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Contribution of Hierarchical Clustering Techniques to the Modeling of the Geographic Distribution of Genetic Polymorphisms Associated with Chronic Inflammatory Diseases in the Québec Population

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Key Words
Asthma · Cardiovascular diseases · French Canadian population · Genetic risk factors

Abstract
Objectives: The purpose of this project was to evaluate the potential of the downward hierarchical clustering analysis (DHCA) for studying genetic heterogeneity, i.e. differences in allele frequency in subpopulations, such as the 15 public health regions of the province of Québec (Canada). ***Methods:*** The study relied on an anonymized sample of 1,680 individuals who had participated in the Québec Heart Health Survey in 1990–1991. The genotyping of 11 variants in 8 candidate genes known to be involved in chronic inflammatory diseases, namely asthma and cardiovascular diseases, was performed using the amplification refractory mutation system and restriction fragment length polymorphism techniques. Only variants showing an allelic frequency >2% in the Québec Heart Health Survey (n = 8) were selected. DHCA techniques were then applied to model the geographical distribution of these 8 genetic variants in 15 Québec public health regions and to study genetic heterogeneity. ***Results:*** The DHCA allowed to group public health regions and gene variants on the basis of genetic variability. For both asthma and cardiovascular diseases, 3 significant clusters of public health regions and 1 cluster of gene variants were identified. ***Discussion:*** This study suggests that DHCA might be useful in studying genetic heterogeneity at the population level and for public health activities.

Introduction

The demographic history of the province of Québec (Canada) is well documented. At the present time, the Québec population numbers more than 7 million people. Approximately 55% of the population lives in the southwestern part of the province, which is ethnically more heterogeneous than the rest of the Québec population, composed mainly of French Canadians [1]. It has been estimated that 25,000 settlers came from various provinces of France, especially from the northwestern part, between the beginning of the 17th century and the British conquest of 1763 [2, 3]. The population size increased steadily after the institution of a settlement plan in 1660, and reached over 70,000 inhabitants by the time of the

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British conquest. The subsequent growth of the French Canadian population is notably due to the high reproduction rate of the following generations [2]. Immigration waves of people from the British Isles as well as American loyalists also contributed to the population increase [3]. In recent decades, however, immigration has occurred from all continents, explaining the increasing diversity of the Québec population.

Researches on the introduction and dissemination of monogenic traits in the French Canadian population of Québec have underlined a genetic variability at the regional level [4]. Moreover, the study of the worldwide distribution of single nucleotide polymorphisms (SNP), associated with phenylalanine hydroxylase and alleles at the cystic fibrosis transmembrane conductance regulator and human leukocyte antigen loci point toward a genetic differentiation between the regional populations of the province of Québec [5]. This genetic diversity is also observed for variants in the lipoprotein lipase (LPL) and low-density lipoprotein receptor (LDLR) genes that cause hypertriglyceridemia and familial hypercholesterolemia, respectively [6–10].

Even though the studies described above demonstrate a certain genetic diversity at the regional level, little is known about the distribution of SNP involved in complex traits or biological processes throughout regional populations. Moreover, these studies were limited to the French Canadian component of the Québec population. In the pilot research presented herein, we wanted to verify whether it is possible to identify public health regions at higher risk for common chronic inflammatory diseases such as allergic asthma and cardiovascular diseases (CVD), 2 proinflammatory phenotypes [11]. We selected these 2 common diseases because they have an important morbidity/mortality rate and represent a large proportion of the health expenses in our society. Throughout the world, approximately 300 million persons are asthmatic [12]. For the year 2004, the prevalence in Canada was estimated at 14.1% and the fatality rate at 1.6% [12]. CVD are also spread widely around the world, with 16.7 million deaths each year [13]. CVD account for 36% of the global mortality in Canada [14]. Thus, asthma and CVD are common worldwide and increasingly contribute to the public health burden of diseases. It has been recently proposed that a significant portion of common diseases such as asthma and CVD are the result of common genetic variants, and that susceptibility mutations that occur at a relatively high frequency are responsible for much of the clinical and economic burden of diseases seen today [15–17]. While there is some controversy as to the magnitude of this burden, there are good reasons to expect that at least some of the genetic risks of asthma, CVD and other chronic inflammatory diseases are due to common variants, based on evolutionary arguments and the fact that most of the human genetic variation is common. The list of common variants that cause common diseases is growing steadily. There is a growing list of examples of common variants that predispose to, or are associated with, common inflammatory diseases. Among the significant ones, known to be associated with asthma or CVD, are: interleukin-4 (IL-4) and IgE Fc receptor subunit β (MS4A2), which are known for their involvement in the Th2 pathway of allergic respiratory diseases [18, 19]; the peroxisome proliferator-activated receptor α (PPARα), the LPL and the LDLR genes, which are known for their association with CVD [20, 21]; the apolipoprotein E (APOE) gene known for its pleiotropic influence on CVD and other inflammatory diseases, among which is its ability to inhibit lymphocyte proliferation and activation [22]. Finally, the peroxisome proliferator-activated receptor γ 2 (PPARγ2) and the angiotensin 1 converting enzyme (ACE) genes have been reported to be involved in the inflammatory pathway of both allergic respiratory diseases and CVD, among others [23–25]. Not all of these genes are directly causing inflammation. However, they are all associated with biological processes leading directly or indirectly to chronic inflammation. Their association with inflammatory diseases and their suspected high frequency of allele at risk (FAR; based on several case-control studies) of documented SNP in these genes explain their selection for the purpose of the present study. Although all these genes contribute to the chronic inflammatory disease relative risk, their contribution to the population-attributable risk is not well documented and no population-based samples have yet been used for estimation. By virtue of its stratified random sampling design, the Québec Heart Health Survey (QHHS) cohort contains a mix of individuals from different ethnic backgrounds, with and without disease. It has the power to estimate the prevalence of variants with an FAR of ≥5% in the general population. From the list of genes listed above, we selected 11 common variants which were tested in the present study (table 1). All those SNP are known to be associated with cardiovascular and/or respiratory inflammatory diseases and all are documented to have an FAR higher than 5% in a cohort of diseased people originating from Quebec except IL4-C-589T, LPL-D9N and LPL-N291S, for which we only have data from a Canadian or a Caucasian cohort [6, 8, 9, 36, 42–48]. LPL-D9N and LPL-N291S were selected even if their documented FAR was lower than 5% on the basis of clinical observations (D.G.).

Table 1. References and allelic frequencies for all SNP tested

Genes	Variants	Allelic frequency, % in tested sample
IL-4 [26]	C-589T [27]	11.4
MS4A2 [19, 28]	E237G [29]	2.6
ACE [30, 31]	I/D [32]	55.2
PPARγ2 [33]	P12A [34]	12.2
PPARα [35]	L162V [36]	7.9
LPL [21, 37]	D9N [38]	2.3
	G188E [38]	0[1]
	P207L [38]	0.1[1]
	N291S [38]	1.3[2]
APOE [39]	E2/E3/E4 [39]	9.2/75.7/15.1
LDLR [40, 41]	W66G [42]	0[1]

[1] Those variants were not considered because their allelic frequencies were lower than 2%.

[2] This variant was conserved because in some regions its allelic frequency reached 2%.

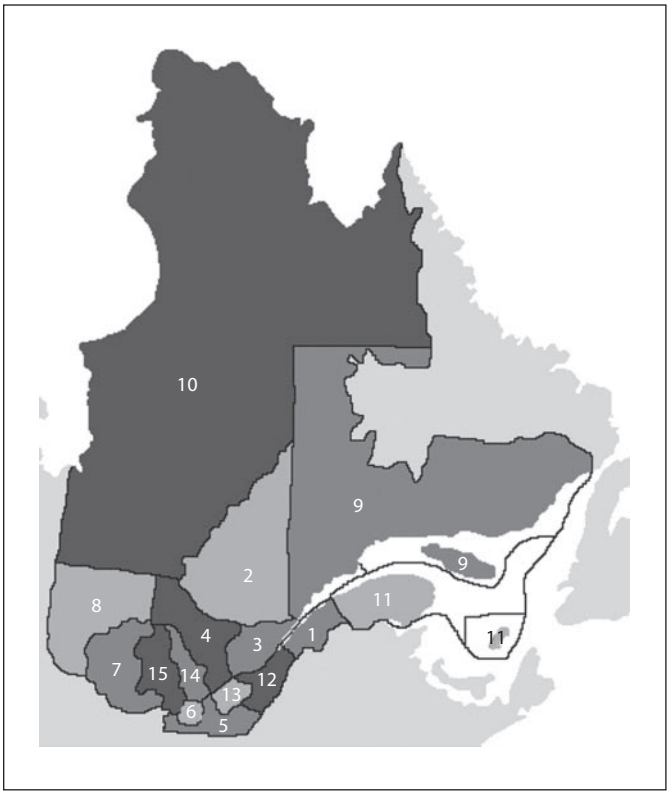

Fig. 1. Public health regions of the province of Québec. This figure shows the public health regions of the province of Québec according to the divisions of 1990, the year in which our samples were collected and classified. 1 = Bas-Saint-Laurent (n = 342, number of alleles given as the size of the cohort for each region); 2 = Saguenay-Lac-Saint-Jean (n = 616); 3 = Québec (n = 340); 4 = Mauricie (n = 258); 5 = Estrie (n = 204); 6 = Montréal (n = 248); 7 = Outaouais (n = 144); 8 = Abitibi-Témiscamingue (n = 158); 9 = Côte-Nord (n = 112); 10 = Nord-du-Québec (n = 70); 11 = Gaspésie-Îles-de-la-Madeleine (n = 104); 12 = Chaudière-Appalaches (n = 112); 13 = Centre-du-Québec (n = 156); 14 = Lanaudière (n = 492); 15 = Laurentides (n = 130).

Obviously our selection of gene variants is not exhaustive and much genetic information has already been collected about asthma and CVD [49, 50]. This study is designed to explore ways of describing genetic heterogeneity, i.e. differences in allele frequency in subpopulations, and geographical clusters in a manner that would be helpful and meaningful for population studies and public health management. Within the limits of our sample, we were able to observe the genetic heterogeneity of the Québec population at the sociogeographical regional level, using the selected variants. Regardless of the bias of the study, we tried to find out if it was possible (a) to group geographical regions on the basis of their allelic structure and (b) to make clusters of 11 selected susceptibility SNP for chronic inflammatory diseases, particularly asthma and CVD, according to their geographical distribution patterns. The primary goal was to evaluate the possibility to model genetic and sociogeographic information at the community and population levels.

Methods

Sample

The sample used in this study is composed of anonymized DNA drawn from 1,680 individuals having participated in the QHHS conducted in 1990 [51] as part of a pan-Canadian survey. The data collection was anonymous and performed with appropriate stratification for age (between 18 and 74 years old) and sex and without selection bias for CVD. The QHHS sampling was representative of the Québec population and covered the 15 Québec public health regions (fig. 1).

DNA Genotyping

DNA was extracted from blood lymphocytes using the guanidine hydrochloride-proteinase K method [52]. Genotyping was done using the standard polymerase chain reaction with the amplification refractory mutation system [53] for C-589T-IL4 and E237G-MS4A2 variants as described by Hill and Cookson [29] and Sandford et al. [48] or with the restriction fragment length polymorphism technique [54] for P12A-PPARγ2, D9N-LPL, G188E-LPL, N291S-LPL, P207L-LPL, I/D-ACE, E2/E3/E4-APOE, W66G-LDLR, L162V-PPARα SNP. The denaturation, annealing and ex-

tension temperature of standard polymerase chain reaction and the quantities for the amplification refractory mutation system or restriction fragment length polymorphism mixes are subject to little changes according to the SNP tested (data not shown).

Data Analysis

For the 11 selected SNP, the FAR was calculated at the provincial level and for each public health region separately. Each public health region corresponds to a territory under the jurisdiction of a regional Board of Health and Social Services. We used the FAR to verify whether the study sample was in Hardy-Weinberg equilibrium in the different public health regions. Three SNP known to be associated with increased cardiovascular risk (G188E-LPL, P207L-LPL and W66G-LDLR) had an FAR lower than 2% in this sample and were not used in subsequent analyses. However, these variants might contribute to the relative and attributable risk of disease in certain regions of the province, particularly the Saguenay-Lac-Saint-Jean and Charlevoix regions [6–10, 55, 56]. The N291S-LPL variant had a provincial FAR of 1.25% but was included in the analysis because its FAR reached 2% in many regions.

Since our starting hypothesis was that genetic pools of all regions were similar, and considering that χ^2 is the best adapted test to compare the FAR, the downward hierarchical clustering analysis (DHCA) was used to study the genetic heterogeneity of the regions of Québec. A contingency table was formed with the FAR obtained for each SNP in each public health region to use the DHCA in order to characterize the distribution patterns of the variants [57]. The principle is to separate the initial group into 2 subgroups on the χ^2 distance basis and to recursively apply the procedure downward until every element forms its own group. It means that regions are first considered as being part of the same group. Then, χ^2 is calculated for each possibility of division of this first group into 2 subgroups and divisions are done until each region is alone. The same method is applied for the division of SNP. At each step of division, the best grouping or cluster is obtained by maximizing the distance over every possible grouping, i.e. using the χ^2 results, the division giving the greater intergroup but the lower ingroup difference for each step of division is selected [57]. The p value is evaluated as an indicator of the relevance of splitting the groups into 2 different subgroups. However, the p value is influenced by the statistical power of this analysis. During the procedure, it is impossible to reconsider previous steps of division. The contingency table is formed by the geographical regions and the SNP FAR, so it can be used to group regions or cluster variants. Analyses were also performed separately for variants known to be specifically involved in respiratory diseases or CVD.

Results

Eight SNP of 11 chosen for their biological relevance, with one or both of the complex diseases selected, were used for statistical analyses. They all had an FAR ranging between 1.3 and 55.2% with a completion rate of 93 ± 5% (table 1). The Hardy-Weinberg equilibrium was tested for conserved SNP in all regions (8 × 15 = 120 combinations). Only 11 combinations were in disequilibrium ($p < 0.05$). Two major reasons can explain the disequilibrium observed for some SNP in certain regions, namely the multiethnicity present in the region or the small size of the cohort representing the region.

The DHCA was then performed and allowed the identification of 3 clusters significantly associated with asthma SNP (fig. 2a): (1) Nord-du-Québec, Gaspésie-Îles-de-la-Madeleine, Québec, Mauricie and Centre-du-Québec; (2) Estrie, Lanaudière and Laurentides; (3) Outaouais, Saguenay-Lac-Saint-Jean, Abitibi-Témiscamingue, Bas-Saint-Laurent, Côte-Nord and Chaudière-Appalaches. Montréal was not included in any group of regions by the DHCA. Applying the same analysis to CVD SNP, 3 different clusters were identified (fig. 2b): (1) Nord-du-Québec, Montréal, Chaudière-Appalaches, Bas-Saint-Laurent and Laurentides; (2) Saguenay-Lac-Saint-Jean and Outaouais; (3) Estrie, Gaspésie-Îles-de-la-Madeleine, Côte-Nord, Centre-du-Québec, Mauricie, Québec and Abitibi-Témiscamingue. The DHCA did not include Lanaudière in any of these groups.

Then, using the DHCA, we clustered together gene variants among the 8 SNP used for statistical analysis that had a similar distribution pattern throughout the regions. This led to the identification of a genetic cluster for asthma (I/D-ACE and P12A-PPARγ2 SNP; fig. 2c) and another one for CVD (I/D-ACE, P12A-PPARγ2, E4-APOE and N291S-LPL and L162V-PPARα variants; fig. 2d).

Discussion

Our results illustrate the genetic heterogeneity of the Québec population at the regional level for common variants associated with chronic inflammatory diseases. This study also suggests that DHCA might be useful for grouping regions and variants on the basis of genetic variability at the population level.

To underline the genetic variability of the population, the sample was divided according to public health areas following the 1990 cardiovascular survey division [58]. To group regions according to their FAR composition and variants on the basis of their distribution pattern, we used the DHCA. With the use of the p values given by the χ^2 distances calculated for each separation step, this analysis is appropriate to obtain groups of similar regions or variants. The downward classification is an exploratory analysis. Therefore, p values should be interpreted with caution and the size of the cohort of each group should be considered.

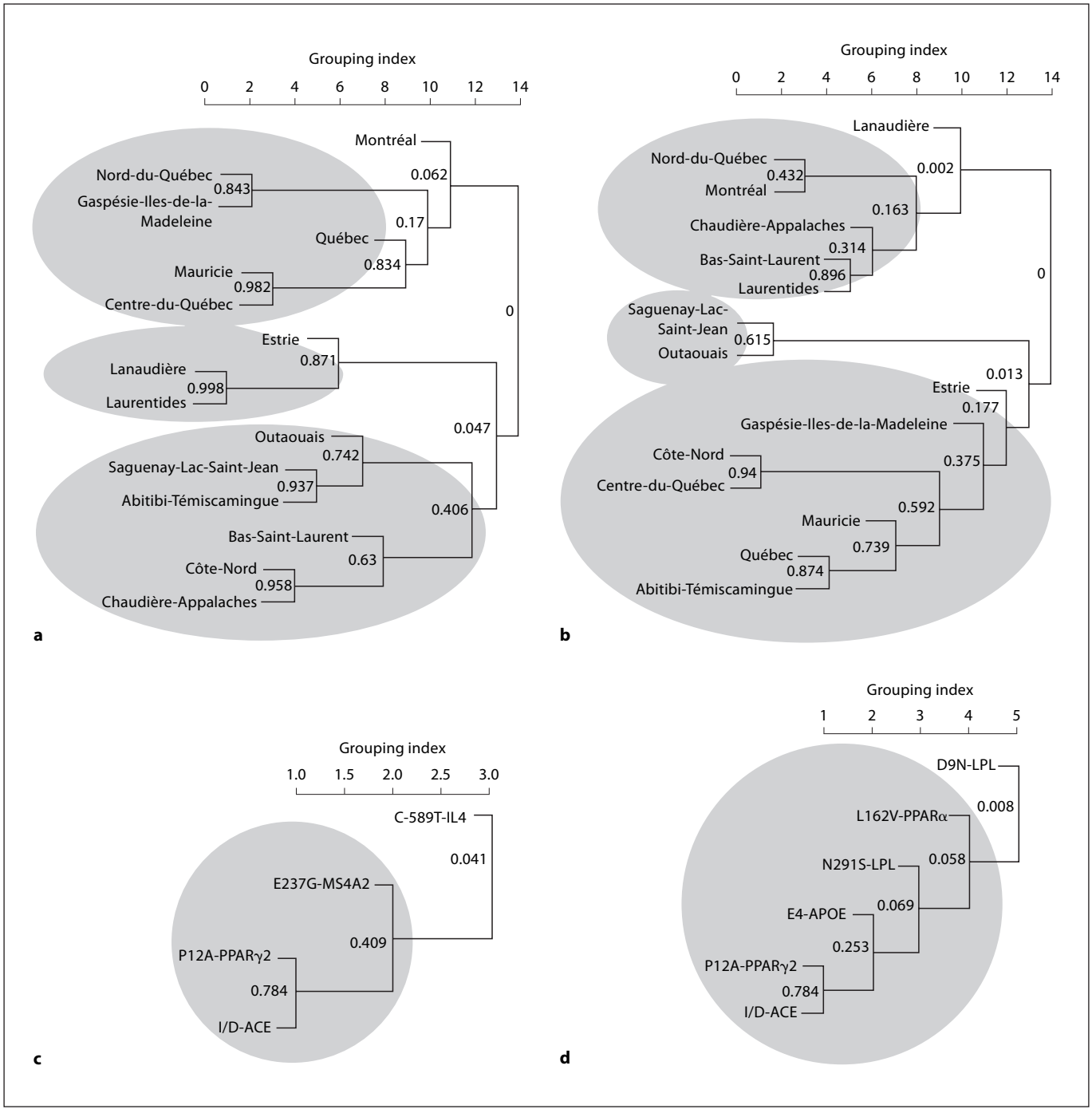

Fig. 2. Groups of the Québec regions according to the allelic frequencies of SNP involved in asthma (**a**) and CVD (**b**) and clusters of SNP involved in asthma (**c**) and CVD (**d**) according to their distribution into the regions of Québec. The 3 groups of regions obtained by the DHCA are not composed by the same regions for asthma (**a**) and CVD (**b**). The presence of most of the regions in each cluster cannot be explained by historical or geographical information. The presence of Mauricie, Centre-du-Québec and Québec in the first cluster, that of Lanaudière and Laurentides in the second one and that of Bas-Saint-Laurent and Chaudière-Appalaches and also Saguenay-Lac-Saint-Jean and Abitibi-Témiscamingue in the third one can be explained by geographic proximity. This figure also illustrates one cluster of variants for asthma (**c**) and CVD (**d**) that have a similar distribution pattern. A part of the cluster of variants for asthma (I/D-ACE and P12A-PPARγ2) is found again in the cluster of CVD variants. All groups of regions and clusters of SNP are separated by $p < 0.05$. The small size of the cohort and, for some SNP (D9N-LPL, N291S-LPL and E237G-MS4A2), the small allelic frequencies decrease the power of this analysis.

Indeed, although DHCA provided interesting and promising results, its potential for population studies and the validity of these results, as applied to the QHHS sample, have limitations due to potential *selection bias*, *regression-dilution bias, population admixture and multiple testing*. The majority of participants in the QHHS represent a population-based sample. It is likely that those who agreed to be screened represent a selected group of 'interested volunteers', and if they are more health conscious than those who did not agree to be screened, our estimates of relative risks may 'underestimate' the true population FAR of inflammatory disease-associated variants. *Selection bias* applies to the criteria used to select the gene variants. The list of selected variants is not exhaustive and the panel only represents a subset of genome variations potentially associated with asthma, CVD or other chronic inflammatory diseases. Indeed, the use of other SNP would produce different groupings. *Regression dilution* is associated with an underestimation of the true association between a given factor and its outcome over time when the classification of individuals is based on only one measurement taken at baseline. Since our analysis is solely based on cross-sectional data, we cannot statistically adjust the FAR coefficients over time. While the population is always in movement, modifying at the same time the genetic pool of regions, the results obtained in this pilot study with the QHHS samples cannot systematically be applied to the present population. *Population stratification* could also occur. This phenomenon is caused by undetected population substructures that can potentially produce a false estimation of the FAR. The way the sample was divided could also produce the same kind of error. In this pilot study the divisions were made according to the public health regions of the 1990 cardiovascular survey, but other kinds of division, for example based on the principal known population movements, would probably give other groupings. Another obstacle in the analysis of the FAR in different regions of a given population is the *multiple testing* problem. In the present study the analysis relies on the number of geographical subregions (n = 15) and variants (n = 8) as well as on the criteria used to define them.

However, the Hardy-Weinberg equilibrium tests demonstrated that only 11 of 120 combinations of the frequency distribution of an SNP in a particular region are in disequilibrium with significant results. This was expected with the level used for the tests and the size of the cohort within each region.

As with historical studies that have underlined the existence of a demographic heterogeneity of founders and epidemiogenetic studies of the contemporary population of Québec, the results obtained with the DHCA reveal the presence of heterogeneity between certain regions or groups of regions [2, 4, 5, 58–60]. We used this analysis to group regions according to the allele frequencies of susceptibility variants for asthma and CVD separately and to detect modeling possibilities.

Taking into consideration major events of Québec settlement history such as the founder effect or geographic proximity may partly explain our findings, but in each of our generated groups the presence of some regions cannot be explained by those factors. A possible explanation is the presence of specific migration movements between regions during settlement processes. For instance, Heyer hypothesized that, considering all demogenetic events that occurred in the Saguenay-Lac-Saint-Jean region (such as differential fecundity and a high birthrate), if only 2% of the first founders coming from Charlevoix (68 individuals) were carriers of the same deleterious gene, this was sufficient to account for the FAR of the common monogenic diseases in today's population [61]. Moreover, with more variants to represent each type of disease, we could probably obtain a better overview of the possible groups of regions. Another explanation could be the size of the cohort. After the distribution of the samples, some regions were represented by a very small cohort (only 70 alleles for the Nord-du-Québec region). This could induce allele frequencies that do not really represent the population of the region and provoke a wrong grouping of regions or SNP. It also decreases the power of this analysis, increasing the chances that the DHCA cannot detect differences between regions.

The DHCA was also performed for the clustering of genetic variants, giving us one cluster for each type of disease. The regional profile of the FAR obtained for each SNP did not validate all p values for each cluster. The lack of power can be a factor because of the small size of the cohort and because the number of low frequencies (under 5%) has a high influence on the χ^2 distance. Like the grouping of regions, the small size of our cohort per region could affect the way in which clusters of variants were made.

The genes selected in our investigation were all associated with an increased risk for asthma and/or CVD [19, 30, 33, 35, 37, 41, 62–64]. Consequently, developing a genetic map of the regional populations of Québec and grouping similar regions could provide a very useful tool to target zones at higher risk in the province and to calculate relative risks, taking into account both environmental and genetic factors. Clustering se-

lected variants with similar distribution patterns is also a way to better describe a relative risk. Moreover, it could make it possible to design a screening procedure that includes susceptibility variants and clustering information.

Conclusions

We found that it is possible to organize a great quantity of data in a comprehensive and practical way based on statistical tools such as the DHCA to model genetic information. The optimal use of genetic information in health care requires an understanding of (1) genetic risk alleles, (2) the distribution of these risk factors in a population and (3) the environmental factors that interact with genetic variants. In the last decade, substantial progress was made to understand highly penetrant monogenic diseases. Conversely, our understanding of the genetics of complex disorders that depend on the contribution of multiple genes and environmental determinants, such as inflammatory diseases, remains limited. The discovery of common risk alleles is consistent with the common disease-common variant hypothesis for complex diseases, which proposes that common disorders are primarily due to the segregation of hundreds or thousands of common genetic variants [65]. Common and rare diseases that have an underlying genetic etiology can systematically be studied with tools afforded by the Human Genome Project and large-scale epidemiological approaches. The latter require an understanding of genetic diversity at the population level. Research initiatives are needed to translate the knowledge on genetic diversity into individual genetic predispositions and collectively into determinants of public health. Furthermore, they will allow the integration of genomics into medical services, health promotion and the prevention of diseases.

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