone often are insufficient to cause disease; they need to

be in the right environment to be causative [185]. The 'right' environment can refer to exposures to 'environmental' and non-genetic factors such as nutrition [414], prenatal conditions [415], microbes [37], and others[185]. Several different models for gene – environment interactions have been suggested [185,388]: (1) a given susceptibility genotype and a specific environmental exposure must be present to confer risk of disease, (2) environmental exposure causes an increased risk of disease in all individuals but a much greater risk in individuals with the susceptible genotype, (3) environmental exposure itself does not confer a risk, but will increase the risk of disease in individuals with the susceptible genotype only, (4) either the environmental or the genotype produce an excess risk, and the presence of both elevate the risk even higher, (5) the genotype may switch between being protective and susceptible depending on the presence (or absence) of the environmental exposure. The last model may be the case for the genetic effect of *VDR* in TB susceptibility. The associations of opposite alleles of the translation start site variant, *Fok*I C>T, in adult and pediatric cohorts suggest that an associated genotype may behave differently in the presence of high mycobacterial exposure, as perceived by the immature immune system of pediatric patients, than that of adult cases.

Failure to consider environmental risk factors may jeopardize the search for genetic risk factors. An obvious scenario would be a cohort consisting of subjects with different degree of environmental exposures, and the genetic effect of the variants would be concealed if the environmental factor were to be disregarded in the analysis [413].

5.2.3 Phenotype heterogeneity

The present definitions for many common diseases in humans

encompass many subclasses, and each is likely to follow different pathways of pathogenesis. For example, although both are diagnosed as asthma, the clinical symptoms of atopic and non-atopic asthma are different. Atopic asthmatics suffer elevated IgE levels [416] while their non - atopic counterparts do not [160]. Hence genes influencing IgE levels would have no effect in the pathogenesis of non – atopic asthma. If cases are recruited only based on their status as asthmatics, then the frequencies of the causal genetic variants will be diluted and render the study underpowered. In the Québec asthma cohort, by recruiting children as probands, we reduced the likelihood of phenotype heterogeneity since childhood asthma is mostly atopic.

Clinical differences are obvious indications of different pathogeneses, other differences among cases which may suggest different disease etiologies, include gender [417,307], age-of-onset [160], and environmental exposures [418-419]. One reason for the inconclusive results from searches of genetic risk factors for asthma / atopy is that different phenotype definitions were studied. For example, many different markers including eosinophil counts, total IgE level, allergen specific IgE level and skin prick test positivity have been used as measures for atopy [387], and exclusive associations suggest that these markers [319-320,] , although related, may associate with atopy differently.

Heterogeneity in phenotype definition also applies to TB susceptibility. In addition to the clinical subphenotypes of pulmonary and extrapulmonary TB in adults and children [45], the phenotype 'TB susceptibility' can also refer to different stages of pathogenesis. By taking into account the subphenotypes in the *VDR* - TB study, the analyses revealed that different *VDR* variants affect different stages of TB pathogenesis. By recruiting 2 groups of controls in the adult TB cohort, we have dissected the phenotype

TB susceptibility further into susceptibility to *M. tuberculosis* infection and disease progression from infection.

The search and subsequent replication of genetic risk factors will be difficult to achieve, if not impossible, if cases in consist of individuals with different 'diseases' or phenotypes [400].

5.3 Future directions

5.3.1 Identification of the causative genetic variants of *VDR* in asthma / atopy susceptibility

At present, the association study of *VDR* in asthma / atopy susceptibility identified an asthma risk haplotype of 9 SNPs spanning approximately 28kb in length. Since none of the 9 SNPs resulted in an amino acid change, the causative variant(s) are likely to be regulatory. The next task is to identify the causative variant(s) and the type of regulation exerted.

Within the 28kb region, the majority of SNPs showing the most significant associations clustered within a 22kb region; sequencing of exons and surrounding introns in this region yielded 66 SNPs in the French Canadian asthma cohort. To select the most likely candidate SNPs that might have regulatory functions, one can exclude rare variants [184]. In addition, to increase the likelihood of finding the causative regulatory SNP(s), genomic regions can be compared across species since regulatory elements are likely to be conserved through evolution [420-421]. The most comprehensive approach is to test each SNP within and around the 22kb region for regulatory functions such as changes in mRNA transcription,

mRNA degradation, location of splice sites and binding affinity to nuclear proteins.

5.3.2 Identification of the causative genetic variants of *VDR* in TB susceptibility and related phenotypes

The next task for the *VDR* - TB susceptibility study is to further characterize the region of association. At present we observed associations between the three phenotypes (TB susceptibility, *M. tuberculosis* infection and disease progression from infection) and the 5' end variants. The promoter region of *VDR* has many potential regulatory elements: (1) *VDR* has multiple transcripts derived from differential splicing of various exons 1, potentially leading to structurally different VDR proteins [422], and (2) a SNP located at the translation site (variant *Fok*I T>C), gives rise to a truncated protein product. Hence, the immediate step is to see how far upstream of *Fok*I the signal of association extends by analyzing additional SNPs in the promoter region of *VDR.* Once the area of association has been localized, a list of candidate SNPs to be tested for causation can be generated by sequencing DNA from selected individuals within the cohorts to identify all variants in the *VDR* promoter region. Similar to the identification of the *VDR* causative variants in asthma, SNPs in the region would need to be tested for regulatory functions.

5.3.3 Additional association studies to identify genetic variants in both TB and asthma / atopy susceptibility

The search for genetic variants common to both TB and asthma / atopy

susceptibilities should continue, especially at a time when various technologies are becoming available to study diseases at the genomic level. For example, genes involved in disease pathology can be identified using microarrays [423]. By the method of oligonucleotide hybridizations onto microarrays (also known as chips), genes which are expressed differently in a disease environment are detected and their expression differences are quantified [424]. Hence, the list of candidate genes to be tested is growing immensely as more phenotypes are being investigated at the genomic level. In addition, methodologies of genome-wide association scans using SNPs are under intense development [425-426], made possible by advances in high throughput genotyping technologies [424].

5.4 Conclusion

Assuming that the hygiene hypothesis is valid, and that *M. tuberculosis* is the infective agent whose absence (due to better hygiene) is promoting susceptibility to allergic conditions such as asthma and atopy in developed countries, we hypothesized that common genetic variants that contribute to TB risk may be protective against asthma / atopy and vice versa. We conducted association testings for a number of candidate genes in three study cohorts, and did not identify such common variants. However, we identified different variants of the *VDR* gene to be associated with both TB and asthma / atopy in the study cohorts. Although our data do not support a protective role of *M. tuberculosis* against asthma / atopy, a better understanding of both diseases in the future will provide stronger candidate genes for re – testing of the initial hypothesis.

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Original Contributions to Knowledge

1. Identification of seven genetic variants of *VDR* associated with asthma, atopy and serum IgE level in the French Canadian population.
2. Identification of new polymorphisms within the *VDR* gene in the French Canadian population.
3. Establishment of LD pattern and construction of haplotypes of the *VDR* gene in the French Canadian population.
4. Confirmation of the *VDR* gene as a TB susceptibility gene in the Mexican adult population.
5. Identification of three genetic variants of *VDR* associated with TB susceptibility, susceptibility to *M. tuberculosis* infection and disease progression in the adult Mexican population.
6. Identification of 2 genetic variants of *VDR* associated with TB disease progression in the Texan pediatric population.
7. Establishment of LD patterns and construction of haplotypes of the *VDR* gene in the Mexican and Texan populations.
8. Demonstrating absence of a *NRAMP1* effect on asthma risk in the French Canadian population.

Appendix 1. Research Ethics Board Approval Letters

Centre universitaire de santé McGill
McGill University Health Centre

November 16, 2004

Dr. Thomas Hudson
Human Genome Laboratories
Montreal General Hospital

RE: REC. 97-035 entitled "Genomic approaches to Identify Genes Predisposing to Asthma."

Dear Dr. Hudson:

We have received an Application for Continuing Review of the Research Ethics Committee of the research study referenced above and the report was found to be acceptable for ongoing conduct at the McGill University Health Centre. At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

We are pleased to inform you that your study continues to meet with the guidelines of the McGill University Health Centre and we hereby grant you he re-approval for the study via review of the Chair on November 16, 2004, valid until October 2005. It was cited in your report that forty (40) participants have been accrued since the last review, and that no revision to the approved consent document is required at this time.

All research involving human subjects requires review at a recurring interval. It is the responsibility of the principal investigator to submit an Application for Continuing Review to the REB prior to the expiration of approval to comply with the regulation for continuing review of "at least once per year".

However, should the research conclude for any reason prior to the next required review, you are required to submit a Termination Report to the Committee once the data analysis is complete to give an account of the study findings and publication status.

Should any revision to the study, or other unanticipated development occur prior to the next required review, you must advise the REB without delay. Regulation does not permit initiation of a proposed study modification prior to REB approval for the amendment.

Sincerely,

Denis Cournoyer, M. D.
Chairman
MUHC-Montreal General Hospital
GEN (Genetics/Population Research/Gen Investigator Initiated Studies)
(formerly Research Ethics Committee)

Cc: REC#97-035

Centre universitaire de santé McGill
McGill University Health Centre

February 07, 2005

Dr. Erwin Schurr
Research Institute
Montreal General Hospital

RE: REC. 98-040 entitled "Genetic Epidemiology of Tuberculosis."

Dear Dr. Schurr:

We have received an Application for Continuing Review of the Montreal General Hospital Research Ethics Committee for the research study referenced above. The report was found to be acceptable for continued conduct at the McGill University Health Centre. At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

We are pleased to inform you that the Chair provided re-approval for the study until February 2006. It is noted in your report that the study is closed permanently to subject recruitment, however, genetic analysis are ongoing.

Please take note that all research involving human subjects requires review at a regular interval and it is the responsibility of the principal investigator to submit an application for Continuing Review before the expiration of the study approval. However, should the research be concluded for any reason prior to the next review, a Termination Report is required for submission to the Committee once the study analysis is complete, to give an account of the study findings and publication status.

Should any revision to the research or other unanticipated development occur prior to the next required review, please advise the REB promptly and prior to initiating a proposed revision.

We trust this will prove satisfactory to you.

Sincerely,

Denis Cournoyer, M. D.
Chairman
GEN-Research Ethics Board
(formerly Research Ethics Committee)
MUHC-Montreal General Hospital

Cc: REC#98-040

Appendix 2. Publication list, paper reprints and permissions

Publication list

Poon A and Schurr E (2004), The NRAMP genes and human susceptibility to common diseases. **In**: Cellier M, Gros, P (eds) The Nramp family. Landes Bioscience and Kluwer Academic/Plenum Publishers, New York, USA. pp 29-43.

Poon AH, Laprise C, Lemire M, Montpetit A, Sinnett D, Schurr E and Hudson TJ. Association of Vitamin D Receptor Genetic Variants with Susceptibility to Asthma and Atopy. *Am J Respir Crit Care Med* 2004; 170: 967-973

Poon AH, Laprise C, Lemire M, Hudson TJ and Schurr E. *NRAMP1* is not associated with asthma, atopy, and serum immunoglobulin E level in the French Canadian population. *Genes Immun* advance online publication, 30 June 2005; doi:10.1038/sj.gene.6364238

Poon AH, Malik S and Schurr E. Association Studies of Vitamin D Receptor Genetic Variants with Tuberculosis. *Manuscript in preparation.*

Behr A, Semrat M, Poon A, and Schurr E. Crohn's disease, mycobacteria, and NOD2. *The Lancet Infect D*is 2004; 4: 136-137

Malik S, Abel L, Tooker H, Poon A, Simkin L, Girard M, Adams GF, Starke JR, Smith KC, Graviss EA, Musser JM and Schurr E. Alleles of the *NRAMP1* Gene are Major Risk Factors for Pediatric Tuberculosis Disease. *Proc Natl Acad Sci USA* Published online before print *August 15, 2005, 10.1073/pnas.0503368102*

e-mail: audrey.poon@mail.mcgill.ca

June 28, 2005

American Thoracic Society
Editorial Department
61 Broadway, 4th Floor
NY, NY 10006

Dear Mr.Pack

I'm writing to request a permission for submitting a previously published article in the American Journal of Respiratory and Critical Care Medicine as part of my doctorate thesis (McGill University, Montreal, Québec, Canada). The article is titled **Association of vitamin D receptor genetic variants with susceptibility to asthma and atopy** by Poon A [illegible], Laprise C, Lemire M, Montpetit A, Sinnett D, Schurr E, Hudson TJ. It was published in Am J Respir Crit Care Med. 2004 Nov 1;170(9):967-73.

Thank you for your help.

Permission granted for the purpose indicated.
Cite: author(s) / year / title /
American Journal of Respiratory and Critical Care Medicine
/ volume / pages.
Official Journal of the American Thoracic Society
©American Thoracic Society
Christina Shepherd, Managing Editor, JUN 27 2005

Sincerely,

Audrey Poon
Ph.D candidate

Audrey Poon

From: "ajpermissions" <ajpermissions@nature.com>
To: "Audrey Poon" <audrey.poon@mail.mcgill.ca>
Sent: June 7, 2005 6:32 AM
Subject: RE: permission

Dear Ms Poon -

You have asked for permission to reprint your NPG journal paper (either partly, or in whole) in a printed volume of your own work. I am happy to tell you that you do not need our permission to do this.

In February 2003 year we changed our rights policy, and we no longer require copyright from authors of research papers. Instead, we ask for an exclusive licence, in return for which authors are free to re-use their papers in printed volumes of their own work (provided the appropriate NPG title is credited as the journal of first publication), and to make copies for teaching purposes. We have also declared that we will extend these rights retrospectively to all articles published before February 2003, regardless of the copyright agreements that were signed at the time.

With best regards –

Jo Webber

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ajpermissions@nature.com

From: Audrey Poon [mailto:audrey.poon@mail.mcgill.ca]
Sent: 02 June 2005 15:04
To: ajpermissions@nature.com
Subject: permission

To permission controller,

I am writing to request a permission in the form of a signed waiver to reuse an accepted manuscript # 2005-GAI-066 in my thesis. Please instruct me on how to obtain it.

Thank you for your attention.

Cheers,
Audrey Poon
Montreal General Hospital Research Institute
1650 Cedar Ave.
Montreal, Quebec
H3G 1A4 Canada
Tel: 514 825 5800

Audrey Poon

From: "Cynthia Conomos" <cynthia@eurekah.com>
To: "Audrey Poon" <audrey.poon@mail.mcgill.ca>
Sent: August 23, 2005 2:51 PM
Subject: Re: publication permission

Hi Dr. Poon,
Because you were an author for the material, you may use the material without fee. You may use a copy of this email as your permission letter.
Please be sure to cite the full book information in your thesis (please remember that it was co-published by Landes Bioscience and Kluwer Academic/Plenum Publishers in New York, so both publisher names should be included). Of course if any of the material you published with us was previously published, you will have to go to the original publisher for permission.
Thanks - and good luck to you,
Cynthia

On 8/23/05 1:32 PM, "Audrey Poon" <audrey.poon@mail.mcgill.ca> wrote:

> Hello Cynthia,
>
> We have corresponded back in 2003 on the publication of **The Nramp family, [edited by] Mathieu Cellier, Philippe Gros.** I am writing to request a permission for submitting the chapter 'The NRAMP genes and human susceptibility to common diseases' as part of my doctorate thesis (McGill University, Montreal, Québec, Canada). Could you please let me know the procedure to obtain this permission?
>
> Thanks alot.
>
> Cheers,
> Audrey Poon
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> L1-413 Montreal Quebec
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Association of Vitamin D Receptor Genetic Variants with Susceptibility to Asthma and Atopy

Audrey H. Poon, Catherine Laprise, Mathieu Lemire, Alexandre Montpetit, Donna Sinnett, Erwin Schurr, and Thomas J. Hudson

McGill Centre for the Study of Host Resistance, Research Institute of the McGill University Health Centre, Montreal; Université du Québec à Chicoutimi; Community Genomic Medicine Centre, University of Montreal, Chicoutimi Hospital, Chicoutimi; McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada

Genome scans for asthma have identified suggestive or significant linkages on 17 different chromosomes, including chromosome 12, region q13–23, housing the vitamin D receptor (VDR) gene. Through interaction with VDR, 1,25-dihydroxyvitamin D3 mediates numerous biological activities, such as regulation of helper T-cell development and subsequent cytokine secretion profiles. Variants of the VDR have been found to be associated with immune-mediated diseases that are characterized by an imbalance in helper T-cell development, such as Crohn's disease and tuberculosis. The VDR, hence, is a good candidate to be investigated for association with asthma, which is characterized by enhanced helper T-cell type 2 activity. Here, we examined VDR genetic variants in an asthma family-based cohort from Quebec. We report six variants to be strongly associated with asthma and four with atopy ($0.0005 \leq p \leq 0.05$). Analysis of the linkage disequilibrium pattern and haplotypes also revealed significant association with both phenotypes ($0.0004 \leq p \leq 0.01$). The findings have been replicated by another research team in a second but not in a third cohort. These results identify VDR variants as genetic risk factors for asthma/atopy and implicate a non-human leukocyte antigen immunoregulatory molecule in the pathogenesis of asthma and atopy.

Keywords: genetic predisposition; polymorphism; vitamin D receptor

The interaction of 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) with the vitamin D receptor (VDR) modulates many biological activities of the neural, immune, and endocrine systems, including calcium and phosphorous homeostasis, apoptosis, and cell differentiation (reviewed in 1, 2). Once bound to $1,25(OH)_2D_3$, VDR binds to specific DNA sequence elements in vitamin D-responsive genes, termed VDR response elements, to influence the rate of RNA polymerase II–mediated transcription (3–5). Vitamin D–dependent rickets and osteomalacia are classic manifestations of vitamin D deficiency (OMIM 601769).

(Received in original form March 25, 2004; accepted in final form July 27, 2004)

Supported by Fonds pour la recherche en santé du Québec (C.L.), a Clinician-scientist Award in Translational Research from the Burroughs Wellcome Fund (T.J.H.), the Canadian Institutes of Health Research (T.J.H.), and the Canadian Genetic Diseases Network.

A.H.P. and C.L. contributed equally to this work.

This article is a companion article to Raby BA, Lazarus R, Silverman EK, Lake S, Lange C, Wjst M, Weiss ST. Association of Vitamin D Receptor Gene Polymorphisms with Childhood and Adult Asthma. The article will appear in the November 15, 2004 issue of the *Journal*. The published-ahead-of-print version of the article may be accessed now at http://dx.doi.org/10.1164/rccm.200404-447OC

Correspondence and requests for reprints should be addressed to Thomas J. Hudson, M.D., McGill University and Genome Quebec Innovation Centre, 740 Penfield Avenue, Room 7105, Montreal, PQ, H3A 1A4 Canada. E-mail: tom.hudson@mcgill.ca

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

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Originally Published in Press as DOI: 10.1164/rccm.200403-412OC on July 28, 2004
Internet address: www.atsjournals.org

Abnormalities related to the pleiotropic functions of VDR underlie the pathogenesis of several diseases. Table E1 in the online supplement summarizes results of association studies of four *VDR* variants (*Fok*I, *Bsm*I, *Apa*I, and *Taq*I) with serum osteocalcin levels, bone mineral density, prostate cancer, hyperparathyroidism, insulin-dependent diabetes mellitus, Crohn's disease, leprosy, tuberculosis, and acquired immunodeficiency syndrome (6–13). Among the biological effects of ligand-bound VDR, its influence on Th cell development (14) is of particular interest for diseases involving the immune system. In the mouse, $1,25(OH)_2D_3$ has been shown to inhibit Th1 development and interferon-γ production and to stimulate Th2 cell development and the production of interleukin-4 and interleukin-10 (15–17). In humans, although vitamin D has been shown to inhibit Th1 responses, Th2 enhancement has not been demonstrated (18). However, several of the associated phenotypes in Table E1 are characterized by an imbalance in Th1/Th2 cell activity, for example, acquired immunodeficiency syndrome progression (19, 20), tuberculoid leprosy (12), lepromatous leprosy (12), Crohn's disease (11), and tuberculosis (13, 21–23). Because of the well-known immunoregulatory role of VDR and its known association with several immune-mediated diseases, *VDR* presents itself to be a candidate gene for asthma susceptibility. Because asthma is characterized by a shift of Th cell responses toward type 2, we hypothesized that VDR may function as a regulator of asthma and atopy susceptibility. We investigated this hypothesis through the characterization of genetic variants of *VDR* in an asthma family-based cohort from northeastern Quebec. Some of the results of this study have been previously reported in the form of an abstract (24).

METHODS

Cohort Description

Families are from the Saguenay-Lac-St-Jean region of northeastern Quebec, Canada. Probands were recruited if they fulfilled at least two of the following three criteria: (*1*) a minimum of three clinic visits for acute asthma within 1 year, (*2*) two or more asthma-related hospital admissions within 1 year, or (*3*) steroid dependency, as defined by either 6 months of oral or 1 year of inhaled corticosteroid use. Families were included for study if at least one parent was available for phenotypic assessment, at least one parent was unaffected, and all four grandparents were of French Canadian origin. When possible, grandparents and other relatives were also recruited to the study.

After recruitment of probands and their family members, the affection status of all study participants was determined by clinical evaluation and the completion of a standard respiratory questionnaire that was modified to include questions about asthma and atopy severity, family history of asthma and/or atopy, age of onset, and the presence of other respiratory failure diagnoses (25). In 41 cases, the age of onset described by parents was below 2 years; because of the uncertainty of this information, we used a default class of 2 years. We defined participants as having asthma if (*1*) a reported history of asthma (questionnaire based) and a history of physician-diagnosed asthma (past/current) were available or (*2*) confirmation by a positive methacholine provocation

test was done only on subjects older than 12 years of age (*see* online supplement methods section for a description of the clinical tests performed). Subjects were deemed atopic if they had at least one positive response (wheal diameter ≥ 3 mm at 10 minutes) to skin-prick tests. The family participation rate was approximately 60%, and all subjects gave informed consent. A total of 223 independent families (1,139 individuals) with family size ranging from 3 to 17 and the number of affected family members (including probands) ranging from 1 to 10 were analyzed.

Single Nucleotide Polymorphism Selection and Genotyping

We investigated 93 kb of genomic DNA harboring *VDR*, spanning from chromosome 12 position 46586093 to 46492363 on build 34 hg16 genome assembly released by the National Center for Biotechnology Information. An initial panel of 20 single nucleotide polymorphisms (SNPs) was selected from public databases (National Center for Biotechnology Information and the SNP Consortium) based on (*1*) the location in the gene, (*2*) relative distances to each other, (*3*) compatibility with the genotyping methods employed, which is dependent on types of base change and flanking sequences, and (*4*) the known associations with diseases. A final collection of 12 SNPs was tested for associations. The remaining eight SNPs were discarded because of low information content or unreliable genotyping data. SNPs described in this report are cited using their reference SNP identifier from the National Center for Biotechnology Information database, except for *VDR* SNPs having commonly used aliases (*Apa*I, *Bsm*I, *Fok*I, and *Taq*I).

SNP genotyping was performed using SNPstream UltraHigh Throughput Genotyping System (Orchid Biosciences, Princeton, NJ) (26). All protocols and reaction conditions are available in this journal's online supplement (*see* Table E3 in the online data supplement for oligonucleotides used in genotyping and Table E4 for those used in sequencing).

Statistical Analysis

Hardy-Weinberg equilibrium was tested in a subset of DNA samples using MERLIN (27). These samples correspond to parents of probands whose DNA are available. These samples are independent so that unbiased estimates of Hardy-Weinberg equilibrium for the variants can be obtained.

Allele distribution patterns were assessed by the family-based association test (FBAT, version 1.4) (28, 29). This software uses an empirical variance–covariance estimator to account for the possibility of nonindependent allelic transmission to affected sibs (30). Asthma and atopy phenotypes were tested separately under an additive genetic model.

Associations between *VDR* variants were assessed. Strength of linkage disequilibrium (LD) between pairs of SNPs was measured as D′ (31), using Haploview (http://www.broad.mit.edu/personal/jcbarret/haplo/documentation.php). Regions of strongly associated markers (LD blocks) were inferred and modified from the definition proposed by Gabriel and colleagues (32) as implemented in Haploview. Specifically, the parameter confidence interval minima for strong LD was relaxed from the definition of Gabriel and colleagues, and the upper confidence level was set from 0.98 to 0.90.

Haplotype-specific associations were investigated using the "hbat" command implemented in FBAT (version 1.4) (28, 29). An empirical variance estimator was used (30). Asthma and atopy phenotypes were tested separately under an additive genetic model.

VDR Resequencing

The promoter region and all exons were sequenced to detect novel coding SNPs using ABI PRISM BigDye Terminator (version 2) kit on an ABI 3,700 DNA sequencer (Applied BiosSystems, Foster City, CA) as described elsewhere (33). The protocols, reaction conditions, and primers used are described in this article's online data supplement and at http://www.genomequebec.mcgill.ca/VDR.

RESULTS

Patient Characteristics

Clinical characteristics were obtained for 1,139 individuals (Table 1). At the time of recruitment, study participants were aged 3 to 88 years. There were 570 subjects with asthma, of which 223 are probands. The median age of onset for cases and their affected siblings is 5 years (2–46 years). We note that 419 of the 570 subjects (74%) with asthma and 218 of the 569 subjects (38%) without asthma were atopic. The sex ratio (male:female) for probands is 1:1.2, for affected family members is 1:1.4, and for unaffected family members is 1:1.2. Compared with their affected family members, the probands with asthma had higher immunoglobulin E levels and coexistence of atopy.

TABLE 1. CLINICAL CHARACTERISTICS OF SUBJECTS

	Families Probands (*n* = 223)	Members Affected (*n* = 347)	Members Unaffected (*n* = 569)
Male:female ratio	1:1.2	1:1.4	1:1.2
Mean age in years (range)	18 (3–46)	40 (2–83)	48 (3–96)
Median age in years	16	41	48
Mean age of onset in years (range)	7.8 (2–46)	7.5 (2–75)	Not applicable
Median age of onset in years	5	5	Not applicable
Smoking status			
Never	186 (83.4%)	176 (50.7%)	243 (42.7%)
Ex-smoker	12 (5.4%)	105 (30.3%)	199 (40%)
Smoker	25 (11.2%)	66 (19%)	127 (22.3%)
FEV_1 as percentage predicted (SD)	92.2 (16.3)	88.9 (22.7)	98.9 (17.01)
PC_{20} in mg/ml (SD)*	2.66 (3.33)	3.36 (4.53)	26.91 (3.04)
Serum IgE in mg/L (SD)*	229.09 (4.61)	157.4 (4.55)	80.9 (3.72)
Number of persons with subphenotypes			
Asthma	223 (100%)	347 (100%)	Not available
Atopy	182 (82%)	237 (68.3%)	218 (38.3%)
AHR†	169 (90%)	217 (82.2%)	64 (11.2%)
IgE > 100 mg/L	141 (63%)	185 (53.3%)	155 (27.2%)
IgE > 280 mg/L	95 (43%)	106 (30.5%)	70 (12.3%)

Definition of abbreviations: AHR = airway hyperreactivity according to American Thoracic Society criteria; PC_{20} = provocative concentration of methacholine inducing a 20% fall in FEV_1.

Evaluated for 188 probands, 486 unaffected family members, and 264 affected family members.

* Geometric mean

† Present asthma or past documented clinical history of asthma.

Genetic Analysis of the *VDR* Locus

A final panel of 12 SNPs was used from an initial collection of 20 obtained from public databases for *VDR* characterization and association testing (*see* Table E2 in the online supplement for SNPs characteristics). Of the eight discarded SNPs, seven are uninformative, with minor allele frequencies of less than 0.025, and one SNP failed the genotyping assay. Among the 12-SNP panel are four widely studied variants: *FokI* C>T (rs2228570) (13, 22, 34), *ApaI* A>C (rs7975232) (35, 36), *Bsm*I G>A (rs1544410) (7, 37–40), and *TaqI* C>T (rs731236) (13, 21, 23). Of the 12 SNPs, *Fok*I and *Taq*I reside in the coding region, whereas the remaining 10 SNPs are located in noncoding regions, between 4.8 kb upstream of the translation start site and 32 kb downstream of exon 9 (Figure 1A).

Of the 12 SNPs, only rs2239182 gave a significant deviation from Hardy-Weinberg equilibrium (p = 0.039); given the number of SNPs tested, this deviation can be expected to occur by chance alone.

Family-based Association Analysis of *VDR* with Asthma and Atopy

The 12 SNPs were tested individually for association with asthma and atopy (Figure 1B). In the absence of *a priori* evidence for transmission models at this locus, we tested allelic associations under an additive genetic model. Six alleles (rs3782905C, rs1540339A, rs2239185C, rs2239185G, *Bsm*IG, and *Taq*IT) and four alleles (rs2239185C, *Bsm*IG, *Apa*IC, and *Taq*IT) were significantly overtransmitted to offspring with asthma and offspring that were atopic ($p < 0.05$), respectively.

IgE data were collected and analyzed both as quantitative and dichotomous traits, independent of atopy status. In a dichotomous trait model, subjects can be classified as either high or low responders according to their IgE levels. In this cohort, log (IgE) values were normally distributed, and a cut-off point of 100 mg/L divided the subjects into low (two-thirds of participants) and high (one-third) responders. Under an additive model, three alleles (rs2239185C, *Apa*IC, and *Taq*IT) are associated with being a high responder at 0.006 less than p less than 0.02. The alleles associated with high IgE are the ones also seen associated with asthma and atopy. As a quantitative trait, the phenotype investigated is high IgE responder. When regressed on age and sex, two alleles (rs2239185C and *Taq*IT) are associated at p of less than 0.005.

Intragenic LD Structure of *VDR*

Associations among the 12 SNPs were assessed by measuring pairwise LD using D′ (Figure 2A). The two flanking upstream SNPs (rs2238136, *Fok*I) and the most downstream SNP (rs757344) are not in significant LD (D′ ≤ 0.30) with any other marker tested. Thus, the LD of the two central *VDR* blocks is unlikely to have extended to these SNPs, and they are likely to be outside the core *VDR* blocks. The remaining nine SNPs are distributed within a

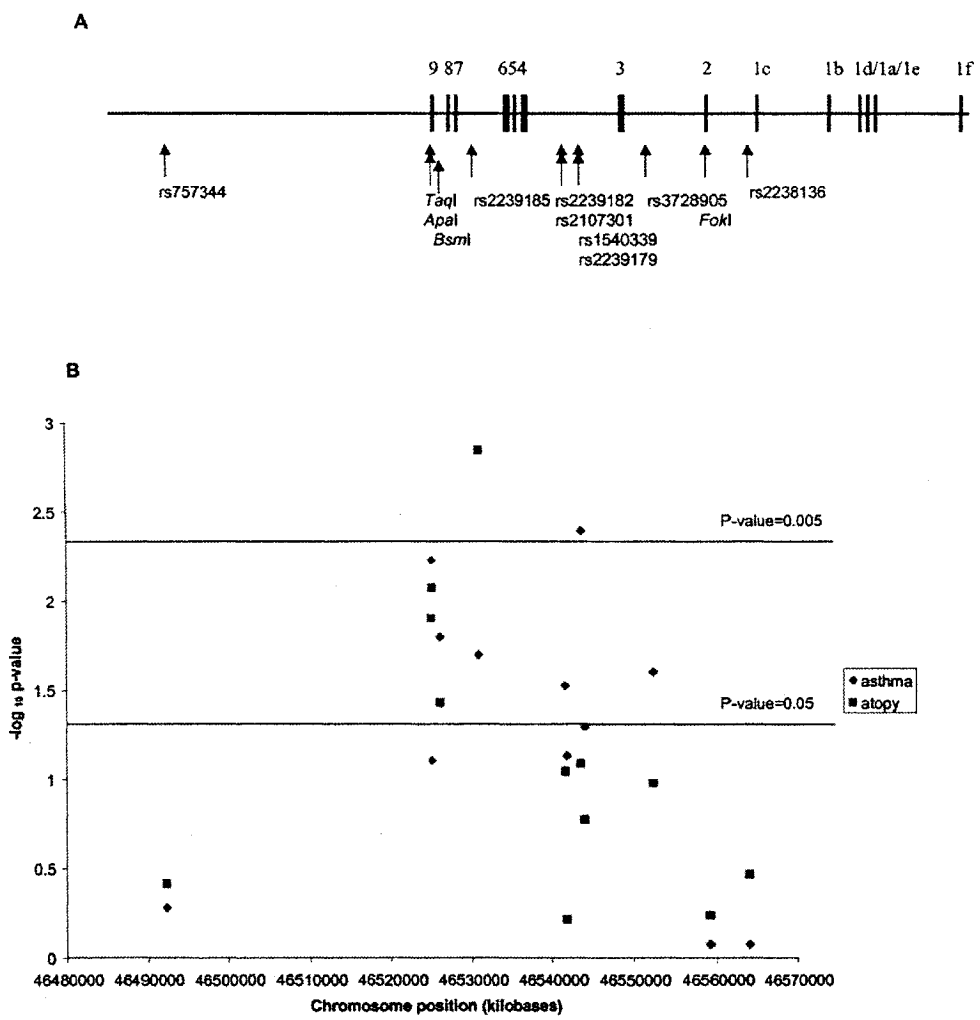


Figure 1. (*A*) Genomic organization of *VDR* and (*B*) association plot between *VDR* variants and the two phenotypes: asthma and atopy. Exons are represented by *black boxes* connected by a *straight line* representing introns and 3′ and 5′ noncoding regions. Locations of exons 1a–1f are described elsewhere (54). Positions and names of the 12 single nucleotide polymorphisms (SNPs) analyzed are represented by *arrows* below the gene structure. (*B*) Significance of association given as $-\log_{10}$ (p value) is plotted against relative SNP position given in kilobases. Three levels of significance (p = 0.05, p = 0.005, and p = 0.0005) are indicated by *straight lines* drawn across the plot. Association with asthma is depicted by *blue diamonds* and atopy by *pink squares*. Chromosome positions are based on the July 2003 freeze of the University of California Santa Cruz genome browser (http://genome.ucsc.edu/).

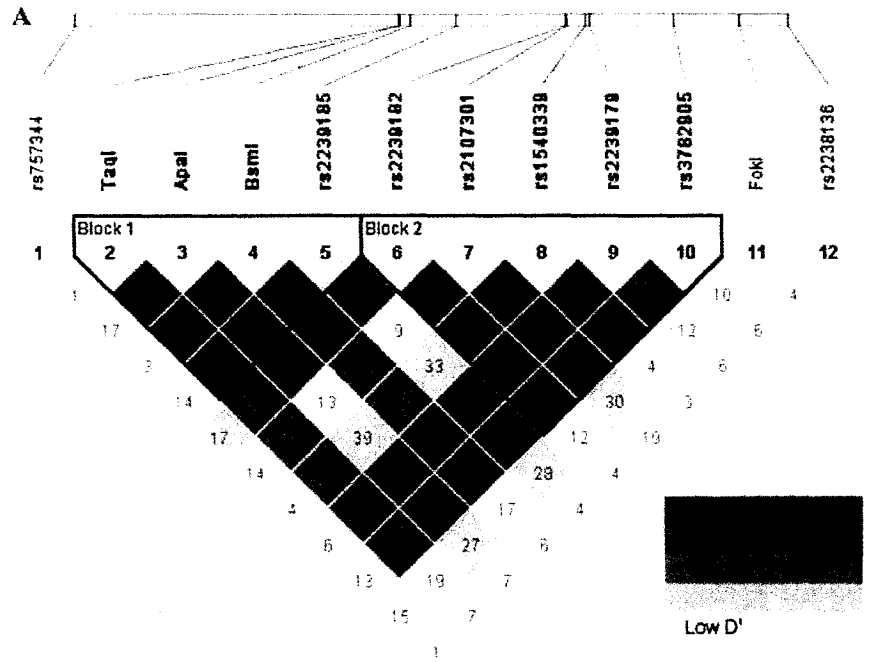
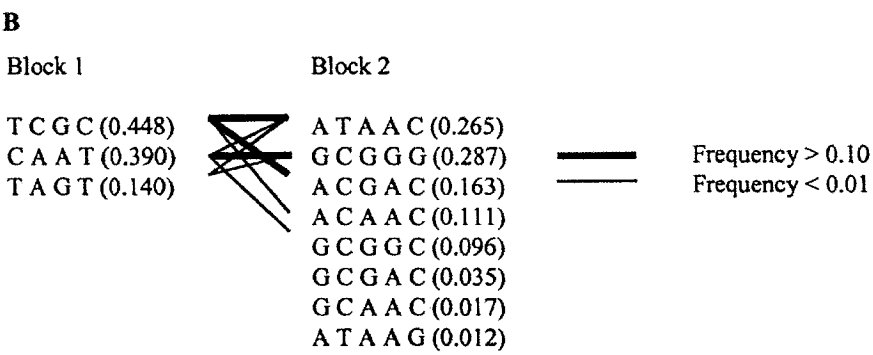


Figure 2. (*A*) Pairwise linkage disequilibrium (LD) pattern of *VDR* measured by D′ and (*B*) the common haplotypes of the 28-kb LD region. The location of each tested SNP along the chromosome is indicated on top. The number in each *diamond* indicates the magnitude of LD (D′ × 10^{-2}) between respective pairs of SNPs. For example, the pairwise D′ for SNPs rs1540339 and rs2107301 is 0.96. Squares without D′ written on them represent perfect LD (D′ = 1.0). The strength of LD is depicted by progression of color, for all D′ with LOD of more than two, the color moves from red to light blue as D′ runs from 1 to 0; for D′ with LOD of less than two, it is represented by white. (*B*) Common haplotypes of the two blocks are listed with their frequencies within parentheses. *Thick lines* joining haplotypes from each block represent combined haplotypes with a frequency of more than 0.1 and *thin lines* for a frequency of less than 0.01.



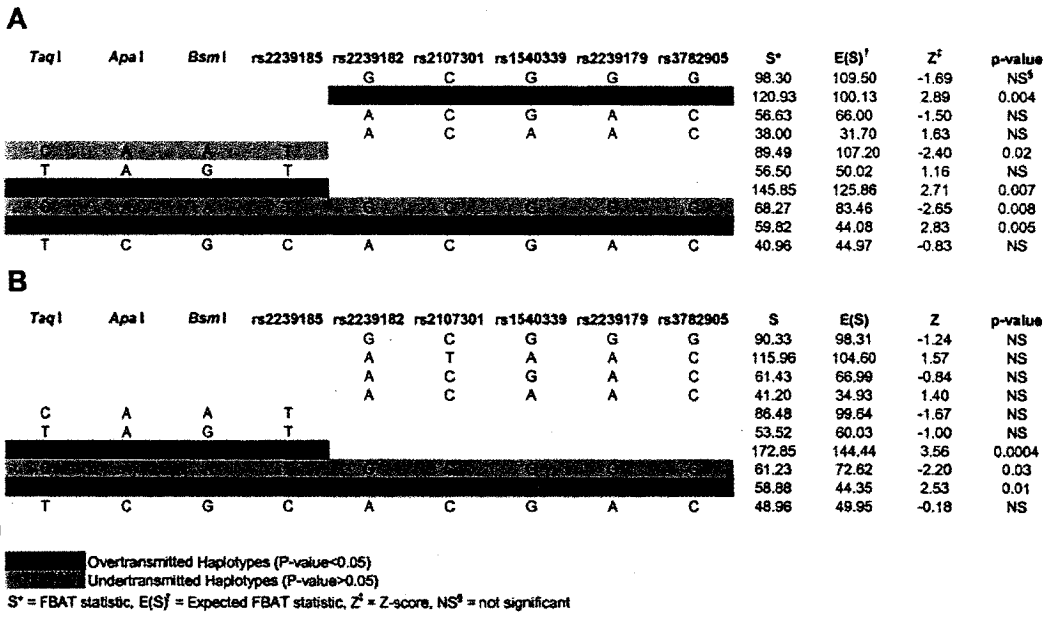


Figure 3. Haplotype transmission patterns for (*A*) asthma and (*B*) atopy. E(S) = expected FBAT statistic; S = FBAT statistic; Z = Z score.

28-kb region, between intron 2 and exon 9 of *VDR*, and are in strong LD ($D' \geq 0.8$) with at least one additional SNP (80% of the pairwise LDs are $D' \geq 0.80$). Of the 36 pairwise LDs calculated between these nine SNPs, eight D's are low ($0.09 \leq D' \leq 0.73$), and they further separate the region into two blocks of tightly associated SNPs. We relaxed Gabriel's criteria for haplotype block definition: the outermost marker pair was required to be in LD with an upper confidence limit that exceeds 0.90 and a lower confidence limit that exceeds 0.7. Block 1 locates toward the 3′ end of *VDR* and consists of SNPs *Taq*I, *Apa*I, *Bsm*I, and rs2239185, whereas block 2 locates toward the 5′ end of *VDR* and comprises SNPs rs2239182, rs2107301, rs1540339, rs2239179, and rs3782905. Block 1 spans approximately 5.8 kb, and block 2 spans roughly 8.4 kb. The two blocks, which are separated by 10.8 kb, show moderate LD between blocks ($D' = 0.77$, data not shown). Three common haplotypes (frequency > 0.1) are observed within block 1: haplotypes TCGC (frequency = 0.45), CAAT (0.39), and TAGT (0.15); within block 2, four common haplotypes are observed: haplotypes GCGGG (0.29), ATAAC (0.27), ACGAC (0.16), and ACAAC (0.11). Three common haplotypes of the nine SNPs that extended across the two blocks were observed: (3′ to 5′ SNPs) CAATGCGGG, TCGCATAAC, and TCGCACGAC (Figure 2B).

Haplotype-specific Association Analysis

FBAT results and LD patterns indicate that associations between *VDR* variants and asthma/atopy occur across the two blocks within a 28-kb region. To characterize haplotype transmission in this region, 10 common haplotypes previously inferred using Haploview were assessed for nonrandom transmissions using FBAT, version 1.4 (28, 29) (Figure 3). For asthma, 5 of the 10 common haplotypes show nonrandom distribution of haplotypes. In block 1, the nonrandomly transmitted haplotypes were TCGC (overtransmitted, $p = 0.007$) and CAAT ($p = 0.02$, undertransmitted). In block 2, haplotype ATAAC was overtransmitted ($p = 0.004$). In the combined block, haplotype TCGCATAAC was overtransmitted ($p = 0.005$), and haplotype CAATGCGGG was undertransmitted ($p = 0.008$).

For atopy, 3 of the 10 common haplotypes show nonrandom distribution of haplotypes. In block 1, haplotype TCGC was overtransmitted ($p = 0.0004$). In the combined block, haplotype TCGCATAAC was overtransmitted ($p = 0.01$), and haplotype CAATGCGGG was undertransmitted ($p = 0.03$).

VDR Resequencing

To exclude the presence of a coding *VDR* polymorphism that was not seen in previous analyses of *VDR*, we sequenced the proximal promoter region housing the six alternatively spliced exons 1 (1a–1f), exons 2–9 and intron/exon boundaries of *VDR* locus from genomic DNA obtained from 24 cases; the selection was based on their haplotype diversity. A total of 15 SNPs were identified, including *Fok*I, *Apa*I, and *Taq*I. The remaining 12 SNPs were only seen in noncoding regions; these SNPs are listed in Table E5 in the online supplement.

DISCUSSION

Using a family-based cohort, we observed association between common allelic variants of *VDR* and phenotypes of asthma and atopy in a French-Canadian founder population. Six SNPs, in introns 2, 3, 6, and 8 and exon 9 of *VDR* spanning approximately 28 kb of genomic sequence, were associated with asthma. These markers fall into two haplotype blocks, with very substantial LD among markers of both blocks. With our present LD pattern obtained from 12 SNPs, the precise boundaries of the two blocks are unknown, and additional SNPs might be needed to represent better the LD pattern at this locus. Nevertheless, significant nonrandom segregation of marker haplotypes spanning this 28-kb region of *VDR* further confirmed association of this region with asthma and atopy.

Interestingly, the same 28-kb haplotypes spanning both core *VDR* LD blocks are associated with asthma and atopy. If considering the blocks individually, the same haplotypes show the same direction of over or undertransmission, but some of these haplotypes do not reach statistical significance for association with one of the phenotypes. At this point, the current level of information is not sufficient for us to assign a greater likelihood of association for any of the two haplotype blocks to either of the phenotypes. Given the high clinical correlation between both phenotypes and the similarity of their haplotype associations, we believe it likely that there is one or more functional variants at the 3′ end of the *VDR* locus responsible for susceptibility to both asthma and atopy.

In a separate association study of *VDR* variants described in a companion article (Raby and colleagues) (41), association with asthma was evaluated in two study populations: a family-based cohort and a case control cohort. The family cohort is part of the Childhood Asthma Management Program (CAMP) study (42, 43), with families being recruited from eight centers across North America (www.jhucct.com/camp/open/sites.htm). The case control cohort is part of the Nurses' Health Study (44) and included only women.

In the Nurses' Health Study cohort, four of the five SNPs tested are associated with asthma (Table 2). Of these four SNPs, three are with the same alleles found to be associated in the Quebec cohort. For the remaining associated SNP *Apa*I, the C allele was overtransmitted in both the Quebec and the Nurses' Health Study cohorts but only reaches the 95% significance level in the Nurses' Health Study cohort. In the Quebec cohort, SNP *Apa*I showed significant association with the atopy phenotype. SNP *Apa*I is the only tested SNP being associated with asthma in the CAMP cohort, but the distribution pattern of its alleles was different from both the Quebec and the Nurses' Health Study cohorts: allele A was overtransmitted in the CAMP cohort and was undertransmitted in the other two cohorts. Hence, the same *VDR* alleles were observed to be associated with asthma in two of three study populations.

We note that the CAMP study recruited patients with median age of onset of three (boys) and four (girls) (ranging from 2 to 6 years), comparable to that of the Quebec probands (5 years, with a wide range of 2 to 46 years). However, the median age of recruitment of the Quebec probands was older (16 years),

TABLE 2. COMPARISON OF ASSOCIATIONS OBSERVED IN THE QUEBEC, CHILDHOOD ASTHMA MANAGEMENT PROGRAM, AND NURSES' HEALTH STUDY COHORTS

Variant	Quebec	Childhood Asthma Management Program	Nurses' Health Study
Rs3782905	S	NS	S*
Rs1540339	S	NS	NS
Rs2239182	S	NT	NT
Rs2239185	S	NS	S*
*Bsm*I	S	NS	NT
*Apa*I	NS (S for atopy)	S†	S
*Taq*I	S	NS	S*

Definition of abbreviations: NS = not significant; NT = not typed; S = statistically significant association ($p < 0.05$).

* Same allele of association as the Quebec cohort.

† Different allele of association from both the Quebec and the Nurses' Health Study cohorts.

which correlates with a longer duration of disease and/or recurrence in adulthood. The Nurses' Health Study cohort includes female nurses with asthma present in adulthood (44). The lack of *VDR* associations in the CAMP study may be explained by differences in phenotypes related to age or duration of disease and/or sample size issues (45, 46).

By sequencing the promoter, exons, and their surrounding regions, we excluded novel missense polymorphisms that could have been responsible for the observed associations. Of the SNPs associated with asthma/atopy, only *Taq*I resides in the coding region (exon 9), and the polymorphism does not result in an amino acid change. *Taq*I has been shown to be associated with many metabolic and immune-mediated diseases. The T allele, which is associated with asthma and atopy in our cohort, is also associated with tuberculosis (13, 21–23), chronic hepatitis B infection (23), lepromatous leprosy (12) and type 1 diabetes in Eastern Europeans (47) and South Indians (39). The C allele, on the other hand, is associated with Crohn's disease (11), tuberculoid leprosy (12), and type 1 diabetes in Germans (48). The functional consequence of this polymorphism is not fully understood. In lymphocytes, the *Taq*IC allele is 30% less abundant (49), whereas in pituitary adenomas and transfected green monkey kidney (COS-7) cells, the same allele is associated with higher mRNA levels (7, 37).

Similar to variant *Taq*I, SNPs *Apa*I and *Bsm*I have been widely studied and have been shown to be associated with many diseases. Alleles *Bsm*IG and *Apa*IC are associated with asthma/atopy in this cohort; the same alleles are also associated with high bone mass density (7) and sporadic hyperparathyroidism in females (37), whereas allele *Bsm*IA is associated with fast acquired immunodeficiency syndrome progression (6) and type 1 diabetes risk per se and acute-onset type 1 diabetes (40).

Given that association of asthma with *VDR* involves variants from intron 2 to exon 9, spanning approximately 28 kb and that no SNPs giving rise to an amino aid change were found, it is possible that the functional variant that confers susceptibility to asthma is a regulatory SNP (50), located in a *VDR* intron. Because VDR is a known immunoregulatory switch molecule and many of the associated phenotypes have the characteristics of Th1/Th2 imbalance, the mechanism of VDR in immune-mediated diseases may involve varying levels of VDR in immune cells on stimuli.

In summary, we identified a strong association between genetic variants at the *VDR* locus and asthma/atopy in a Quebec cohort. Along with other known asthma risk genes identified such as *ADAM*33 (51), *TNFA* (52), *RANTES* (53), and *GPRA* (54), the addition of *VDR* involvement in the understanding of asthma/atopy pathogenesis will shed light for better control and treatment.

Conflict of Interest Statement: A.H.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; C.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.J.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A report of invention is being reviewed by McGill University Office of Technology Transfer.

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experiments that have already been done elsewhere in the world.

A library would have to be an international effort because of the rarity, increasing complexity, and multidisciplinary nature of the evidence. In addition, recent bioterrorist threats have made the maintenance of a knowledge base on vaccines against diseases that have been eradicated (smallpox), or have been abandoned for lack of a lucrative "market" (plague), or have a small market (anthrax) important to facilitate swifter reactions to future unexpected natural or man-made threats.

Most of all, evidence needs to be analysed and interpreted in an unbiased manner to allow those who receive vaccines or their guardians to make an informed choice. If we already have the knowledge, why should we ignore it?

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Crohn's disease, mycobacteria, and NOD2

In a recent Personal view paper, Robert J Greenstein presented the rationale behind efforts to elucidate a mycobacterial cause for Crohn's disease.[1] Since the scientific case for such an association derives mostly from publications on mycobacteria, it is interesting that Greenstein also introduced the issue of host genetics, most notably as it pertains to susceptibility to mycobacterial diseases. We have been reading the same publications from a combined host-pathogen perspective and wondered about the impact of new developments in Crohn's genetics and innate immunity, with specific reference to whether they contribute to or contradict the role of mycobacteria in Crohn's disease. These considerations have stimulated preliminary investigations; the following case report suggests a unified approach to exploring Crohn's disease pathogenesis.

A 21-year-old Canadian-born man with no known exposure to mycobacterial infection was referred because of persistently active Crohn's disease despite maximum medical therapy (including mesalazine, mercaptopurine, corticosteroids, and infliximab). Crohn's disease had been diagnosed 3 years earlier, after a clinical presentation of severe abdominal pain and weight loss. Ileocolonoscopy and biopsy sampling showed terminal ileitis and granulomatous colitis. An enteroclysis showed diffuse jejunal thickening and dilated small-bowel loops. Mesenteric adenopathy was shown by computed tomography (CT) scan and laparoscopic biopsy showed non-necrotising granulomata on histopathology.

A block of the pathology sample from a diseased mesenteric node was retrieved, deparaffinised, and DNA was extracted for bacteriological study. *Mycobacterium avium paratuberculosis* (MAP) DNA was present on direct PCR for the specific insertion sequence IS900. The PCR product was sequenced and confirmed to be identical to IS900 and different from closely related sequences of other mycobacteria.[2] To avoid potential contamination nested PCR was not done. Controls including tissue processed in the same batch were all negative and PCR of the same sample was negative for IS6110 of *Mycobacterium tuberculosis*. This same DNA was also tested for the genetic region of MAP that distinguishes cattle from sheep strains; PCR was positive for the cattle type of MAP.[3]

To further define his illness, blood was drawn, lymphocytes separated and stimulated in vitro with tuberculin, paratuberculin, and a non-specific mitogen (phytohaemaglutinin). Unstimulated cells were also studied as a control. RNA extracted from these cells was studied by quantitative RT-PCR and twice showed increased interferon γ message in both paratuberculin and tuberculin-stimulated cells compared with unstimulated cells (induction ratio of 25–100 fold).

Since the patient was in chronic abdominal pain and unable to function in daily activities, anti-MAP treatment was offered, consisting of clarithromycin and rifabutin. After 7 days, the patient developed a febrile, influenza-like illness, similar to the lepra reaction reported with antibiotic treatment of leprosy. There was no evidence of intercurrent illness. This reaction subsided within 4–6 weeks with paracetamol for symptomatic relief. After 12 months of treatment, the patient is tolerating the medications well. A repeat enteroclysis revealed marked improvement, with most of the intestine reported as normal, save for one isolated segment of inflamed ileum. A follow-up abdominal CT scan showed for the first time a mild reduction in the quantity of lymph nodes. These findings suggest that in this individual there is compelling evidence of MAP disease, based on microbiological, immunological, and therapeutic observations.

Because of the recent published material showing genetic susceptibility to Crohn's disease, we also did genetic testing, targeting documented susceptibility alleles of the *NOD2/CARD15* gene (G908R, R702W, and 3020ins). The patient was heterozygous for the G908R substitution and homozygous wild-type for the other alleles. Thus, this man has evidence of typically defined Crohn's disease with a Crohn's disease susceptibility mutation, but also has evidence of human MAP disease. Applying the principle of Occam's razor, the most parsimonious explanation in a patient without other illness is

that MAP infection in a genetically susceptible host resulted in the Crohn's disease phenotype.

Genome-wide searches for irritable-bowel-disease-susceptibility genes have implicated several loci for Crohn's disease. Of these *NOD2/CARD15* has received the most attention.[4] The NODs are a family of cytosolic proteins implicated in intracellular recognition of bacterial components, and the bacterial motif recognised by NOD2/CARD15 is muramyl dipeptide (MDP) derived from the peptidoglycan of bacteria.[5] Several independent groups have documented variants of this gene predisposing to Crohn's disease, including the G908R substitution for which heterozygosity was associated with a relative risk of 6·3 for Crohn's disease.[6] Peripheral blood monocytes from people with Crohn's disease-susceptibility alleles have been shown to be defective in their ability to respond to MDP in vitro including healthy individuals without Crohn's disease.

This finding indicates that the presence of certain bacteria in the face of permissive NOD2 variants is necessary for development of Crohn's disease.[7] Since *NOD2* is expressed in the monocyte lineage,[8] an invasive intracellular pathogen, such as a mycobacterium, becomes an attractive candidate. While the intracellular receptors needed for mycobacterial recognition are not yet fully determined, it is known that the immuno-adjuvant effect of mycobacteria is dependent on MDP.[9] These lines of evidence suggest that in an individual with a NOD2 variant, the innate response to mycobacterial exposure would be inadequate, permitting establishment of a persistent mycobacterial infection. Chronic infection could then activate the inflammatory response characteristic of Crohn's disease.

Since we have only documented the coexistence of MAP disease and a permissive *NOD2/CARD15* mutant in one individual, it is premature to speculate on the epidemiological effect of these findings. For instance, *NOD2/CARD15* mutations have only been documented in around one-third of Crohn's disease patients; therefore, in theory, one might predict that different forms of genetic susceptibility may predispose to distinct pathogens, thereby complicating the search for the "cause" of Crohn's disease. However, we believe that this case illustrates a potential conceptual approach to Crohn's disease aetiology, which involves a tandem search for bacterial trigger and host susceptibility. The proportion of Crohn's disease cases potentially attributable to MAP and the clinical/epidemiological consequence of MAP exposure among human beings are the focus of continuing studies.

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Directly observed therapy for HIV/tuberculosis co-infection

The South African government recently unveiled plans for provision of antiretroviral therapy (ART) to HIV-infected individuals, which includes a commitment to provide ongoing treatment for 50 000 people by the end of 2004, and for as many as 1·4 million by 2009.[1] With drug prices declining and the availability of drugs increasing, through the Global Fund to Fight AIDS, Tuberculosis, and Malaria and other sources, the question arises of when and how ART will be delivered in the developing world.

The introduction of highly active antiretroviral therapy in the developed world in 1996 transformed the course of HIV infection, turning it into a chronic, long-term, manageable disease.[2] Conversely, in Africa, where 70% of the world's HIV-infected population lives, ART is still largely unavailable. With nearly 30 million people living with HIV worldwide, there is a critical need to understand better how ART can be delivered efficiently and cost-effectively. One strategy that may be appropriate in settings where individuals are co-infected with HIV and tuberculosis is to use the existing tuberculosis diagnosis and treatment infrastructure to deliver and monitor ART.

Tuberculosis is the most common serious infectious complication associated with HIV in sub-Saharan Africa and the most common cause of death among patients with HIV in many developing countries.[3–6] Coinfection with HIV results in substantially higher fatality rates among those infected with tuberculosis, irrespective of the administration of effective tuberculosis chemotherapy.[7,8] Among populations with high HIV burdens in Malawi and South Africa tuberculosis mortality rates are 31% and 24·7%, respectively.[9,10] The high prevalence of HIV among those with active tuberculosis may make it easier to identify individuals with HIV in the developing world who are likely to benefit most from ART. Harnessing current tuberculosis-treatment programmes is, therefore, likely to be an effective strategy for identifying patients who are most in need of ART.

In many developing countries, an established, acceptable, and familiar infrastructure is available to provide

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CHAPTER 3

The *NRAMP* Genes and Human Susceptibility to Common Diseases

Audrey Poon and Erwin Schurr

Abstract

Results obtained in murine models have raised the hope that human *NRAMP* genes could be important determinants of susceptibility for common human diseases. There is good agreement among studies conducted in racially vastly different populations that *NRAMP1* alleles are risk factors for tuberculosis. However, the extent of the *NRAMP1* mediated risk may vary according to the specific epidemiologic setting. There is also good evidence that *NRAMP1* is involved in susceptibility to autoimmune diseases, such as rheumatoid arthritis, especially for patients who present early onset of disease. A large number of additional diseases with either known or suspected infectious etiology have also been investigated for a possible contribution of *NRAMP1* to risk of disease. In most examples, replication studies are needed before firm conclusions can be reached. In contrast to *NRAMP1*, no disease associations have been identified for *NRAMP2*.

Introduction

It is becoming increasingly evident that human genetic variability is an important modulator for the risk of acquiring common human diseases. Unfortunately, the identity of genetic variants that impact on risk of disease is largely unknown. A major hurdle in identifying genetic risk factors in human infectious disease is the complex genetic control of such phenotypes, and susceptibility to infectious diseases is frequently referred to as a "complex trait." Complex traits are characterized by incomplete penetrance, polygenic contribution to trait expression and genetic heterogeneity.[1] To identify susceptibility genes, geneticists are employing linkage and/or association studies.[2] If the correct genetic model is known, parametric linkage studies are the most powerful means of identifying disease variants. However, the mode of inheritance, the penetrance of predisposing alleles, the frequency of disease alleles and other pertinent parameters are usually not known for susceptibility to infectious diseases and non-parametric linkage methods are employed. These methods, especially if used for genome wide searches, have limited sensitivity to detect genes impacting on disease susceptibility and usually only allow the identification of genes that exert strong effects on susceptibility.

To identify genes that exert moderate genetic effects on susceptibility to disease candidate genes are employed. Candidate genes are selected based on their known or proposed function in humans or animal models. Specifically, animal models can provide useful candidates to be tested in human populations. For example, the mouse model of susceptibility to early resistance/susceptibility to infection with *Mycobacterium bovis* (BCG) led to the identification of the human *NRAMP1* gene as a risk factor in susceptibility to mycobacterial disease.[3-6] Most commonly candidate genes are employed in association studies. By comparing the frequencies

The Nramp Family, edited by Mathieu Cellier and Philippe Gros.

of candidate gene alleles in affected and non-affected individuals, or by analysing the number of transmissions of such alleles from parents to affected children, association of the studied allele with susceptibility to disease can be established. Association between allele(s) and susceptibility to infectious disease may imply that either the allele is the disease causing variant or that it is in physical proximity to the allele causally involved in disease development.[1,7] Presently much of the research aimed at deciphering the genetic component of infectious disease susceptibility is employing association studies. It is noteworthy that association studies while providing a powerful means of analysis also present distinct disadvantages. Specifically, small numbers of individuals enrolled, undetected population substructures, allelic heterogeneity, failure to correct for multiple testings or for not reported testings as well as publication bias have led to the publication of false positive and false negative studies. Hence, replication of association findings (or the absence of such associations) is critical for a full appreciation of the role of studied genetic variants as risk or protective factors for infectious diseases.

NRAMP1 Gene Location and Structure

The official nomenclature for the human natural resistance-associated macrophage protein 1 (*NRAMP1*) gene is solute carrier family 11 (protein-coupled divalent metal ion transporter) member 1 (*SLC11A1*) gene. The rationale for renaming commonly known genes with essentially meaningless and highly obscure complex gene family designations has been questioned in the past and the discussion of the pros and cons is outside the scope of this review.[8] In this review, we will adhere to the originally assigned gene designation *NRAMP1*. *NRAMP1* was cloned and mapped to chromosomal region 2q35, in close proximity to the interleukin 8 receptor gene (*IL8R*), the villin gene (*VIL*) and the recently identified Nuclear LIM Interactor-Interacting Factor gene (*NLI-IF*).[4,6,9-12] Interestingly, the *NRAMP1* gene is located in a genomic region with extreme density of Alu- and other genomic repeat clusters.[12,13]

The *NRAMP1* gene consists of 15 exons and spans 13kb.[4,10] An alternative splice site exists within intron 4. The regular spliced gene is predicted to encode a 550 amino acid protein, with 12 putative transmembrane domains, 2 N-linked glycosylation sites and 1 evolutionary conserved consensus transport motif. The alternatively spliced gene is predicted to encode a truncated protein due to a premature stop codon in alternatively spliced exon 5.[4,10] This truncated protein is assumed to be nonfunctional. The transcription start site has been mapped at 148 bp or 175 bp 5'of the translation codon.[10,14] Regulatory motifs within the *NRAMP1* promoter include 1 TATA box element, 6 IFNγ response elements, 3 W-elements, 3 NFκB binding sites, 1 AP-1 site, 1 Z-DNA forming enhancer element and 9 purine-rich GGAA core motifs for the myeloid-specific PU.1 transcription factor.[4,10,11] The presence of these consensus and binding motifs for transcriptional activation is consistent with the effects of known immune modulators of *NRAMP1*.

NRAMP1 Polymorphisms

A total of 11 polymorphisms have been identified and verified in the *NRAMP1* gene.[6,10,15,16] One repeat polymorphism and one single nucleotide polymorphism (SNP) are located in the promoter region, (designated as 5'(CA)n and –236C/T, respectively), while nine biallelic polymorphisms are found in the remainder of the gene. Four of the eight biallelic polymorphisms occur in the coding region of which two introduce an amino acid change (A318V in exon 9 and D543N in exon 15); three polymorphisms occur in intronic regions (469+14G/C in intron 4; 577-18G/A in intron 5 and 1465-85G/A in intron 13); two insertion/deletion polymorphisms are found in the 3'UTR of *NRAMP1* (1729+55delTGTG and 276insCAAA280; Fig. 1).

Specifically, the 5' (CA)n polymorphism has been the focus of intensive study in efforts to link select gene polymorphisms to altered *NRAMP1* functional activity. The 5'(CA)n polymorphism has been proposed to function as an enhancer element due to its predicted Z-DNA

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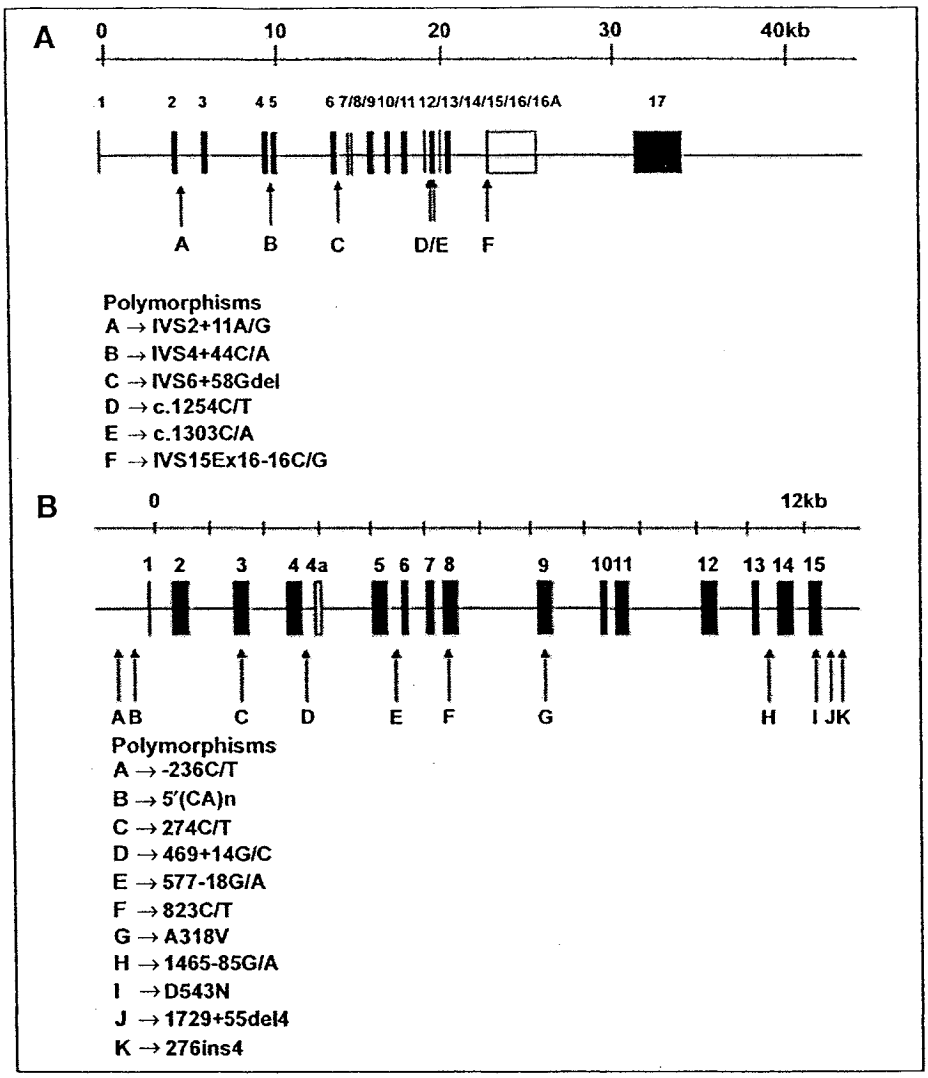

Figure 1. Schematic presentation of the intron-exon organization of the two human *NRAMP* genes. A) Location of polymorphisms in the *NRAMP1* gene. Adapted from references 4 and 6. B) Location of polymorphisms in the *NRAMP2* gene. Adapted from references 72 and 82.

forming properties.[5] DNA segments in a "Z" secondary structure interact with a distinct class of binding proteins to regulate transcriptional activity. Four alleles of the 5'(CA)n polymorphism have been observed in 36 Brazilian multicase families of leprosy, tuberculosis (TB) and visceral leishmaniasis. The four alleles are $T(GT)_5AC(GT)_5AC(GT)_nG$, n = 11 (allele 1), 10 (allele 2), 9 (allele 3) and 4 (allele 4).[10] Four additional alleles have been observed in different populations; n=8 was observed in a Texas TB study[17], alleles 5 ($T(GT)_4AC(GT)_5AC(GT)_{10}G$ and 6 ($T(GT)_5AC(GT)_5AC(GT)_4AT(GT)_4GGCAGA(G)_7$) were observed in a primary biliary cirrhosis case control study, and allele 7 ($T(GT)_5AC(GT)_5AT(GT)_{11}GGCAGA(G)_6$) was observed in a Japanese study investigating

NRAMP1 polymorphisms in inflammatory bowel disease (IBD).[17-19] In all populations studied, allele 3 (n=9) is the most frequent representing 75% to 80% of all chromosomes followed by allele 2 (n=10) which is found in approximately 20% to 25% of all chromosomes. The remaining promoter alleles are rare variants.

The four promoter repeat alleles identified in the Brazilian study were shown to drive differential expression of a luciferase reporter construct following transfection into myeloid U937 cells.[5] In the absence of exogeneous stimuli, alleles 1, 2 and 4 were poor promoters; while allele 3 drove high luciferase expression. In the presence of interferon γ (IFNγ), all four alleles showed enhanced gene expression. Such enhancement after stimulation with IFN-γ implies that multiple γ-IREs flanking the Z-DNA forming polymorphic repeats are important in enhancing gene expression across all 4 alleles. Addition of bacterial antigen lipopolysaccharide (LPS) in the presence of IFN-γ had no effect on alleles 1 and 4 expression while it caused significant expression reduction driven by allele 2 and enhanced expression driven by allele 3.[5] These results supported the view that NRAMP1 protein would be synthesized by cells belonging to the monocyte-macrophage lineage in response to microbial assault and that certain individuals, depending on their promoter *NRAMP1* alleles, would be able to mount a more vigorous production of NRAMP1 protein resulting in increased disease resistance. If and to what extent this expectation will hold true is presently unknown.

NRAMP1 Function

In humans, *NRAMP1* mRNA is expressed in spleen, lung, liver, and most abundantly in peripheral blood leukocytes.[4,20] The function of human NRAMP1 is not known. Consequently, it is presently unclear if known *NRAMP1* polymorphisms impact on the function of NRAMP1 protein, and this is clearly a question that requires further study. It appears likely that NRAMP1 protein has a function that is closely related to the one of its mouse ortholog Nramp1. The mouse protein is targeted to the phagosomal membrane where it controls pathogen replication by transporting divalent cations across the phagosomal membrane.[3,21] The direction of transport is still controversial but most likely proceeds from the vesicular lumen into the cytoplasm. Absence of Nramp1 in the phagosomal membrane results in altered cation fluxes that in turn trigger substantial changes in intracellular vesicle trafficking.

NRAMP1 and Infectious Diseases Susceptibility

The World Health Organization (WHO) reported that in the year 2000, one third of total global deaths were caused by infectious diseases. Epidemiologic and genetic studies suggest that susceptibility to and progression of infectious diseases are determined by the genetic makeup of the host, pathogen factors and the interplay between environment, host and pathogen. Infectious diseases caused by mycobacteria are one of the most challenging global health problems. Consequently, enormous efforts have been directed to the investigation of susceptibility to TB and leprosy, the two most prevalent human mycobacterial diseases.

TB is an infectious disease caused by *Mycobacterium tuberculosis*. *M. tuberculosis* transmission occurs via aerosols and the main site of infection and disease manifestation are the lungs. Other sites that may show pathological changes in response to *M. tuberculosis* infection include the larynx, lymph nodes, pleura, brain, kidneys or bones and joints (often summarily referred to as extrapulmonary TB). The mycobacteria can also enter the bloodstream and spread to all parts of the body (miliary TB). If an infected individual develops TB within approximately one year of infection, the disease is often categorized as primary TB. If disease develops years after infection, it is categorized as reactivation TB.

Leprosy, caused by *Mycobacterium leprae*, exhibits a spectrum of clinical phenotypes, ranging from localized, paucibacillary forms (tuberculoid leprosy) to disseminated, multibacillary forms (lepromatous leprosy). *M. leprae* induced pathological changes occur mainly in the skin and peripheral nerves, but can also involve other areas such as the eyes, nose, or testicles.[22] It is important to realize that severe neurological damage frequently occurs in patients that have

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been microbiologically cured of the disease. Following cure, in particular multibacillary patients may suffer from vigorous, neuro-destructive auto-inflammatory reactions, so called reversal reaction, and hence require lengthy follow-up after antibiotic treatment.

NRAMP1 *and Susceptibility to Tuberculosis*

Linkage studies aimed at providing evidence for *NRAMP1* as a susceptibility gene in TB have been done in human populations of varying racial and ethnic backgrounds and epidemiological settings. Generally speaking, linkage studies have provided inconsistent evidence for a role of *NRAMP1* in TB susceptibility. For example, a genome scan in South African and Gambian sibpairs did not detect any significant evidence for linkage between the *NRAMP1* region and TB.[23] Interestingly, an earlier study in a large number of Brazilian TB families provided evidence for linkage of two markers tightly linked to *NRAMP1*, but not for *NRAMP1* itself.[24,25] In striking contrast to the above linkage studies, a genetic analysis of a TB outbreak in an aboriginal Canadian community provided strong evidence for linkage between *NRAMP1* and TB susceptibility.[26]

Differences in markers employed, patient recruitment schemes, population histories and/or analytical methodologies may have contributed to the different results in the above linkage studies. For example, the Canadian study investigated the role of *NRAMP1* in rapid progression of infection to disease by recruiting cases who developed TB shortly after infection. In contrast, the other studies investigated the role of *NRAMP1* in TB infection susceptibility by recruiting patients who represented an unknown mix of rapid and slow progression from infection to TB. Likewise, the Canadian study considered the exposure history of patients to model gene-environment interactions while other studies solely considered the impact of *NRAMP1*, or closely linked genetic markers, on TB. Interestingly, by neglecting exposure histories evidence for linkage of *NRAMP1* with TB dissipated even in the Canadian study. Lastly, human populations are evolving under different environmental pressures, and genetic variability among races may result in genetically heterogeneous control of TB susceptibility.

In addition to linkage studies, many association studies have been conducted to investigate the role of *NRAMP1* in TB susceptibility. In a large population based association study done in The Gambia, four polymorphisms (5'(CA)n, 469+14G/C, D543N and 1729+55del4) displayed association with tuberculosis.[27] For each of these polymorphisms, the rare alleles increased the risk of developing TB, with odd ratios of 1.5-1.9. Further analyses on Gambian TB patients showed that promoter alleles 1 and 2 conferred risk to TB while allele 3 offered protection.[28] The two 5' polymorphisms (5'(CA)n and 469+14G/C) are in strong linkage disequilibrium, as are the two 3' polymorphisms (D543N and 1729+55del4). However, there is only limited linkage disequilibrium between the 5' and 3' ends of *NRAMP1*, and 5'and 3' *NRAMP1* polymorphisms were independently associated with TB susceptibility. As a consequence, the risk to develop TB increased dramatically for individuals who were heterozygous for both the 5'and 3'polymorphism (OR=4.07; 95% CI 1.86-9.12).

Following the Gambian study, *NRAMP1* polymorphisms have been investigated by various groups in other populations. Confirmations and contradictions exist. Associations of 5' polymorphisms with TB have been confirmed in Japanese TB patients but not in a Cambodian population.[29,30] In addition, a family based association study done in Guinea-Conakry confirmed the association of the 469+14G/C polymorphism with TB.[31] The Japanese study confirmed increased risk of TB associated with alleles 1 and 2 and a protective effect associated with allele 3 of the 5'(CA)n polymorphism. A study conducted among Caucasian TB patients in Denmark failed to detect an association of *NRAMP1* polymorphisms with TB.[32] However, in this study, among patients of divers racial backgrounds, an association with the 469 + 14G/C polymorphism was observed in patients with microscopy-positive TB as compared to microscopy-negative cases. It is not clear if this finding was confounded by the unbalanced distribution of patients belonging to different racial groups among microscopy-positive and -negative TB cases. The 3' *NRAMP1* polymorphism associations with TB have been confirmed

in Korean and Japanese TB populations, however, studies conducted in Guinea-Conakry, Denmark and Taiwan did not detect any 3'polymorphism association with TB.[31-33] The study in Cambodian patients detected a protective effect of the rare allele of the D543N polymorphism, contrary to the findings in the Gambian, Japanese and Korean populations.[27,29,30,34] Additional contrasting findings came from a study of Caucasian TB patients in Houston that, in contrast to the Danish study, observed an excess of promoter allele 3 in non TB controls compared to pulmonary TB, extrapulmonary TB and HIV-positive TB patients (p=0.004).[17] Within the different clinical forms of TB, the Houston study observed a significant increase of allele 2 in extrapulmonary as compared to pulmonary cases. This observation raised the enticing possibility that an effect of *NRAMP1* on TB susceptibility may be more pronounced among extrapulmonary cases of TB.

The notion that *NRAMP1* variants are genetic risk factor in TB susceptibility is mostly agreed upon. It is difficult to envision that *NRAMP1* polymorphisms are in linkage disequilibrium across racial boundaries with unknown disease causing polymorphisms in a gene other than *NRAMP1*. The only gene that one could suspect of being in linkage disequilibrium with *NRAMP1* is *NLI-IF* and a contribution of this gene to TB susceptibility has been ruled out.[35] Hence, the repeated reports in different ethnic populations of *NRAMP1* polymorphisms being associated with TB are strong evidence to implicate *NRAMP1* in TB susceptibility. What is not clear is the causal relationship between these *NRAMP1* risk factors and TB disease, and the question if *NRAMP1* measured risk of disease is more pronounced for specific forms of TB. For example, the finding that *NRAMP1* variants are associated with microscopy-positive TB and extrapulmonary TB suggests that *NRAMP1* is involved in control of bacillary growth and possibly dissemination rather than susceptibility to TB *per se* or susceptibility to infection with *M. tuberculosis*.[17,32]

NRAMP1 *and Susceptibility to Leprosy*

In addition to classifying forms of leprosy according to number of skin lesions and counts of bacilli in skin smears, the disease can be classified more accurately according to motor and sensory changes and biopsy findings. These findings can classify the disease, as indeterminate, tuberculoid, borderline tuberculoid, mid-borderline, borderline lepromatous, and lepromatous leprosy.[22] The role of *NRAMP1* in susceptibility to leprosy has been investigated. A linkage study done on French Polynesian leprosy families failed to detect linkage between *NRAMP1* and susceptibility to leprosy.[36,37] By contrast, a linkage study performed on 20 Vietnamese multiplex leprosy families revealed significant linkage between an *NRAMP1* haplotype and leprosy per se, i.e., leprosy regardless of the clinical leprosy manifestation.[38] The reason for the differences in *NRAMP1* linkage to leprosy in Vietnam and French Polynesia could be indicative of genetic heterogeneity of leprosy susceptibility. However, it is noteworthy to realize that the French Polynesia families did not provide the power to detect modest genetic effects on leprosy susceptibility. Considering that in a recent large scale genetic study of leprosy susceptibility in Vietnam the *NRAMP1* region had only a modest impact on susceptibility (Lod score = 1.0; $p < 0.02$), it is clear that such a moderate genetic effect of *NRAMP1* in the French Polynesian families could not have been detected.[39]

In addition to linkage studies, association studies have been carried out to investigate the role of *NRAMP1* in leprosy susceptibility. A study in Mali did not detect any association between the tested polymorphisms and leprosy susceptibility per se.[40] However, when substructured according to leprosy type, the leprosy population had an excess of heterozygotes for the *NRAMP1* 1729+55del4 polymorphism in the multibacillary group compared to the paucibacillary group; i.e., the risk of developing multibacillary leprosy is greater than paucibacillary leprosy if an individual carries at least one copy of the deletion allele (OR=5.79).[40] Questions remain about the validity of stratifying patient populations in the absence of an overall effect. An association study done in an Indian population failed to detect associations

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between the *NRAMP1* 5'polymorphisms and leprosy, keeping in mind that the polymorphisms tested were not very polymorphic in this Indian population.[41]

In contrast to the tuberculin skin test that is being used to establish infection by *M. tuberculosis* and measured at 48 hrs to 72 hrs after injection, the Mitsuda reaction, measures the granulomatous specific immune response against intradermally injected heat-killed *M. leprae* bacilli (lepromin) at 28 days post infection. The Mitsuda reaction has a reasonably good prognostic value for susceptibility to lepromatous forms of leprosy. Complex segregation analysis done in a Brazilian population suggested major gene control of Mitsuda reactivity.[42] A linkage study of 20 nuclear Vietnamese families detected strong linkage of *NRAMP1* with extent of Mitsuda reactivity; suggesting that *NRAMP1* alleles influence the acquired anti-mycobacterial immune response.[43] Interestingly, the linkage of *NRAMP1* to Mitsuda reactivity appeared to be independent of the clinical phenotype of tested persons. Since granulomatous responses are indicative of a TH1-type immune response it is possible that *NRAMP1* alleles impact on the TH1/TH2 balance of anti-mycobacterial immunity. This suggestion would be consistent with the observed association of *NRAMP1* polymorphisms with leprosy type in the Mali study.[40] Although a follow up study of the Brazilian population failed to detect linkage between *NRAMP1* polymorphisms and Mitsuda reactivity, the very small sample size and failure to identify alleles by identity-by-state vs identity-by-descent may have contributed to the negative result.[44]

NRAMP1 *and Susceptibility to Other Infectious Diseases*

Positive evidence supporting the role of *NRAMP1* in susceptibility to TB and leprosy prompted its candidacy as susceptibility gene in other infectious diseases. For example, the possible role of *NRAMP1* polymorphisms in HIV susceptibility has been investigated in a Columbian population.[45] The results of this study indicated allele 3 of the 5' $(CA)_n$ polymorphism to be a protective factor for HIV infection (RR=0.35 95%CI 0.14-0.91), along with the two linked SNPs (274C/T and 469+14G/C), while the 823C/T polymorphism is a risk factor (RR=2.29, 95%CI 1.06-4.92). The reason why the high expressing *NRAMP1* promoter allele is associated with HIV infection susceptibility is not known but likely is a consequence of the pleiotropic effects of the *NRAMP1* gene on macrophage physiology. Specifically, the recent finding that promoter allele 3 homozygotes drive increased TNFA production could offer a functional explanation since TNFA is known to drive high levels of HIV transcription and production of infectious particles.[46] A Taiwanese study which investigated the role of *NRAMP1* polymorphisms and susceptibility of TB in HIV seropositive patients did not detect any association with TB susceptibility; yet the 5'(CA)n polymorphism was not tested.[33] In a similar study of HIV/TB co-infection in Caucasian subjects, it was observed that homozygotes and heterozygotes for promoter allele 3 were at increased risk for coinfection of HIV and TB (OR=6.86 95%CI 1.55-30.21).[17] Hence, it appears that allele 3 of the 5'(CA)n repeat is a protective factor for TB in the absence of HIV infection, but becomes a risk factor for HIV/TB co-infection.

Recently a Japanese study showed an association between the 5'promoter (CA)n repeat of *NRAMP1* and Kawasaki disease in Japanese children.[47] Although the causative agent of Kawasaki disease has not been identified, epidemiology and clinical features of the disease suggest an infectious etiology. The study detected allele 1 of the polymorphism to be a risk factor for Kawasaki disease in Japanese children. The results of the study are unusual since this is the only report associating allele 1 with disease susceptibility. Due to an increased frequency of allele 1 among Asians (3%-4%) as compared to non-Asians (<2%), a risk modulating effect of this allele may be easier to detect in Asian populations. Finally, evidence is now emerging that links *NRAMP1* with susceptibility to visceral leishmaniasis.[48] Earlier studies had failed to detect a link between visceral leishmaniasis and cutaneous leishmaniasis in Brazilian and Ethiopian patients, respectively.[49,50]

The possible role of *NRAMP1* polymorphisms in *Mycobacterium avium-intracellular* complex (MAC) disease was investigated in two studies.[51,52] Both studies failed to detect significant evidence for *NRAMP1*-mediated susceptibility. However, the sample sizes employed

would have only allowed to detect Mendelian effects of *NRAMP1*, a situation that is unlikely to occur. Other studies which investigated the role of *NRAMP1* in infectious diseases and failed to detect associations include a South Indian study of *Wuchereria bancrofti* infection, a study of Vietnamese typhoid fever patients, and a study among Peruvian patients suffering from *Trypanosoma cruzi* infection.[53-55]

NRAMP1 and Autoimmune Diseases Susceptibility

The finding of differential *NRAMP1* expression driven by promoter alleles 2 and 3, respectively, is consistent with the hypothesis that alleles that are detrimental in relation to autoimmune disease susceptibility may be maintained in the population because they improve the survival following infectious challenges. The pleiotropic effects of *NRAMP1*, such as macrophage activation and inflammation regulation, and its likely function as an iron transporter further support the hypothesis of *NRAMP1* alleles as risk modifiers of both autoimmune and infectious diseases.

Many autoimmune diseases are characterized by chronic inflammation in specific organs. Rheumatoid arthritis (RA) is characterized by increased iron deposits in the synovial membrane and the presence of a chronic inflammatory response. A British study has investigated the linkage and association between *NRAMP1* polymorphisms and RA susceptibility.[56] Linkage and association were not detected with the 5'(CA)n polymorphism due to its low information content in the study population. However, *D2S1471*, a marker located an estimated 200 kbp from *NRAMP1*, was weakly linked to RA in a human lymphocyte antigen (HLA) discordant subgroup (p=0.05). Another UK study detected linkage (LOD=1.01, p=0.024) for a gene in the *NRAMP1* area and a trend in nonrandom transmission of promoter allele 3 in RA affected offspring (χ^2 = 3.9, p=0.048).[57] By contrast, *NRAMP1* promoter allele 3 was identified as a protective factor in Spanish RA patients who did not possess any HLA risk factor for RA.[58] In addition to being a risk factor for RA susceptibility (OR=3.74; CI=1.31-10.72), allele 2 increases the risk of developing severe form of RA (OR=5.45). Association of the 5'(CA)n polymorphism with RA could not be found in Korean and Canadian patients.[59,60] The Korean study, however, detected genotypic and allelic association between the *NRAMP1* 823C/T, D543N and 1729+55del4 polymorphisms with RA. The rare alleles of these polymorphisms were found more frequently in patients than in controls (allelic, p=0.006, genotypic, p<0.05). The Canadian study found the 543N allele and the deletion allele of 1729+55del4 polymorphisms to confer some protection against RA (p=0.014 for both polymorphisms), regardless of the *HLA-DRB1* genotypes. Finally, a study in Latvia investigated the role of *NRAMP1* in juvenile rheumatoid arthritis (JRA).[61] This Latvian study observed allele 3 of the 5'(CA)n polymorphism to be a risk factor (OR=2.26), and allele 2 to be a protective factor for JRA (OR=0.44).

Similar to RA, inflammatory bowel disease (Crohn's disease (CD) and ulcerative colitis (UC)) is characterized by a chronic inflammatory response. CD is a particularly interesting phenotype to investigate for association with *NRAMP1* because of cases of colonic TB that had been misdiagnosed as CD.[62] The similarity in clinical presentation between CD and colonic TB may suggest a similar genetic influence on pathogenesis. Two microsatellites markers, *D2S434* and *D2S1323*, which localize to the *NRAMP1* gene region on human chromosome 2q35, were found to be associated with CD.[63] These results are unlikely to implicate *NRAMP1* in CD susceptibility since there is presently no data supporting the assumption that the two microsatellite markers are in linkage disequilibrium with *NRAMP1*. The 5'(CA)n polymorphism was investigated for its role in CD and UC in a Japanese case control study.[19] A novel promoter repeat allele, allele 7, was found to be a risk factor for CD and UC when compared to two separate control groups. (OR=2.67, 2.25 (p=0.015, 0.023) for CD, OR=2.7, 2.28 (p=0.018, 0.027) for UC) A Dutch population-based case-control study did not detect association with the two markers tested (274C/T and 823C/T).[64] To some extent the Dutch

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results are not consistent with the Japanese findings since at least *NRAMP1* 274C/T is known to be in strong linkage disequilibrium with the promoter polymorphisms.

Also clinically similar to TB is sarcoidosis. It is characterized by a hypersensitivity response to unknown antigens. A population based case control study was carried out in an African American population to investigate the role of *NRAMP1* in sarcoidosis.[65] Compared to the common promoter allele 3, all other alleles of 5'(CA)n decreased the risk of sarcoidosis (OR=0.48, 95%CI 0.28-0.81, p=0.014). When tested in high risk homozygotes for allele 3, the N allele of the D543N morphism confers protection when present with two copies (OR=0.33, CI95% 0.13-0.83, p=0.003). Although clinically and histologically similar to TB, sarcoidosis seems to differ from TB in the genetic control of susceptibility since promoter allele 3 is a protective factor for TB but a risk factor for sarcoidosis.

In a Japanese case control study that investigated early onset of type 1 diabetes, allele 1 and 3 increased the risk of the disease (OR=1.8, CI95% 1.1-2.9).[66] In a UK family based case control study, allele 3 was nonrandomly transmitted to the affected offspring (p=0.04) who had a first or second-degree relative with RA.[67] These data provide some evidence that allele 3 could be a weak risk factor for type I diabetes in defined clinico-epidemiologic settings. However, given the weak effect of tested alleles these results need to be replicated.

When chronic inflammation occurs in the central nervous system, multiple sclerosis (MS) may be diagnosed. A case control study was carried out in South Africans subjects of European ancestry.[68] Three groups of controls were included in this study: general, parental nontransmitted alleles and elderly. The study observed the allelic distribution of the 5'(CA)n polymorphism to be different between the MS patients and the control groups, both individually and as one group ($p<0.05$). The very rare promoter allele 5 described by Graham et al, [18] was found more frequently in patients than in controls ($\chi2 = 35.2$, 2 *df*, $p<0.01$). Interestingly, when compared to the younger control group, in the elderly control group *NRAMP1* promoter alleles 3 and 5 are overrepresented ($\chi2 = 16.6$, 2 *df*, $p<0.01$). The latter finding gave rise to the interesting speculation that alleles considered detrimental in relation to autoimmune diseases may prove to be beneficial for longevity via protection against infection, iron overload and oxidative processes that result in cellular aging.

Primary biliary cirrhosis (PBC) is a chronic slowly progressive cholestatic liver disease involving the formation of granulomas and tissue damage. Autoimmune mechanisms are believed to be the culprit, however, an infectious origin cannot be excluded. A population based case control study was carried out to investigate the role of the 5'(CA) polymorphism of *NRAMP1* in PBC susceptibility.[18] A novel allele 5 was found more frequently in the PBC patients when compared to normal controls ($p<0.024$), to alcoholic liver disease patients ($p<0.012$), or to hepatitis C patients ($p<0.012$). Importantly, this study pointed out the technical possibility of mistaking allele 5 for allele 3, possibly confounding the findings from previous studies.

The heart lesions and tissue damage of Chagas' disease are thought to be the result of autoimmune processes. Infection with *Trypanosoma cruzi* has been identified to be the trigger. A Peruvian study investigated the role of *NRAMP1* polymorphisms in the susceptibility of *T. cruzi* and development of Chagasic cardiac disease.[55] The study did not find any of the tested polymorphisms to be associated with *T. cruzi* infection susceptibility. However, the study observed with borderline statistical significance more cardiomyopathic patients being homozygous for allele 3 of the 5'(CA)n polymorphism than asymptomatic patients ($\chi^2 = 3.30$, $p=0.07$). The latter finding strengthens two hypotheses: chronic hyperactivation of macrophages associated with allele 3 is functionally linked to autoimmune disease, and heart damage in chronic Chagas'disease is due to an autoimmune process.

Atopy is characterized by elevated serum IgE levels upon trigger by common environmental allergens. To study a possible contribution of *NRAMP1* alleles to atopy, a Swedish study investigated the role of the 5' (CA)n promoter repeat polymorphism in atopy of BCG vaccinated and non-vaccinated children.[69] The study did not find an association between atopy,

BCG vaccination and *NRAMP1* promoter alleles. However, when investigating association of *D2S1471* alleles with atopy a borderline association (p=0.07) was detected. The risk of atopy for *D2S1471* allele 5 carriers appeared more significant for vaccinated children (OR=2.6, 95% CI 1.3-5.5, p=0.01). Amazingly, the same allele provided protection against food allergy (OR=0.49, 95%CI 0.27-0.91, p=0.03) in BCG non-vaccinated children but increased the risk of positive IgE responses against air borne allergy in vaccinated children (OR= 4.3, CI 95% 1.7-10.7, p=0.002). The interpretation of these results is difficult since the *D2S1471* alleles were found not to be in linkage disequilibrium with the *NRAMP1* promoter alleles. Possible linkage disequilibrium of D2S1471 with 3' *NRAMP1* alleles was speculated but not shown. It also must be considered that the overall evidence for an association in the vaccinated group was weak and absent in the non-vaccinated group. This raises the common problem of interpreting stratification in the absence of an overall effect.

NRAMP2 Location and Genomic Structure

There are two members of the human NRAMP gene family, *NRAMP1* and *NRAMP2*. The cDNA clone corresponding to the second member of the NRAMP gene family, *NRAMP2* (now also called *SLC11A2*), was mapped onto chromosomal region 12q13.[70] The *NRAMP2* coding nucleotide sequence is 64% identical to *NRAMP1*.[71] However, the *NRAMP2* gene contains one additional 5'exon, and one additional alternative spliced 3' exon.[72] Indeed, alternative splicing appears unusually extensive for *NRAMP2*.[73] The 5'regulatory region contains five potential metal response elements (MRE's), three potential SP1 binding sites and a single γ-interferon responsive element.[72] Tissue expression specificity of *NRAMP2* mRNA has not been firmly established,[70,74] although its expression in the duodenum has been shown.[75,76] In non-intestinal cells, NRAMP2 is found to co-localize with LAMP2 to the membranes of the late endosome/lysosome compartment.[77] Analysis of the *NRAMP2* cDNA clones identified two splice forms differing at the 3'end.[72] The two forms are designated as *NRAMP2*- (iron responsive element) IRE and *NRAMP2* non-IRE. The IRE form contains an AT rich 3'UTR with one classical iron responsive element. The non-IRE form substituted 18 amino acids at the carboxyl terminal with novel 25 amino acids, and a different 3'UTR lacking a classical IRE.[72] To what extent iron regulates mRNA stability of the non-IRE form remains to be investigated.

NRAMP2 Functions

In vitro studies using *Xenopus* oocytes injected with rat DCT1 (divalent cation transporter 1) demonstrated DCT1 to be a pH-coupled divalent cations transporter. Northern blotting indicated that *DCT1* mRNA was upregulated in most tissues when challenged with iron deficiency.[78] Comparative sequence analysis revealed 92% identity between rat *DCT1* and human *NRAMP2* genes. Independently, it was shown that two single mutations at the homologous position in rat and mouse *Nramp2* give rise to microcytic anemia in *mk/mk* mice and underlie the defect in iron transport in the Belgrade *b* rat.[79,80] Together with extensive transport studies in mammalian cells these findings clearly establish that Nramp2 proteins are capable to transport a broad range of divalent cations including iron.[81] Specifically, it has been shown that Nramp2 is a pH-dependent iron/proton symporter. It is likely that human NRAMP2 will have a similar function consistent with its localization in the membranes of acidified vesicles.[77]

NRAMP2 Polymorphisms

Amongst the large number of genetic variants detected in the *NRAMP2* gene, 7 polymorphisms have been investigated for their roles in human diseases susceptibility. Two of the 5 SNPs are in the coding regions (c.1254T/C and c.1303 C/A), with the latter SNP resulting in an amino acid change of leucine to isoleucine. The remaining four SNPs are intronic (IVS2+11A/G, IVS4+44C/A, IVS6+538G/Gdel and IVS15Ex16-16C/G).[72,82] A microsatellite

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TATATCTATATATC$(TA)_{6-7}(CA)_{10-11}$CCCCCTATA$(TATC)_3(TCTG)_5$TCCG$(TCTA)_6$ was detected in intron 3 and 4 alleles have been identified in a Japanese study population (allele 1: 247bp, allele 2: 245bp, allele 3: 243bp and allele 4: 239bp).[83]

NRAMP2 Polymorphisms and Human Diseases Susceptibility

Mouse and rat models indicated hypochromic anemia to be associated with mutation in Nramp2.[79,80] A G/A mutation resulting in an glycine to arginine substitution at codon 185 in both mouse and rat is associated with failure of iron transport out of endosomes within the transferrin cycle, leading to abnormal reticulocyte iron uptake and gastrointestinal iron absorption. In humans, a disease intimately connected to disturbed iron transport pathways is hereditary haemochromatosis. It is an autosomal recessive disorder characterized by excessively high iron accumulation in tissues caused by excessive intestinal iron absorption. The phenotype was found to be associated with mutations in the haemochromatosis gene (*HFE*).[84,85] However, mutations in the *HFE* gene only accounted for roughly 85% of the cases suggesting the involvement of additional genes in the control of haemochromatosis.[84,85] The fact that iron uptake is also disturbed in *mk/mk* mice and *b* rats, albeit these animals are characterized by deficient rather than excess iron uptake, prompted investigations for a role of *NRAMP2* in haemochromatosis. To investigate the role of *NRAMP2* polymorphisms in haemochromatosis susceptibility, a case control study was carried out on Caucasian haemochromatosis subjects.[86] The study did not find a significant difference in 1254G/C and IVS6+538G/Gdel allelic distributions between the patients and the controls, whether analyzed separately or combined. Likewise, stratification by *HFE* genotypes did not produce significant differences in allelic or haplotypic distributions. Another study investigated the coding region of the *NRAMP2* gene in haemochromatosis probands who did not carry any *HFE* mutations on both chromosomes.[73] Surprisingly, a total of 17 splice mRNA variants were found; 5 due to exon skipping and 12 due to cryptic splicing sites. Eight of these cryptic splicing site activations were between exons 3 and 4 and observed in a majority of haemochromatosis probands and control subjects, implying splicing instability in the region. The study did not find any evidence for *NRAMP2* involvement in hereditary haemochromatosis.[73]

In a functional case control study, expression of *NRAMP2* in haemochromatosis patients was compared to controls.[75] The patients were all homozygous "G" at the *HFE* C282Y mutation and carried no H53D mutation; all controls were negative for the C282Y and H63D mutations. The study found duodenal *NRAMP2* mRNA expression was a magnitude higher in haemochromatosis patients than in controls, indicated by both Northern blotting and competitive PCR ($p<0.001$). In addition, *NRAMP2* cDNA and serum ferritin concentration were inversely correlated in controls ($r=-0.94$, $p=0.001$) but not in patients. No mutation in the *NRAMP2* coding region of seven hemochromatosis patients was observed. The findings in this study support the proposed mechanism of an initial duodenal iron depletion due to the *HFE* mutation, resulting in iron-regulatory proteins (IRP) mediated increased *NRAMP2* mRNA stability and heightened NRAMP2 transport activity that increases iron absorption.

In humans, iron balance is regulated at the level of intestinal absorption. Since two genome-wide scans located an IBD susceptibility locus on the long arm of chromosome 12 approximately 10 cM proximal of *NRAMP2* and due to the known role of NRAMP2 in intestinal iron metabolism, *NRAMP2* is suspected to play a role in IBD susceptibility.[87,88] A Dutch study investigated the possible role of *NRAMP2* polymorphisms in IBD susceptibility and identified a novel SNP in intron 15 (IVS15Ex16-16C/G) that was neither linked nor associated with IBD.[82] Only homozygotes for the "G" allele of IVS2+11A/G showed a weak increase in the risk of Crohns disease (OR=2.2, CI95% 1.3-3.9, χ^2 =8.4, p=0.013). By contrast, allele sharing methods did not provide evidence for linkage of *NRAMP2* to IBD or any of its clinical entities. The disagreement between the association and linkage studies suggests that the phenotype is under complex genetic control with *NRAMP2* providing at best a small contribution to overall expression of the phenotype.

Conclusion

As has been outlined in this chapter, a contribution of *NRAMP* genes has been studied for a sizeable number of human diseases. The most encouraging results have been derived from a large number of studies in different ethnic groups implicating variants of the *NRAMP1* gene in increased susceptibility to TB. Together, these studies provide very strong evidence for *NRAMP1* being a TB susceptibility locus even in the absence of a clearly identified disease mechanism. The major challenge for the study of *NRAMP1* in human TB will now be to provide the mechanistic link between risk alleles and biological function. This won't be easy considering the difficulties encountered by establishing Nramp1 function in the mouse even though knockout strains had been available to accomplish the task. Perhaps the best strategy will be to apply what is known about Nramp1 function in the mouse to genetically characterized human populations. As for many of the other disease associations, it is likely that some of the reported associations are due to chance while possibly some of the negative findings are a result of under-powered studies. Only carefully designed replication studies will be able to give conclusive answers.

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FULL PAPER

NRAMP1 is not associated with asthma, atopy, and serum immunoglobulin E levels in the French Canadian population

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Reduced infection by mycobacteria, including Mycobacterium tuberculosis, may be partly responsible for increased prevalence of allergic and autoimmune diseases in developed countries. In a murine model of innate resistance to mycobacteria, the Nramp1 gene has been shown to affect asthma susceptibility. From this observation, it was proposed that human NRAMP1 may be a modulator of asthma risk in human populations. To experimentally test the candidacy of NRAMP1 in asthma susceptibility, we characterized five genetic variants of NRAMP1 (5′ CA_{n}, 274C>T, 469+14G>C, D543N, and 1729+del4) in an asthma family-based cohort from northeastern Quebec. We did not observe any significant association between NRAMP1 variants (either allele or haplotype specific) with asthma, atopy, or serum immunoglobulin E levels. These results demonstrate that, in spite of direct involvement of Nramp1 in a murine asthma model, in human populations NRAMP1 is not likely to be a major contributor to the genetic etiology of asthma and asthma-related phenotypes.
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Introduction

In 1989, David Strachan reported the findings of a prospective study of 17 414 British children from birth to 23 years of age.[1] The study found a significant inverse correlation between the number of older siblings in the household and the prevalence of hay fever at age 11 and 23 years. A similar correlation was also observed for eczema in the first year of life. Subsequent epidemiologic studies confirmed the inverse association between family size and atopic markers such as skin prick positivity and specific immunoglobulin (Ig) E titers.[2] To explain the inverse correlation between family size and atopy, it was suggested that declining family size, improved household amenities, and higher standards of personal cleanliness, all associated with improved hygiene, had reduced the opportunities of cross-infection, and this may have increased the prevalence of atopic diseases. This explanation is now known as the *hygiene hypothesis* and it has been extended to include autoimmune diseases in general.

One proposed mechanism underlying the hygiene hypothesis is that microbial exposures to viruses, bacteria, and parasites elicit an immune response towards the maturation of T helper type 1 cells (Th1), and the subsequent production of cytokines such as interleukin (IL)-2 and interferon-γ. This predominating Th1 immune response suppresses the production of Ig E and IgG1, as well as cytokines IL4, IL5, IL9, and IL13, characteristic of type 2 (Th2) responses. The possibility of a Th1/Th2 imbalance and an association between *Mycobacterium tuberculosis* infection and atopy expression was first shown by a study of Japanese school children.[3] The study reported an inverse association between delayed hypersensitivity to tuberculin, a broad measure of exposure to mycobacteria (Th1 response), and serum IgE levels (Th2 response), and concluded that exposure to *M. tuberculosis* may inhibit the development of atopic diseases. In humans, *M. tuberculosis* is the cause of tuberculosis (TB). However, not all individuals exposed to *M. tuberculosis* will become infected, and of those infected only a small proportion will develop clinical disease.[4,5] It is now well established that both genetic and environmental factors determine the progression from exposure to infection and from infection to disease.

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If *M. tuberculosis* infection protects against atopy, then genetic risk factors for TB susceptibility may be protective for asthma and *vice versa*. A well-established TB susceptibility gene is the natural resistance associated macrophage protein 1 gene (*NRAMP1*). In addition to its known role in TB susceptibility, its associations with numerous immune-mediated disorders such as rheumatoid arthritis, type I diabetes, and multiple sclerosis are making *NRAMP1* a prime candidate to test the hypothesis of inverse genetic control of asthma and TB susceptibility.[6,7]

Data from the mouse support the candidacy of *NRAMP1* as an asthma susceptibility gene. It has been shown that *Nramp1*-resistant (*Nramp1*^{r}) mice have a lower IgE and IL-4 response compared to *Nramp1*-susceptible (*NRAMP1*^{s}) mice after infection with an attenuated strain of *Salmonella typhimurium*,[8] demonstrating that Nramp1 can modulate Th1/Th2 host responsiveness. Moreover, in *M. vaccae* and allergen-sensitized mice, subsequent allergen challenge triggered higher levels of Th2 cytokines (IL-4, IL-5, IL-13) and IgE in *Nramp1*^{s} as compared to *Nramp1*^{r} mice.[9] This finding implies that the ability to develop atopy-associated Th2 responses is dependent on susceptibility to infection, dictated in this model by *Nramp1*. Finally, *M. vaccae* is more efficient in lowering allergic and asthmatic symptoms in allergen-challenged *Nramp1*^{s} than in *Nramp1*^{r} mice,[10] directly demonstrating that *Nramp1* can modify immune responses following mycobacterial infection. Taken together, these observations provide a direct experimental link between genetically controlled resistance to infection and altered asthma susceptibility, and constitute the main motivating force for the present study.

Results

Patient characteristics

Clinical characteristics of the study participants have been reported previously.[11] Briefly, 1139 individuals between ages 3 and 88 years were recruited. The median age of onset for index cases and their affected siblings is 5 years (2–46 years). Of the 570 subjects with asthma and 569 without asthma, 419 (74%) and 218 (38%) were atopic, respectively. The male to female ratios in probands, affected and unaffected family members are 1:1.2, 1:1.4, and 1:1.2, respectively. Index cases have higher IgE levels and coexistence of atopy compared to other affected family members.

Genetic variants of the *NRAMP1* locus

We characterized five polymorphisms of the *NRAMP1* gene in the family-based cohort (Figure 1). Variant 5′$(CA)_n$ is a promoter dinucleotide repeat polymorphism; variant rs2276631 (reference SNP identifier from the National Center for Biotechnology Information database) is a synonymous C>T polymorphism in exon 3 (common alias 274 C>T); rs3731865 involves a G>C base change in intron 4 (common alias 469+14G>C); variant D543N is a G>A substitution resulting in an aspartic acid to asparagine amino-acid change in exon 15, and variant 1729+55del4 is a TGTG tetranucleotide deletion polymorphism in the 3′ untranslated region (Table 1). All variants have previously been described.[32] *NRAMP1* variants were selected due to their known associations with susceptibility to infectious and autoimmune diseases. In the French–Canadian families, variants in the 5′ *NRAMP1* region (5′$(CA)_n$, 274C>T, and 469+14G>C) are polymorphic, with minor allele frequencies of 0.31, 0.28, and 0.31, respectively, and all variants are in Hardy–Weinberg equilibrium. Variants 1729+del4 and D543N were uninformative (minor allele frequency = 0.012 and 0.017, respectively). Hence, these two 3′ end variants were not further analyzed.

Family-based association analysis of *NRAMP1* with asthma, atopy, and IgE levels

The three informative *NRAMP1* polymorphisms located in the 5′ region of the gene were tested individually for association with asthma, atopy, and IgE levels (Table 2). IgE serum levels were analyzed as dichotomous trait, independent of atopy status. Subjects were classified either as high or low responders according to their IgE levels. Based on the normal distribution of the log (IgE) values, a cutoff point of 100 mg/l divided the subjects into low (two-thirds of participants) and high (one-third) responders. We tested allelic associations under additive and dominant genetic models. No allele was significantly

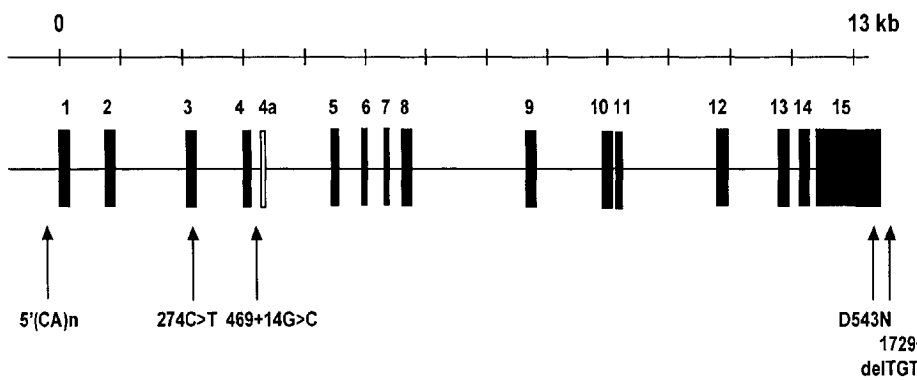

Figure 1 Chromosomal location of *NRAMP1* polymorphisms associated with common diseases. In the schematic presentation of the *NRAMP1* genomic organization, exons are depicted as black boxes with the corresponding exon numbers on top. Introns are depicted as lines between exon boxes. Genomic distances between exons are indicated by the scale in kilobases (kb) above the panel, with 0 kb being the transcription start site of exon 1 and 13.6 kb indicating the end of exon 15. The white box 4a represents the alternatively spliced exon 4A.[10] Positions of variants are indicated by arrows. Names or identification numbers of variants are given underneath the arrows.

Table 1 *NRAMP1* polymorphisms and disease associations

Variant	*Chromosome position*[c]	*Allele*	*Genomic characteristics*	*Human disease associated*	*References*
5′$(CA)_n$	219072130	2[d,e] $t(gt)_5ac(gt)_5ac(gt)_{10}ggcaga(g)_6$ 3 $t(gt)_5ac(gt)_5ac(gt)_9ggcaga(g)_6$	Promoter	TB HIV infection Kawasaki Rheumatoid arthritis (RA) Inflammatory bowel disease Sarcoidoisis Type I diabetes Multiple sclerosis Primary biliary cirrhosis	12–14 15 16 17,18 19 20 21,22 23 24
rs2276631[a], common alias: 274C>T[b]	219074518	G[e] A	Exon 3, synonymous	HIV infection	15
rs3731865, common alias: 469+14G>C	219075508	G C	Intron 4	TB HIV infection	12,25,26 15
Common alias: D543N	219085237	G[e] A	Exon 15, aspartic acid to asparagines	TB RA sarcoidoisis	12,14,27,28 29,30 20
Common alias: 1729+delTGTG	219085319	TGTG[e] -TGTG	3′ untranslated region	TB Leprosy RA	12 31 29,30

[a]Rs numbers are the reference SNP identifiers from the NCBI database.
[b]Common alias perviously described.[32]
[c]Chromosome positions are based on July 2003 freeze of the University of California Santa Cruz genome browser (http://genome.ucsc.edu).
[d]At least nine alleles have been identified in five studies; in all studied populations, allele 3 is the most common allele, followed by allele 2, the remaining seven alleles are all rare variants.[6,7]
[e]Base changes indicated are on the negative strand of July 2003 freeze of the University of California Santa Cruz genome browser.

($P<0.05$) transmitted nonrandomly to offspring with asthma or atopy, or to high IgE responders. The 469 + 14G allele, under an additive model, was non-significantly overtransmitted to asthmatic offspring ($P=0.08$).

Haplotype-specific association analysis

Association among the three variants was assessed by measuring pairwise linkage disequilibrium (LD) using D'. The three variants are strongly associated, as evidenced by a high degree of LD ($D'>0.90$) among them (data not shown). Alleles of the three variants are likely to be transmitted together as groups, or haplotypes. Within this region of high LD, two haplotypes with frequency >0.1 are observed: haplotype (5′$(CA)_n$) allele 3-(274)C-(469 + 14)G (frequency = 0.683) and haplotype allele 2-T-C (0.258). Other less frequently occurring haplotypes are allele 2-C-G (0.021), allele 2-C-C (0.015), and allele 3-C-C (0.010). All haplotypes were assessed for nonrandom transmissions in the asthma families using family-based association testing software (FBAT), version 1.5[33,34] (Table 3). Under additive and dominant genetic models, all haplotypes were randomly transmitted to offspring with either asthma, atopy, or high IgE responsiveness.

Power calculations

We examined the possibility that the failure to detect association of *NRAMP1* variants with asthma and related phenotypes was due to insufficient power of the study sample. Power was calculated for 60 sets of parameters defined by susceptibility allele frequency (q) and genetic attributable fraction (GAF) for an additive disease model with disease prevalence set at 0.10 (Figure 2). The result shows that with the present cohort of 1139 individuals there is excellent power (>80%) to detect variants across a wide allele frequency range (0.20–0.50) for a heterozygotes odds ratio (HET OR) >1.6. For example, for $q=0.20$, power >80% is achieved with GAF ≥0.20, corresponding to a HET OR >1.6 (Figure 2). For the low allele frequencies of 0.05 and 0.10, power >80% is achieved for a HET OR >1.8. For the high allele frequency of 0.70, power >80% is achieved for a HET OR >1.7 (data not shown). By contrast, power to detect variants with a HET OR <1.4 is poor (<60%) for the allele frequency range of 0.10–0.70, although such small-

Table 2 Allele transmission pattern for asthma and related phenotypes

Phenotype/model	*Variant*	*Allele (frequency)*	*S*[c]	*E(S)*[d]	*Z*[e]	*P-value*
Asthma/additive	5′$(CA)_n$	3[a] (0.687)	270	263.33	0.95	NS[f]
		2[b] (0.313)	112	118.67	−0.95	NS
	274C>T	C (0.72)	242	234.41	1.11	NS
		T (0.28)	92	99.58	−1.11	NS
	469+14G>C	G (0.69)	267	254.17	1.74	0.08
		C (0.31)	101	113.83	−1.74	0.08
Asthma/dominant	5′$(CA)_n$	3	61	58.91	0.65	NS
		2	101	105.58	−0.74	NS
	274C>T	C	49	46.16	0.89	NS
		T	80	84.74	−0.80	NS
	469+14G>C	G	66	63.41	0.79	NS
		C	90	100.25	−1.58	NS
Atopy/additive	5′$(CA)_n$	3	286	283.58	0.34	NS
		2	128	130.42	−0.34	NS
	274C>T	C	252	248.50	0.51	NS
		T	104	107.50	−0.51	NS
	469+14G>C	G	298	294.00	0.54	NS
		C	128	132.00	−0.54	NS
Atopy/dominant	5′$(CA)_n$	3	60	58.99	0.31	NS
		2	109	110.41	−0.23	NS
	274C>T	C	52	48.49	1.11	NS
		T	93	92.99	0.002	NS
	469+14G>C	G	66	64.49	0.47	NS
		C	113	115.49	−0.39	NS
IgE/additive	5′$(CA)_n$	3	165	165.50	−0.09	NS
		2	71	70.50	0.09	NS
	274C>T	C	150	154.42	−0.85	NS
		T	70	65.58	0.85	NS
	469+14G>C	G	161	164.42	−0.62	NS
		C	73	69.58	0.62	NS
IgE/dominant	5′$(CA)_n$	3	35	34.16	0.36	NS
		2	62	60.66	0.28	NS
	274C>T	C	26	25.91	−0.04	NS
		T	58	53.50	−0.96	NS
	469+14G>C	G	34	34.41	−0.17	NS
		C	64	61.00	−0.62	NS

[a]Allele 3: $t(gt)_5ac(gt)_5ac(gt)_9ggcaga(g)_6$.
[b]Allele 2: $t(gt)_5ac(gt)_5ac(gt)_{10}ggcaga(g)_6$.
[c]*S* = FBAT statistic.
[d]*E(S)* = Expected FBAT statistic.
[e]Z = Z-score.
[f]NS = not significant.

risk modifiers may still account for a substantial proportion of cases, especially if the risk variant is present at high allele frequencies. For low allele frequencies <0.05, power is poor even for a HET OR between 1.6 and 1.8. Overall, the results of the power calculation argue against *NRAMP1* alleles being asthma susceptibility factors with relative risk >1.8 in the French Canadian population.

Discussion

In light of the hygiene hypothesis and the Th1/Th2 paradigm in TB and asthma pathogeneses, the established TB susceptibility gene, *NRAMP1*, is a strong candidate gene for asthma susceptibility. In mice, the *Nramp1* gene encodes a 90–100 kDa transmembrane protein and its mRNA is expressed in primary macrophages and granulocytes. The Nramp1 protein is found at the late endosomal/lysosomal compartment of macrophages.[35,36] *Nramp1* is a major determinant of innate host resistance to infection. The gene affects intracellular replication of a wide range of pathogens including *S. typhimurium*,[37] *Leishmania donovani*,[38] *M. lepraemurium*,[39] *M. intracellulare*,[40] *M. avium*,[41] and the TB vaccine strain *M. bovis* — Bacillus Calmette-Guérin (BCG).[42] Specifically, a glycine-to-aspartic-acid change at amino acid 169 (G169D), located in the predicted transmembrane domain number 4 (TM 4), has removed the host's ability to inhibit pathogen growth.[43,44] Comparative sequence analysis of the Nramp gene family suggested that Nramp1 functions as a divalent cation transporter.[45] Kinetic studies demonstrated that Nramp1 transports cations out of the phagosomes, and, consequently,

Table 3 Haplotype transmission pattern of *NRAMP1*

5′(CA)$_n$	274C>T	469+14G>C	Frequency	S[a,b]	S[d]	E(S)[a,c]	E(S)[d]	Z[a,e]	Z[d]	P-value[a]	P-value[d]
Asthma											
3	C	G	0.683	221.995	146.995	214.023	145.334	1.242	0.607	0.214	0.544
2	T	C	0.258	101.995	82.995	106.671	85.515	−0.721	−0.462	0.471	0.644
2	C	G	0.021	9.005	9.005	8.01	8.01	0.575	0.575	0.565	0.565
2	C	C	0.015	—	—	—	—	—	—	—	—
3	C	C	0.010	—	—	—	—	—	—	—	—
Atopy											
3	C	G	0.683	241.99	161.99	237.41	160.69	0.729	0.453	0.466	0.650
2	T	C	0.258	109.99	91.99	111.78	91.647	−0.294	0.066	0.769	0.947
2	C	G	0.021	8.01	8.01	7.51	7.51	0.243	0.243	0.808	0.808
2	C	C	0.015	—	—	—	—	—	—	—	—
3	C	C	0.010	—	—	—	—	—	—	—	—
IgE											
3	C	G	0.683	122.99	83.99	124.6	83.918	−0.362	0.033	0.717	0.973
2	T	C	0.258	66.99	54.99	63.887	51.813	0.647	0.768	0.517	0.442
2	C	G	0.021	—	—	—	—	—	—	—	—
2	C	C	0.015	—	—	—	—	—	—	—	—
3	C	C	0.010	—	—	—	—	—	—	—	—

[a]Additive model.
[b]FBAT statistic.
[c]Expected FBAT statistic.
[d]Dominant model.
[e]Z score.
— represents less than 10 informative families.

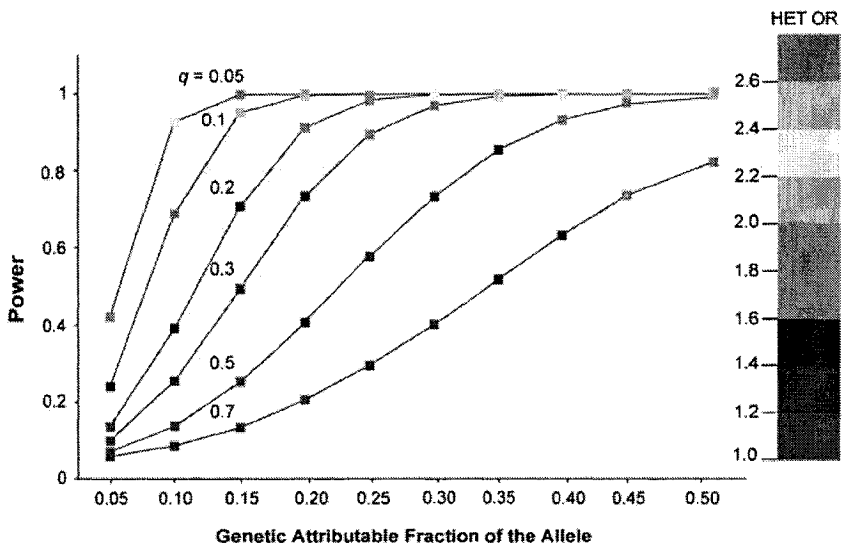

Figure 2 Power estimates. The GAF of a susceptibility allele is plotted on the *x*-axis against power to detect association on the *y*-axis. Susceptibility allele frequencies (*q*) of 0.05, 0.1, 0.2, 0.3, 0.5, and 0.7 are tested. Each line of squares corresponds to the power calculated under an additive disease model with disease prevalence of 0.10, for a specific *q* across a range of GAF. The color of the squares represents the associated odds ratios of the heterozygotes (HET OR), according to the color gradient on the right of the graph. A heterozygote odds ratio of 2 indicates that an individual with one copy of the susceptibility allele has twice the risk to develop the disease as does a person with no copy of the susceptibility allele. For example, if the susceptible allele of frequency 0.30 attributes to 25% of the cases (GAF = 0.25), and exerts a HET OR between 1.6 and 1.8 (light blue), then the present cohort has power >0.80 (80%) to detect the association between allele and disease.

mediates depletion of nutrients potentially essential for survival of pathogens in the host cell phagosome.[46,47] In addition, Nramp1 is critical to overcome pathogen-triggered blockages of intracellular vesicle trafficking.[48] The exact function of human NRAMP1 is not known. However, due to its high sequence homology with mouse *Nramp1* (93% overall sequence similarity and 88% sequence identity), the NRAMP1 protein is likely to be a divalent cation transporter.[49–53] The amino-acid sequences of Nramp1 and NRAMP1 in predicted TM 4

are identical, but the homologous G169D variant in TM 4 has not been found in human NRAMP1.[49] However, genetic variants at the 5′ and 3′ ends of the human gene are associated with infectious and autoimmune disease susceptibility.[6,7]

Based on the previous findings in a murine model of asthma demonstrating that the propensity to mount an atopic immune response is dependent on *Nramp1*, we carried out a family-based association study to test if, in humans, *NRAMP1* genetic variants are associated with asthma and related phenotypes, such as atopy and IgE levels. We selected five *NRAMP1* variants that had previously been shown to be associated with susceptibility to a variety of immune disorders and infectious diseases (Table 1). Specifically, polymorphisms in the 5′ region of *NRAMP1* have been found to be risk modifiers for TB in multiple populations. This suggests that a variant located in the 5′ *NRAMP1* region impacts on NRAMP1 function. The 5′$(CA)_n$ promoter variant has been linked to variable *NRAMP1* mRNA expression.[54,55] Promoter allele 3 drives a higher mRNA expression compared to other alleles such as allele 2 (Table 1) in the absence of any stimulant. When stimulated with interferon-γ, alleles 2, 3, and other rare alleles demonstrate enhanced mRNA expression. When co-stimulated with interferon-γ and bacterial antigen lipopolysaccharide, expression by allele 2 is reduced, while that of allele 3 is further enhanced.[54] Interestingly, allele 3 has been found to be associated with TB protection, and with risk for type I diabetes, an autoimmune disorder.[6]

Despite supportive evidence from a mouse model of atopic disease, we failed to detect an impact of human *NRAMP1* on asthma and related phenotypes. Our findings suggest that *NRAMP1* genetic variants do not play a major role in human atopic disease. Detailed power calculation indicated that our cohort is of sufficient size (power >80%) to detect associations of allelic variants with weak impact on disease risk (HET OR >1.6). However, cohort size is insufficient (power <60%) to detect variants with very low impact on disease risk (HET OR <1.4). The inability to detect genetic risk factors with such low impact on disease risk is not unique to the present study. Due to unfavourable cost–benefit ratios, genetic studies are rarely powered to detect risk variants with OR <2.

The reason why we could not replicate the mouse findings in a human population is unknown. However, it is becoming increasingly clear that the majority of common human diseases are multifactorial and complex, and that animal systems might not be able to accurately model all aspects of human diseases. In the case of *NRAMP1*, differences between the mouse model of innate resistance/susceptibility to mycobacteria and human mycobacterial diseases are well known. In mice, *Nramp1* controls intracellular replication of several atypical mycobacteria and BCG, but does not seem to affect resistance to *M. tuberculosis*.[56,57] In humans, *NRAMP1* has been shown in multiple studies to be a risk modifier of TB. This species-dependent permissiveness in mycobacterial replication may be due to dosage and route of pathogen administration in mice that do not accurately mimic the natural infection in humans. Likewise, if resistance to *M. tuberculosis* is under different genetic controls in mice and humans, it is possible that asthma susceptibility may also be under different control in mice and humans. Another major difference between mouse *Nramp1* and human *NRAMP1* is the tissue-specific gene expression. In humans, *NRAMP1* mRNA expression is more pronounced in the lung than in the spleen and liver,[49] whereas in mice, *Nramp1* mRNA is expressed strongly in the spleen and liver, with almost no detectable expression in the lung.[46,58–60] Since *M. tuberculosis* infection in the mouse manifests itself as progressive lung disease, low or absent *Nramp1* expression in the lung of mice coincides with their inability to control *M. tuberculosis* infection. It is possible that similar, but presently unknown differences, in tissue expression impact on asthma susceptibility in mice and humans. Finally, the mouse findings that provided the rationale for our study used *M. vaccae* as the mycobacterial stimulant. At present, the effect of *NRAMP1* on *M. vaccae* susceptibility in humans is unknown and it is possible that, like *M. tuberculosis*, *M. vaccae* is under different genetic controls in mice and humans.

In conclusion, the results of our study have two implications. First, even in well-developed animal models of complex human diseases such as atopy, the genetic control elements may differ between humans and the model system. Second, it seems unlikely that a single inverse relationship exists between variants that predispose to asthma/atopy and those that predispose to TB. Hence, reduced *M. tuberculosis* infection may not be the driving force behind increased asthma/atopy prevalence in developed countries, and, if it is, the genetic mechanism is not likely to have included *NRAMP1*.

Patients and methods

Populations

Families are from the Saguenay-Lac-St-Jean region of northeastern Quebec, Canada. The recruitment scheme has been described previously.[11] Briefly, probands were recruited if they fulfilled at least two of the following three criteria: (1) a minimum of three clinic visits for acute asthma within 1 year; (2) two or more asthma-related hospital admissions within 1 year; or (3) steroid dependency, as defined by either 6 months of oral, or 1 year of inhaled corticosteroid use. Families were included for study if at least one parent was available for phenotypic assessment, at least one parent was unaffected, and all four grandparents were of French Canadian origin. When possible, grandparents and other relatives were also recruited to the study.

The affection status of all study participants was determined by clinical evaluation and the completion of a standard respiratory questionnaire that was modified to include questions about asthma and atopy severity, family history of asthma and/or atopy, age-of-onset and the presence of other respiratory failure diagnoses.[61] We defined participants as asthmatics if (1) a reported history of asthma (questionnaire-based) and a history of physician-diagnosed asthma (past/current) were available, or (2) confirmation of diagnosis by a positive methacholine provocation test was obtained (only on subjects older than 12 years of age). Subjects were deemed atopic if they had at least one positive response (wheel diameter ≥3 mm at 10 min) to skin-prick tests. The family participation rate was approximately 60% and all subjects gave informed consent. A total of 223

independent families (1139 individuals) with family size ranging from 3 to 17 and number of affected family members (including probands) ranging from 1 to 10 were analyzed.

Polymorphism selection and genotyping

Five polymorphisms of the *NRAMP1* gene were selected based on their known association with disease. Two of the variants have reference SNP identifiers (rs#) from the NCBI database. In this report, we referred to the common aliases of the variants: 5′$(CA)_n$, 274C>T, 469+14G>C, D543N and 1729+del4.[32] Variant 5′$(CA)_n$ was genotyped by length polymorphism analysis using ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA, USA). Primers for amplifying polymerase chain reaction (PCR) products were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCR products of sizes ranging from 168 basepairs (bp) to 182 bp were amplified using primer pair: 5′-AACGAGGGGTCTTGGAACTC-3′ and 5′-gcctcccaagtta gctctga-3′. PCR reactions were carried out in PTC-100® Peltier thermal cyclers (MJ Research, MA, USA) under the following condition: 10 ng of genomic DNA was added to 20 μl reaction mixture containing 1 × PCR buffer, 2.5 mM of $MgCl_2$, 0.5 U of Platinum Taq polymerase (Qiagen, CA, USA), 0.50 mM of dinucleotides and 0.30 μM of primers. PCR was initiated by denaturing the samples at 96°C for 10 min, followed by 30 cycles of denaturation at 96°C for 25 s, annealing at 67°C for 1 min. Final extension was carried out at 72°C for 5 min. Finally, 1 μl of PCR product was mixed with 0.30 μl of formamide and 10 μl of GeneScan™–500 Liz™ Size Standard (Applied Biosystems, CA, USA) before being denatured at 95°C for 5 min and injected into ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA, USA). Fluorescence signal was read and analyzed using ABI PRISM® GeneMapper™ Software version 3.5 (Applied Biosystems, CA, USA).

Variants 274C>T and 469+14G>C were genotyped using HEFP™ (Molecular Devices),[62] a single-base extension (SBE) fluorescence polarization platform, as previously described.[11] Briefly, PCR and SBE primers were designed using the Primer3 software. PCR reactions were carried out using primer set 1 (274C>T): 5′-GCCAGCCTGAAGATCTGACT-3′, 5′-GGACCCCCTCA CTCTACTCC-3′ and set 2 (469+14G>C): 5′-ATCGTG GAAGCTGAAAATGG-3′, 5′-GCGAGGTCTGCCATCTC TAC-3′. A total of 6 ng of genomic DNA was added to 8 μl reaction mixture containing 2.5 mM of $MgCl_2$, 25 mM of dinucleotides, 0.2 U of HotstartTaq DNA polymerase (Qiagen), and 100 nM of primers. PCR was initiated by denaturing the samples at 94°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 56°C (primers specific) for 30 s and extension at 72°C for 30 s. Final extension was done at 72°C for 6 min. PCR products were treated with Exonuclease I and Shrimp Alkaline Phosphatase as recommended by the manufacturer (AcycloPrime-FP SNP Detection Kit, Perkin-Elmer, Wellesley, MA, USA). SBE detection primers used were (274C>T): (sense) 5′-GGAAAGCAATGCTCATGAG-3′, (antisense) 5′-TTCACGGGGCCTGGCTT-3′, (469+14G>C): (sense) 5′-TGGTTCTCCCTGTCCAGG-3′ and (antisense) 5′-TAAGGTGAGCTTGGGGG-3′. FP-SBE reactions were performed in one or both orientations as suggested by the manufacturer (AcycloPrime-FP SNP Detection Kit, Perkin-Elmer, Wellesley, MA, USA). After the addition of reading buffer, the plates were read using the Analyst HT® reader (Molecular Devices, CA, USA) as described previously.[63]

Variants D543N and 1729+55del4 were genotyped by TaqMan assays.[64] Each variant was analyzed using two sets of oligonucleotides (external primers and internal probes) designed using the Primer3 software. The internal probes were labelled with fluorescent dyes: TAMRA (6-carboxytetramethyl-rhodamine) at 3′ ends, FAM (6-carboxy-fluorescein) and TET (6-carboxy-4,7,2′,7′-tetrachlorofluorescein) (one per oligonucleotide) at 5′ ends. For variant D543N, primers set 5′-CCAC CACCACTTCCTGTATG-3′ and 5′-CACGTCATACATG CCACTCC-3′ and probes set 5′-FAM-CCCTTTCTG GTCCTCTTCAAGGA-TAMRA-3′ and 5′-TET-CCCTTT CTGGTTCTCTTCAAGGAGC-TAMRA were used. For variant 1729+55del4, primers set 5′-GGGAGTGGCATG TATGACG-3′ and 5′-TCTATCCTGCTGCCTGCAC-3′, and probes set 5′-FAM-TGGCCTGCTGGATGTGGAG TAMRA and 5′-TET-TGACTGGCCTGCTGGAGAGG TAMRA were used. For both variants, 10 ng of genomic DNA were added to a 20 μl reaction mixture containing 1 × PCR buffer, 5.0 mM of $MgCl_2$, 0.2 mM of each nucleotide, 0.5 U of HotstartTaq DNA polymerase (Qiagen), 0.03 μM of probes, and 0.30 mM of primers. PCR was initiated by denaturing the samples at 96°C for 10 min followed by 40 cycles of denaturation at 96°C for 30 s, annealing and extending at 60°C for 1 min. Final extension was carried out at 72°C for 5 min. PCR endpoint fluorescence reading was carried out using ABI PRISM® 7700 Sequence Detector System (Applied Biosystems, CA, USA). The fluorescence intensity was adjusted and recorded using Sequence Detector Software version 1.7 (Applied Systems, CA, USA).

Statistical analysis

Hardy–Weinberg equilibrium was tested in a subset of independent DNA samples (parents of probands) using HAPLOVIEW.[65] Allele distribution patterns were assessed by FBAT, (version 1.5).[33,34] This software uses an empirical variance–covariance estimator to account for the possibility of nonindependent allelic transmission to affected sibs.[66] Asthma, atopy and IgE level phenotypes were tested separately under additive and dominant genetic models.

Associations between variants were assessed by calculating D', a measurement of the LD strength[67] using HAPLOVIEW.[65] Based on the LD strength of variants, haplotypes were inferred and assessed for nonrandom transmission using the 'hbat' command of FBAT version 1.5.[33,34] An empirical variance estimator was used.[66] Asthma, atopy, and IgE level phenotypes were tested separately under additive and dominant genetic models.

To assess whether the cohort size has sufficient power to detect association between variants and phenotypes, we used Power calculation of the FBAT (PBAT).[68,69] The family design was based on the observed count of nuclear families, according to the number of affected sibs, unaffected sibs, and missing parents. The genetic models considered assumed a constant population prevalence of 0.10, varying susceptibility allele frequencies (q) and varying GAF of the allele GAF, under an additive model for risk. GAF corresponds to a reduction of incidence in the study families when the risk variant is

removed, that is, a GAF of 5% indicates that removal of the risk variant would reduce incidence by 5% in the study families. We used values for q ranging from 0.05 to 0.70 and values for GAF ranging from 0.05 to 0.50. For an additive disease model with prevalence at 0.10, the odds ratio for the heterozygotes was calculated for each q and GAF parameter set. The level of significance was set at 0.05.

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Alleles of the *NRAMP1* gene are risk factors for pediatric tuberculosis disease

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Relatively little is known about the human genetics of susceptibility to common diseases caused by bacterial pathogens. Tuberculosis, caused by *Mycobacterium tuberculosis*, is a major cause of morbidity and mortality worldwide. So far, genetic studies of tuberculosis susceptibility have largely been focused on adult patients despite the fact that tuberculosis is highly prevalent among children. To study the host genetic component of pediatric tuberculosis susceptibility, we enrolled 184 ethnically diverse families from the Greater Houston area with at least one child affected by pediatric tuberculosis disease. Using a family-based control design, we found allelic variants of the natural resistance-associated macrophage protein gene 1 (*NRAMP1*) (alias *SLC11A1*) significantly associated with tuberculosis disease in this pediatric patient population [$P = 0.01$; odds ratio = 1.75 (95% confidence interval, 1.10–2.77)]. The association of *NRAMP1* with pediatric tuberculosis disease was significantly heterogeneous ($P = 0.01$) between simplex [$P < 0.0008$; odds ratio = 3.13 (1.54–6.25)] and multiplex families ($P = 1$), suggesting an interplay between mechanisms of genetic control and exposure intensities. In striking contrast to previous studies in the adult population, we observed that the common alleles of *NRAMP1* polymorphisms were risk factors for pediatric tuberculosis disease. To explain the different direction of allelic association between adult and pediatric disease, we hypothesize that *NRAMP1* influences the speed of progression from infection to tuberculosis disease.

complex traits | host genetics | mycobacterial diseases

The human pathogenic bacterium *Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects an estimated one-third of the world's population, resulting in >8 million tuberculosis cases and 2 million deaths each year (1). The rate of progression from infection to disease is highly variable, and ≈90% of infected individuals never develop clinical disease. Of the 10% of *M. tuberculosis*-infected persons who do develop clinically overt disease, approximately half will be diagnosed within <2 years of infection and are considered to be fast progressors of tuberculosis disease. This so-called primary tuberculosis disease is particularly common among children, and the majority of pediatric cases present with primary tuberculosis disease. Tuberculosis patients who progress more slowly from infection to tuberculosis disease and develop clinical disease >2 years after infection are referred to as "reactivation" cases. Little is known about the mechanisms that influence the rate of progression from infection to disease. For example, it is unknown whether different mechanisms of pathogenesis operate in individuals who progress at different rates from infection with *M. tuberculosis* to clinical tuberculosis disease.

Many lines of evidence support an important role of host genetic variation in tuberculosis susceptibility, including animal models of the disease (2–6), ethnic clustering of tuberculosis cases (7), increased concordance rates of tuberculosis among monozygotic vs. dizygotic twins (8, 9), evidence that certain gene variants are associated or linked with increased risk of tuberculosis (10, 11), and the demonstration that patients with Mendelian disorders of the interleukin 12-IFN-γ axis are hypersusceptible to *M. tuberculosis* (11, 12). However, the great majority of genetic studies have investigated adult pulmonary tuberculosis cases, whereas few have focused on pediatric tuberculosis disease (13). The exclusion from more intense study of primary tuberculosis disease is surprising, because approximately half of all tuberculosis patients are thought to represent primary disease (14). Moreover, contrasting the results of primary and reactivational disease studies will provide a better understanding of the mechanisms that govern progression from infection to disease in two distinct stages of tuberculosis.

The natural resistance-associated macrophage protein gene 1 (*NRAMP1*, alias *SLC11A1*) is the human homologue of the mouse *Nramp1* gene that has been shown to be a critical element in the regulation of intracellular membrane vesicle trafficking of macrophages, a principal cell type expressing Nramp1 (15). In the mouse, it has been shown that absence of mature Nramp1 protein is the result of a G169D polymorphism that causes increased susceptibility to several intracellular macrophage pathogens, including *Mycobacterium bovis* (bacillus Calmette–Guérin), *Salmonella typhimurium*, and *Leishmania donovani* (16). In phagocytosing macrophages Nramp1 is rapidly recruited to the membrane of late endosomal–phagosomal vesicles (17, 18). At the phagosome membrane, Nramp1 functions as a divalent cation pump (19, 20), and Nramp1-altered cation fluxes are thought to abrogate pathogen-induced blockage of phagosome maturation (21–23). The mechanism of NRAMP1 action in human macrophages is not known but is thought to follow similar mechanisms.

Polymorphisms in the *NRAMP1* gene have been found in a number of genetic studies to be risk factors for the development of tuberculosis among adult populations (24). However, except for the study of a tuberculosis outbreak in a Canadian community (25), no distinction was made between primary and reactivational tuberculosis for the patients enrolled in these previous studies. Such a study design might miss or underestimate genetic control mechanisms that differ in the development of primary and reactivational tuberculosis. Hence, we focused our genetic analysis on pediatric cases where patients present with primary tuberculosis disease. We here report strong association between *NRAMP1* alleles and pediatric tuberculosis disease specifically among individuals that are likely to lack previous exposure to *M. tuberculosis*. We also note an inverse association of *NRAMP1*

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Abbreviations: NRAMP1, natural resistance associated macrophage protein 1; TDT, transmission disequilibrium test; LD, linkage disequilibrium; OR, odds ratio; PPD, purified protein derivative.

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polymorphisms among adult and pediatric tuberculosis disease patients. These results shed light on the role of *NRAMP1* in susceptibility to tuberculosis disease and provide a plausible explanation for *NRAMP1* genetic heterogeneity in tuberculosis susceptibility.

Materials and Methods

Families. The diagnostic criteria for pediatric cases were culture confirmation of tuberculosis (78 patients) or clear clinical criteria of disease (26, 27). All parental cases were culture-positive. Information regarding bacillus Calmette–Guérin vaccination and previous tuberculosis disease was obtained by interview or by visual inspection of skin scars. Ethnicity was self-reported. Mantoux status of family members was determined as part of routine patient care and contact tracing. Blood (2–10 ml) was obtained by venipuncture and used for extraction of genomic DNA with the Nucleon extraction kit (Amersham Pharmacia). Written informed consent was obtained from all study participants. The study was approved by the Institutional Review Board at Baylor College of Medicine and the Ethics Committee at the Research Institute of the McGill University Health Centre.

Genotyping. The intragenic *NRAMP1* polymorphisms *274C/T*, *469 + 14G/C*, *D543N*, and *1729 + 55del4* were determined as described (28). The 3′ UTR (N10) insertion/deletion polymorphism was amplified with the ^{32}P-labeled forward primer reported by Buu *et al.* (29) by using 5′-TCAAGCTCCAGTTTGGAGCCT-3′ as reverse primer and resolved as length variants on 6% polyacrylamide gels. The same conditions were used to genotype the promoter $(GT)_n$ (N01) polymorphism except primers 5′-GACATGAAGACTCGCATTAG-3′ and 5′-TACCCCATGACCACACCC-3′ were used as described by Marquet *et al.* (30).

Markers *D543N* and *1729 + 55del4* (N09) were also genotyped with Taqman assays. The primers and probes used in the assays were designed by using the software PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The following primers were synthesized: (c.D543N), 5′-CCACCACCACTTCCTGTATG-3′ and 5′-CACGTCATACATGCCACTCC-3′; (c.1729+del4), 5′-GGGAGTGGCATGTATGACG-3′ and 5′-TCTATCCTGCTGCCTGCAC-3′. The following probes were synthesized and labeled with fluorescent dyes: (D543N), 5′-FAM-CCCTTTCTGGTCCTCTTCAAGGA-TAMRA; 5′-TET-CCCTTTCTGGTTCTCTTCAAGGAGC-TAMRA; (c.1729+del4), 5′-FAM-TGGCCTGCTGGATGTGGAGTAMRA, 5′-TET-TGACTGGCCTGCTGGAGAGG-TAMRA. PCR reactions were performed in a volume of 45 μl containing 5 μl of 10 × PCR buffer (Invitrogen); 5 μl of $MgCl_2$ (50 mM) (Invitrogen); 1 μl of dNTPs (10 mM) (Invitrogen); 0.75 μl of forward primer (20 mM), 0.75 μl of reverse primer (20 mM), and 0.60 μl of FAM probe (2 M) (Research Genetics, Huntsville, AL); 0.60 μl of TET probe (2 M) (Research Genetics); 0.1 μl of Platinum Taq polymerase (Invitrogen); and 5 μl of DNA (10 ng/μl). Three nontemplate controls were included on each plate. All PCRs were carried out in transparent 96-well plates with caps (Applied Biosystems). DNAs were amplified in MJ PT-100 machines (MJ Research, Cambridge, MA) under the following conditions: (*i*) 96°C for 10 min, (*ii*) 96°C for 25 sec, (*iii*) 60°C for 1 min, (*iv*) repeat steps *ii–iii* 39 times, (*v*) 72°C for 5 min, and (*vi*) 10°C ambient time. PCR products were analyzed with an Applied Biosystems 7700 Sequence Detector spectrophotometer equipped with SEQUENCE DETECTOR, Ver. 1.7 software. Fluorescence readings were exported to a spreadsheet and graphed as a scatter plot.

Markers rs2292555, rs1017698, and rs9076 were genotyped by PCR-RFLP under identical conditions. The PCR reaction mixture included 100 ng of genomic DNA, 1× Buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.09 mM dNTPs, 0.2 μM of each primer (rs2292555: 5′-AGCCAGGGTAGGCAGGATAC-3′; GGCATTCACGATTGCTTTTC-3′; rs1017698: 5′-CCACCATAGCCAAACCATTC-3′, 5′-GGGATGTGATACCCTTCCAG; rs9076: 5′-GTTTTATCCGCAGCCCTTTT-3′, 5′-CCAGTCGGAAGAAACAGCAT-3′), and 1 unit of Taq polymerase. Cycling conditions included an initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 50 sec, 50°C for 50 sec, then 72°C for 50 sec, and a final extension at 72°C for 10 min. For marker rs2292555, a total of 5 μl of PCR product was added to 5 μl of digestion mix, which contained 1 × Buffer 4 (NEB, Beverly, MA) and 0.3 units of DdeI, followed by incubation at 37°C for 4 h. Conditions for DNA restriction were identical for markers rs1017698 and rs9076, except that 0.4 units of BtsI were used for marker rs1017698 and 0.1 units of BsgI and 1× were used for marker rs9076. All banding patterns were resolved on a 2% agarose gel stained with ethidium bromide. Markers rs2104615, rs4324314, and rs4674297 were genotyped on the Orchid Biosciences (Princeton, NJ) UHT genotyping platform (31), as described in detail (32).

Statistical Analysis. The association study was mainly performed by the family-based method implemented in the Family Based Association Test (FBAT program (33). The FBAT statistic combines the three different methods described in the text [Transmission Disequilibrium Test (TDT), Reconstruction-Combined TDT (RC-TDT), and SIB-TDT] and allows the use of an empirical variance–covariance estimator for the statistic that is consistent when sib marker genotypes are correlated (e.g., when the analysis include multiplex families) (34). In addition, empirical *P* values ($P_{empirical}$) can be computed by permutation. Exact *P* values also were computed by using the RC-TDT software (35). Finally, alleles with evidence for association also were analyzed by conditional logistic regression as described (36), assuming a multiplicative effect of alleles on the disease relative risk. This analysis allowed us to provide odds ratio (OR) estimates and to test for differences in the regression coefficients associated with selected polymorphisms according to five binary criteria described in the text.

To test for heterogeneity of the sample according to a binary criterion (e.g., simplex/multiplex), the analysis was performed on the whole sample (184 families) and separately on the two subsamples (143 simplex and 41 multiplex families). Under the hypothesis of homogeneity, twice the difference between the likelihood of the whole sample and the summed likelihoods of the two subsamples is distributed as a χ^2 with one degree of freedom.

The Hardy–Weinberg Equilibrium (HWE) was tested at each SNP for the subset of all parents across ethnicities and for the groups of black and Hispanic parents independently. No significant deviations from HWE were observed. The strength of linkage disequilibrium (LD) between pairs of SNPs was measured as D′ (37) by using HAPLOVIEW (www.broad.mit.edu/mpg/haploview). LD blocks were inferred from the definition proposed by Gabriel *et al.* (38), as implemented in HAPLOVIEW with D′ confidence bounds of 0.7–0.92.

Results

Description of Patients and Their Families. All families enrolled in the study were from greater Houston. The Houston metropolitan area historically has had a high rate of pediatric tuberculosis cases and is ethnically very diverse (26). To avoid possible confounding of gene–phenotype associations due to inappropriately chosen controls or population substructures, we conducted a family-based association study. This design is particularly robust in an ethnically and racially mixed community like that of the greater Houston area. We enrolled 184 nuclear families with at least one child with pediatric tuberculosis (Table 1). The majority of families (n = 143) were composed of only a single

Table 1. Characteristics of 184 nuclear tuberculosis families comprising 737 individuals enrolled for the present study

Subjects	Total	Tuberculosis-affected patients				
		All patients	Children	Mean age (years) at diagnosis (±SD)	Parents	Mean age (years) at diagnosis (±SD)
Females	353	118	100	5.3 ± 5.7	18	25.4 ± 6.8
Males	384	116	106	6.2 ± 5.4	10	29.2 ± 6.3
Combined	737	234	206	5.7 ± 5.6	28	28.8 ± 6.6

tuberculosis case (simplex families), whereas more than one case was diagnosed in 41 families (multiplex families). In 73 of the 184 families in our sample, one parent was not available for analysis. With regard to ethnicity, we enrolled 136 Hispanic, 69 black, 13 Asian, 7 white, and 9 tuberculosis patients of mixed ethnic origin. Of these 234 tuberculosis cases, 28 were adult cases, and 206 were children. The disease manifestation was classified as pulmonary in 51.3%, extrapulmonary in 37.5%, and mixed pulmonary and extrapulmonary in 11.2% of all pediatric cases. There were no statistically significant differences in the proportion of simplex vs. multiplex families and in pulmonary vs. extrapulmonary involvement across ethnic groups (data not shown).

***NRAMP1* Is Associated with Pediatric Tuberculosis Disease.** Over all families, the common C allele of the *NRAMP1* N02 polymorphism was significantly associated with increased risk of pediatric tuberculosis disease ($P = 0.01$; Fig. 1). Under a multiplicative genetic model, the OR of tuberculosis for C/C homozygotes vs. C/T heterozygotes was equal to the OR of C/T heterozygotes vs. T/T homozygotes and corresponded to 1.75 (95% confidence interval, 1.10–2.77). In addition, there was weaker evidence ($0.043 < P < 0.075$) in favor of a positive association between the *NRAMP1* N01 promoter polymorphism and tuberculosis (Fig. 1). There was no significant association between the *NRAMP1* polymorphisms located in the 3′ region of the gene and pediatric tuberculosis disease.

LD Among Markers in the *NRAMP1* Genome Region. To better define the observed association of *NRAMP1* alleles with pediatric tuberculosis disease, we computed LD, measured as D′, among the five tested markers in the group of Hispanic parents, the largest ethnic group among the enrolled families. We found that the three 5′ markers (N01–N03) were in strong LD among them but observed only weak to moderate LD with markers N09 and N10 in the 3′ *NRAMP1* region. To better delimit the LD pattern of the tuberculosis-associated markers N01–N03, we genotyped seven additional markers flanking the 5′ *NRAMP1* region. Five of these SNPs were used to tag the *MGC5081* ORF (rs4674297 and rs4324314) and the *MR-1* gene (rs9076, rs2014615, and rs1017698), the two closest neighbors located 15 and 65 kb upstream of *NRAMP1*, respectively. The two remaining SNPs were located in intron 6 (rs2290708) and exon 15 (NRAMP1

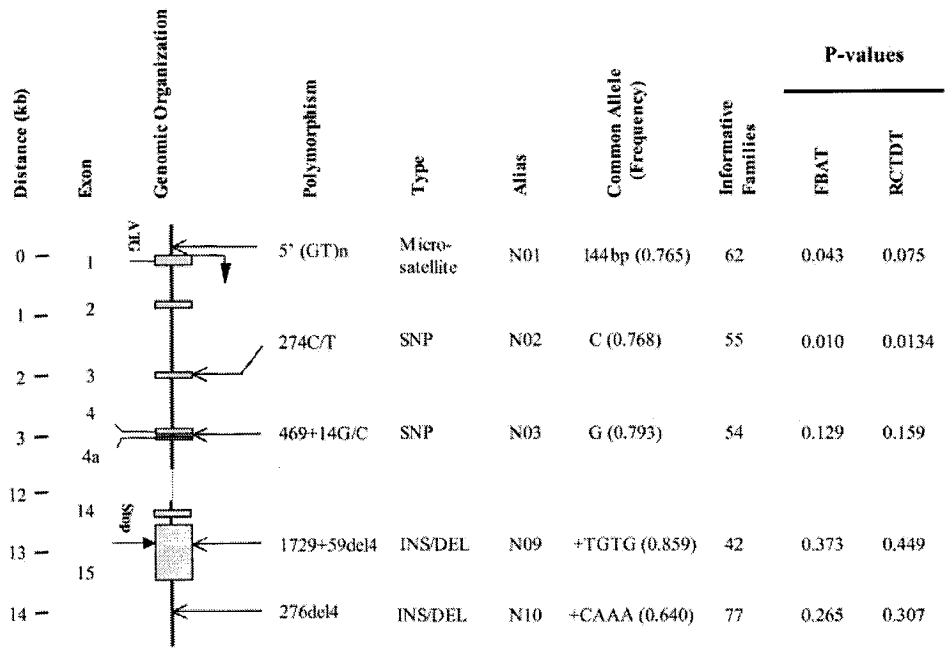

Fig. 1. Schematic presentation of the *NRAMP1* candidate gene and intragenic location of gene polymorphisms. The genomic distance spanned by *NRAMP1* in kilobase pairs (kb), the exon numbers, the translational initiation (ATG), and termination sequences (Stop) and the location of distinct gene polymorphisms with respect to the exon–intron organization are given on the left side of the diagram. Designation of gene polymorphisms either adopted names already established in the literature or followed standard nomenclature rules (51). The type of polymorphism–microsatellite repeat, SNP, insertion/deletion polymorphism (INS/DEL), together with a simple polymorphism alias as well as the identity and frequency of the common allele, are also indicated. Finally, the number of families comprising at least one parent heterozygous for the polymorphisms, i.e., a parent for which preferential allele transmission can be monitored, is given. *P* values indicating evidence for distortion of allele transmissions are given for the Family Based Association Test (FBAT) (33) and the Reconstruction-Combined TDT (RC-TDT) (35) analytical procedures.

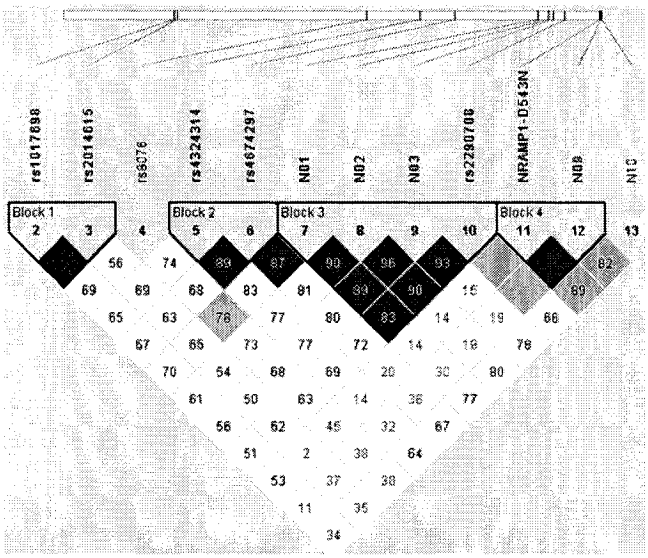

Fig. 2. LD pattern in the Hispanic population between pairs of SNPs spanning the *NRAMP1* gene and its upstream genomic region. The *NRAMP1* 3′ region is located on the right end of the schematic chromosome line indicated on top of the graph. Consequently, the *NRAMP1* gene orientation is in the 3′ to 5′ orientation from right to left. The telomere of chromosome 2q is located toward the right. Names of polymorphisms used for the LD matrix of pairs of markers are given, and their chromosomal locations are indicated by solid lines. Haplotype blocks according to Gabriel *et al.* (38) are indicated, and names of markers that are part of haplotype blocks are indicated in bold. Each square represents the magnitude of pairwise LD. Each pairwise D′ measure is shown as D′ × 10^2 within the corresponding square. Squares without D′ written on them represent D′of 1.0. Black squares indicate pairwise LD that is strong [lower confidence interval (CI), 0.7, upper CI >0.92], light-gray squares represent intermediate strength LD, and white squares represent weak LD.

D543N) of the *NRAMP1* gene. Over the entire interval of ≈80 kb, we were able to identify four haplotype blocks (Fig. 2). Although clearly not part of the same haplotype blocks, there was substantial LD between pairs of SNPs among 5′ *NRAMP1* markers, the two *MGC5081* tag SNPs and, to a lesser degree, the *MR-1* located SNPs (Fig. 2). None of the additional markers showed significant evidence for association with pediatric tuberculosis disease and all pediatric tuberculosis-associated SNPs localized to the 5-kb haplotype block 3 (Fig. 2). The variable strength of association with tuberculosis among those markers is likely explained by the fact that LD is not complete between markers of block 3 and by the differences in allele frequencies (especially between N02 and N03). A similar pattern of pairwise D′ values was also observed for the parents of the black families. However, due to the reduced number of informative chromosomes, confidence intervals were too large to allow for the definition of haplotype block structures (data not shown).

***NRAMP1* Alleles and Association with Pediatric Tuberculosis Disease.** To test whether the association observed between the *NRAMP1* N02 polymorphism and tuberculosis was influenced by family or case characteristics, we performed heterogeneity tests in the conditional logistic regression analysis framework (36). Specifically, we tested for differences in the regression coefficient associated with each of the two polymorphisms according to five binary criteria: family structure (simplex/multiplex), ethnicity of family (Hispanic/other), sex of affected child (male/female), anatomic site of tuberculosis (pulmonary/extrapulmonary), and age of onset (≤5years/>5 years). Because of small numbers, Asian and white families could not be tested independently for *NRAMP1* N02 association heterogeneity. Only the sex of the pediatric patient and the family structure were found to have significant effects. The association of the *NRAMP1* N02 polymorphism and tuberculosis disease was stronger in males [OR for C/C vs. C/T = 2.82 (1.44–5.61)]. The difference in transmission between male and female patients also was significant ($P < 0.04$).

Next, the association of *NRAMP1* with tuberculosis disease was analyzed separately in simplex and multiplex families. Independent of the mode of analysis, there was a highly significant distortion ($P < 0.0008$) of the *NRAMP1* N02 polymorphism transmission in simplex families [OR for C/C vs. C/T and C/T vs. T/T = 3.13 (1.54–6.25)] that was not detected in multiplex families (Table 2). Formal testing of variable strength of association between N02 and pediatric tuberculosis in simplex vs. multiplex families clearly revealed a significant heterogeneity ($P < 0.01$). This result argues that difference in *NRAMP1* N02 transmission to tuberculosis-affected children in simplex and multiplex families represents a true effect and is not simply a reflection of the different numbers of informative simplex and multiplex families leading to loss of significance in the less numerous multiplex families. When focusing only on the 17 informative simplex families with male pediatric patients, the effect of N02 on tuberculosis risk was very highly significant ($P < 0.00004$) with an estimated OR for C/C vs. C/T of 20.0 (2.69–148). Those 17 families of diverse ethnicity include 20 heterozygous C/T parents who transmitted the C allele to their affected child 19 times.

Family Exposure to *M. tuberculosis* and Strength of Association of *NRAMP1* with Pediatric Tuberculosis Disease. To follow up on the restriction of *NRAMP1* tuberculosis association, we investigated the heterogeneity of family structure in *NRAMP1*-mediated risk on pediatric tuberculosis disease (Table 2). The majority (26/41)

Table 2. Association between susceptibility to pediatric tuberculosis disease and *NRAMP1* alleles stratified by family structure

		Simplex families				Multiplex families			
			P value				*P* value		
Gene	Polymorphism	Informative families	FBAT	RC-TDT	Conditional logistic regression	Informative families	FBAT*	RC-TDT†	Conditional‡ logistic regression
NRAMP1	N01	39	0.020	0.048	ND	22	0.594	0.697	ND
	N02	36	0.00045	0.00059	0.00080	19	1.000	1.000	ND
	N03	37	0.014	0.021	ND	17	0.7546	0.876	ND

*FBAT, Family Based Association Test (33).
†RC-TDT, Reconstruction-Combined TDT (35).
‡ND, not done.

Table 3. Family sibship size and proportion of families with at least one unaffected cosib (UCS)

Number of families	Number of UCS	Number of families with UCS-PPD$^+$ ≥1	Number of families with only UCS-PPD$^-$
38	1	14	24
24	2	12	12
6	3	4	2
3	4	2	1

UCS-PPD$^+$, unaffected cosibs that are PPD-positive. UCS-PPD$^-$, unaffected cosibs that are PPD-negative.

of multiplex families in our family collection included adult infectious cases. Children living in close proximity to adult cases are expected to have increased exposure to *M. tuberculosis*. Hence, we used purified protein derivative (PPD) skin-test conversion among all unaffected cosibs as a measure of exposure intensity in individual families and found a substantially higher proportion of cosibs tested PPD+ in multiplex families with at least one affected parent (62.5%) as compared with simplex families (36.5%). Because pediatric cases generally have a low infectious potential, there was no significant difference in the proportion of PPD+ cosibs among multiplex families without adult cases (31%) and simplex families.

To test the resulting hypothesis that *NRAMP1* effects on tuberculosis disease risk are most readily detectable under conditions of low *M. tuberculosis* transmission, we selected all simplex families comprising at least one child in addition to the affected sib. Of the available 71 families, 32 included at least one additional PPD+ cosib ("high-exposure families"), whereas among 39 families, no PPD+ cosib was identified ("low-exposure families"). Family size was not a confounding factor for classification into high- and low-exposure families ($P > 0.9$; Table 3). Among the entire subsample of 71 simplex families with at least one additional cosib, there was strong evidence for an association of *NRAMP1* N02 alleles with pediatric tuberculosis disease ($P_{empirical} = 0.006$). When separated into high- and low-exposure families, there was less evidence of significant distortion of *NRAMP1* N02 allele transmission among high-exposure families ($P_{empirical} = 0.21$) as compared with low-exposure families ($P_{empirical} = 0.011$). Because <10% of cases had been vaccinated with bacillus Calmette–Guérin, these findings strongly suggest that *NRAMP1* alleles have their highest impact on risk of tuberculosis disease under conditions of low transmission/exposure of *M. tuberculosis*.

Discussion

The human *NRAMP1* gene has been implicated in increased risk of tuberculosis disease by a number of studies. For example, polymorphisms in the 5′ and 3′ regions of *NRAMP1* have been linked or associated with tuberculosis disease susceptibility in Guinea Conakry (39), Japan (40), Korea (41), The Gambia (42, 43), Canada (25), Texas (44), Cambodia (45), Denmark (46), and South Africa (24), but not in Taiwan (47) or Morocco (48). The focus of most studies was on susceptibility to smear-positive tuberculosis disease among adult populations. The design of our study was different from previous investigations, because we analyzed the effect of *NRAMP1* alleles on risk of primary tuberculosis disease in a cohort of pediatric tuberculosis disease families. We observed that the *NRAMP1* gene, previously implicated in the genetic control of adult tuberculosis, also influences the risk of pediatric tuberculosis disease. Unexpectedly, we discovered the direction of *NRAMP1* allele association with pediatric tuberculosis disease to be inverted compared with previous studies in adult pulmonary tuberculosis. Although among adult patients the common 5′ *NRAMP1* alleles had been found associated with protection, i.e., depleted among cases, we found that the common alleles are risk factors, i.e., enriched among pediatric tuberculosis disease patients. Consequently, the significant enrichment of the common N02 C allele and, to a lesser degree, the N01 and N03 common alleles in early onset cases and the corresponding depletion of the same allele in late onset patients strongly suggests that the N02 C allele promotes rapid progression from infection to disease.

Importantly, the suggestion that common *NRAMP1* alleles are risk factors for early-onset tuberculosis agrees with the results of a previous genetic analysis of tuberculosis in a large Canadian Aboriginal family that experienced a tuberculosis outbreak (25). In this outbreak, individuals had limited prior exposure to mycobacteria, and all tuberculosis cases were diagnosed within a maximum time of 2 years from the index case. The majority of cases occurred within 6–9 months after diagnosis of the index case (49). Consequently, all patients with clinical tuberculosis could be classified as fast progressors or primary tuberculosis disease cases. Segregation analysis revealed that the common *NRAMP1* alleles were preferentially transmitted to tuberculosis patients (25). Considering that only ≈10% of individuals infected with *M. tuberculosis* advance to clinical forms of tuberculosis, the involvement of genes controlling the rate of progression rather than bona fide susceptibility to tuberculosis may offer an effective genetic control of disease risk. Whether the N02 polymorphism is itself the cause of more rapid progression to tuberculosis disease, or whether N02 is in LD with the causative variant is presently not known. However, recent results from our laboratory directly correlated reduced NRAMP1 functional activity with the high-risk C allele of N02 (unpublished work).

Among pediatric tuberculosis disease cases, *NRAMP1* alleles have different strength of association among different patient subgroups. The observation that *NRAMP1* association with tuberculosis disease is more readily detected among male than female pediatric cases is interesting in light of the known gender-specific differences in frequencies among adult tuberculosis cases. However, because evidence for such a gender-specific effect of *NRAMP1* alleles on pediatric disease was significant but weak in our sample, additional studies are required to confirm this observation. By contrast, our data suggest that gene–environment interactions are critical for the appropriate selection of efficient host responses and hence genetic control mechanisms. This is illustrated by our observation that genetic control of tuberculosis disease by *NRAMP1* was most easily detected in families that experienced low exposure intensities to *M. tuberculosis*. Under conditions of increased exposure, the *NRAMP1* effect became less strong, suggesting that mechanisms independent of the *NRAMP1* gene (e.g., pathogen factors or different sets of genes) are more prominent in this instance.

In the study families, the *NRAMP1* gene clearly is a strong risk factor for pediatric tuberculosis disease under conditions of low exposure to *M. tuberculosis*. Specifically, among simplex families, the population-attributable risk of the *NRAMP1* N02 C risk allele is estimated to be 85% (49–96%). Assuming the N02 polymorphism is the only cause of increased tuberculosis in this area, this means that the incidence of pediatric tuberculosis would be 85% lower if the *M. tuberculosis*-infected Houston population were monomorphic for the protective N02 T allele. Although the actual impact of the N02 polymorphism depends on other risk factors not yet identified, the combination of a relatively strong genetic effect (OR = 3.13) of a risk allele present at high frequency (≈0.76), as described for type II diabetes (50), is expected to make a substantial contribution to the occurrence of pediatric tuberculosis in the Houston area.

The present study is one of an increasing number of positive-association reports between risk of tuberculosis disease and *NRAMP1* alleles in populations of vastly different ethnic

background. Results obtained in the present investigation provide an important complement for a better understanding of the setting in which *NRAMP1* exerts its influence on tuberculosis susceptibility. Specifically, our results suggest that *NRAMP1* effects are most pronounced in the absence of prior exposure to mycobacteria, and that *NRAMP1* is a modulator of the speed of progression from infection with *M. tuberculosis* to tuberculosis disease. The detailed molecular events that prevent rapid progression to clinically defined disease are presently unknown. However, a protein that limits multiplication of ingested *M. tuberculosis* bacilli by antagonizing pathogen-triggered blockage of phagosome maturation offers a reasonable mechanism for containing the spread but not the initial infection by the bacterium.

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