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GENETIC SUSCEPTIBILITY TO THE METABOLIC SYNDROME

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Résumé

Le syndrome métabolique est caractérisé par un regroupement de facteurs de risque présents chez un même individu et augmentant ainsi ses chances de développer le diabète de type 2 et les maladies cardiovasculaires. Il est donc important de comprendre l'étiologie génétique de ce trait. Dans cette thèse, une multitude d'approches génétiques ont été utilisées afin d'apporter un brin de connaissance sur l'architecture génétique du syndrome métabolique et de ses composantes individuelles. Trois gènes candidats ont été testés incluant le récepteur activé par les proliférateurs de péroxisomes (PPAR) α et PPARγ ainsi que la protéine de transfert des phospholipides (PLTP). Les gènes PPARα et PLTP ont tous deux été associés significativement avec plusieurs variables d'adiposité. Des effets significatifs d'interaction entre les gènes PPARα et PPARγ ont été obtenus pour les paramètres de glucose et d'insuline. Il a aussi été démontré que le polymorphisme PPARα L162V influence les changements de cholestérol-HDL_2 suite à un traitement au gemfibrozil. Par la suite, des criblages génomiques ont été effectués sur les concentrations de lipides et de lipoprotéines plasmatiques. Plusieurs régions chromosomiques ont été identifiées incluant 1q43, 11q13-q24, 15q26.1, et 19q13.32 pour le cholestérol-LDL, 12q14.1 pour le cholestérol-HDL, 2p14, 11p13, et 11q24.1 pour les triglycérides, 18q21.32 pour l'apolipoprotéine (apo) B-LDL, et 3p25.2 pour l'apoAI. La contribution génétique à la variation du diamètre principal des particules LDL (DP-LDL) a aussi été étudiée. Les résultats démontrent une forte ressemblance familiale avec des coefficients d'héritabilité de plus de 50%, la présence d'un gène à effet majeur, et une forte évidence de liaison sur le chromosome 17q. Le gène de l'apoH, localisé à cet endroit, a par la suite été significativement associé au DP-LDL, suggérant que ce gène est responsable du signal de liaison observé sur le chromosome 17. Finalement, une variable quantitative du syndrome métabolique a été construite à l'aide d'une analyse factorielle. Un criblage génomique effectué sur cette variable a démontré une évidence de liaison sur le chromosome 15q, suggérant la présence d'un gène à cet endroit contribuant au regroupement des facteurs de risques caractérisant le syndrome métabolique. Plusieurs de ces résultats devront être répliqués, alors que d'autres méritent d'être suivis.

Abstract

The metabolic syndrome is a cluster of interrelated cardiovascular risk factors co-occurring in the same individual. People with this syndrome are at increased risk for developing diabetes mellitus and cardiovascular diseases. Accordingly, it is important to elucidate the genetic aetiology governing this trait in order to better comprehend its pathogenesis. In the present thesis, heritability and complex segregation analyses as well as candidate gene and genome-wide scan approaches have been applied to shed some lights on the genetic architecture of the metabolic syndrome and its individual components. A total of three candidate genes have been investigated including peroxisome proliferator-activated receptor (PPAR) α and PPARγ as well as phospholipid transfer protein (PLTP). It has been shown that polymorphisms in both PPARα and PLTP genes are significantly associated with several indices of adiposity. In addition, significant gene-gene interactions have been observed between PPARα and PPARγ on glucose/insulin parameters. It has also been shown that the HDL_2-cholesterol response to gemfibrozil therapy is modulated by the PPARα L162V polymorphism. Genome-wide linkage scans have been performed on lipid and lipoprotein concentrations. Many chromosome regions harbouring lipoprotein/lipid genes have been identified including 1q43, 11q13-q24, 15q26.1, and 19q13.32 for LDL-cholesterol, 12q14.1 for HDL-cholesterol, 2p14, 11p13, and 11q24.1 for triglycerides, 18q21.32 for LDL-apolipoprotein (apo) B, and 3p25.2 for apoAI. The genetic contribution of the variation in LDL peak particle diameter (LDL-PPD) has been also investigated. Overall, the results indicate: 1) that LDL-PPD strongly aggregates within families with heritability estimate above 50%; 2) the existence of a major gene effect affecting the phenotype; and 3) the presence of a major quantitative trait locus located on chromosome 17q. The apo H gene, a positional candidate gene, was then significantly associated with LDL-PPD, suggesting that this gene is responsible for the linkage signal observed on 17q. Finally, factor analyses have been used to construct a quantitative metabolic syndrome variable and a genome-wide linkage scan has been conducted to identify the genomic regions underlying this trait. A major quantitative trait locus has been observed on chromosome 15q suggesting a gene within this region contributing to the clustering of the metabolic syndrome-related phenotypes. Many of these findings must go through independent replication, while others produced new leads that deserve follow-up.

Avant-Propos

La présente thèse est constituée de dix manuscrits sous la forme d'articles scientifiques en plus d'une introduction et d'une conclusion générale sur la susceptibilité génétique au syndrome de résistance à l'insuline. Les trois premiers manuscrits traitent d'un polymorphisme dans le gène du récepteur activé par les proliférateurs des péroxysomes alpha (PPARα) et de : 1-son rôle sur les effets métaboliques d'un traitement au fibrate; 2-son association avec les mesures d'adiposité; et 3-son effet d'interaction avec un polymorphisme dans un second gène impliqué dans l'homéostasie du glucose et de l'insuline. Le quatrième manuscrit rapporte l'association significative obtenue entre des variations génétiques dans le gène de la protéine de transfert des phospholipides (PLTP) et les mesures d'adiposité. Les cinquième et sixième articles sont des criblages génomiques effectués sur les niveaux de lipides, lipoprotéines et apolipoprotéines plasmatiques permettant ainsi d'identifier les régions chromosomiques contenant les gènes impliqués dans la variabilité inter-individuelle de ces phénotypes. Les trois manuscrits suivants traitent de la génétique de la taille des lipoprotéines de faible densité (LDL). Ainsi le septième quantifie la contribution génétique de la taille des particules LDL, le huitième identifie les régions chromosomiques contenant les gènes en cause et le neuvième permet d'identifier un gène significativement associé avec le phénotype. Finalement, le dernier manuscrit combine analyse factorielle et criblage génomique afin d'identifier les régions chromosomiques contenant les gènes du syndrome métabolique.

Tous les articles présentés dans cette thèse sont publiés ou soumis pour publication. Le tableau ci-dessous indique l'état de chacun des manuscrits et leur référence. Une partie de l'introduction et de la conclusion a aussi fait l'objet d'un article de synthèse publié dans la revue « Journal of Lipid Research 2004; 45 :1008-1026. ».

Chapitre 1

Bossé Y, Pascot A, Dumont M, Brochu M, Prud'homme D, Bergeron J, Després JP, Vohl MC. Influences of the PPARα-L162V polymorphism on plasma HDL_2-cholesterol response of abdominally-obese men treated with gemfibrozil. Genetics in Medicine 2002; 4 (4) : 311-5.

Chapitre 2

Bossé Y, Després JP, Bouchard C, Pérusse L, Vohl MC. The peroxisome proliferator-activated receptor α L162V mutation is associated with reduced adiposity. Obesity Research 2003; 11 (7): 809-16.

Chapitre 3

Bossé Y, Weisnagel JS, Bouchard C, Després JP, Pérusse L, Vohl MC. Combined effects of PPARγ$_2$ P12A and PPARα L162V polymorphisms on glucose and insulin homeostasis: the Québec Family Study. Journal of Human Genetics 2003; 48 (12) : 614-21.

Chapitre 4

Bossé Y, Bouchard L, Després JP, Bouchard C, Pérusse L, Vohl MC. Haplotypes in the phospholipid transfer protein gene are associated with obesity-related phenotypes: The Québec Family Study. International Journal of Obesity (soumis).

Chapitre 5

Bossé Y, Chagnon YC, Després JP, Rice T, Rao DC, Bouchard C, Pérusse L, Vohl MC. Genome-wide linkage scan reveals multiple susceptibility loci influencing lipid and lipoprotein levels in the Québec Family Study. Journal of Lipid Research 2004; 45 (3): 419-26.

Chapitre 6

Bossé Y, Chagnon YC, Després JP, Rice T, Rao DC, Bouchard C, Pérusse L, Vohl MC. What have we learned from genomewide scans on lipid-related phenotypes so far? Fixing perspective with a new genomewide search on apolipoprotein levels in the Québec Family Study. Journal of Lipid Research (sous presse).

Chapitre 7

Bossé Y, Vohl MC, Després JP, Lamarche B, Rice T, Rao DC, Bouchard C, Pérusse L. Heritability of LDL peak particle diameter in the Québec Family Study. Genetic Epidemiology 2003; 25 : 375-81.

Chapitre 8

Bossé Y, Pérusse L, Després JP, Lamarche B, Chagnon YC, Rice T, Rao DC, Bouchard C, Vohl MC. Evidence for a major quantitative trait locus on chromosome 17q21 affecting LDL peak particle diameter. Circulation 2003; 107 : 2361-8.

Chapitre 9

Bossé Y, Feitosa MF, Després JP, Lamarche B, Rice T, Rao DC, Bouchard C, Pérusse L, Vohl MC. Is the major gene effect for LDL peak particle diameter on 17q caused by the apolipoprotein H gene. Atherosclerosis (soumis).

Chapitre 10

Bossé Y, Després JP, Chagnon YC, Rice T, Rao DC, Bouchard C, Pérusse L, Vohl MC. Genome-wide linkage scan for the metabolic syndrome reveals a major quantitative trait locus on chromosome 15q: The Quebec Family Study. Diabetes (soumis).

Les dix manuscrits ont été réalisés en collaboration avec de précieux collaborateurs. Les résultats rapportés au premier chapitre proviennent des données de l'étude « Gemfibrozil-Exercice-Lipides » (GEL). Les Drs Denis Prud'homme et Jean-Pierre Després sont les principaux investigateurs de cette étude. Le Dr Martin Brochu et Mme Martine Dumont ont assuré la coordination et la réalisation de l'étude GEL. Le Dr Jean Bergeron et le Dr Marie-Claude Vohl étaient pour leur part responsables de la mesure des concentrations plasmatiques des lipides et des lipoprotéines et du génotypage du polymorphisme PPARα L162V, respectivement. Finalement, le Dr Agnès Pascot a réalisé les mesures de la taille des particules HDL. Pour les neuf autres études les données ont été obtenues à partir de l'Étude des Famille de Québec (QFS). Les Drs Claude Bouchard, DC Rao, Louis Pérusse, Jean-Pierre Després, Treva Rice et Marie-Claude Vohl ont agit comme principaux investigateurs de cette étude. Le Dr Pérusse a particulièrement été impliqué dans les analyses génétiques présentées aux chapitres 5, 6, 7, 8 et 10. L'expertise du Dr John S Weisnagel sur l'homéostasie de l'insuline et du glucose et du Dr Benoît Lamarche sur la taille des particules de LDL ont été d'une grande utilité au chapitre 3 et aux chapitres 7, 8 et 9, respectivement. Au chapitre 4, M. Luigi Bouchard était responsable du génotypage des polymorphismes du gène de la PLTP. Le Dr Yvon C Chagnon était en charge du

génotypage des marqueurs génétiques nécessaires à la réalisation des criblages génomiques présentés aux chapitres 5, 6, 7 et 10. Finalement, Mme Mary F Feitosa a réalisé les analyses de ségrégation complexe présentées au chapitre 9. Tous les co-auteurs ont également participé activement à la révision des manuscrits. À noter aussi que le Dr Marie-Claude Vohl a supervisé plusieurs étapes du génotypage et du séquençage de même que la réalisation et la préparation de tous les manuscrits. À titre de premier auteur de ces articles, j'ai été impliqué dans toutes les étapes de production. Pour les tâches de laboratoire (cueillette des données), j'ai séquencé le gène de la PLTP et de l'APOH présenté aux chapitres 4 et 9, respectivement. J'ai aussi mesuré la taille des particules LDL dans l'étude QFS, qui a fait l'objet des chapitres 7, 8 et 9. Pour les analyses et l'interprétation des résultats, j'ai effectué la grande majorité des analyses statistiques et synthétisé le contenu. Par la suite, j'ai conceptualisé et rédigé la totalité des manuscrits. J'étais aussi en charge de la soumission et la révision des articles sous les conseils de ma directrice de thèse. Aucune modification des articles originaux n'a été effectuée dans la thèse.

Il est impossible de réaliser un projet de cette envergure sans le soutien nécessaire sur le plan technique et moral. Je profite donc de cette section pour remercier les gens qui de près ou de loin ont contribué à cet ouvrage.

Tout d'abord, je dois remercier ma directrice de recherche, Marie-Claude Vohl, pour l'environnement de travail privilégié qu'elle m'a offert tout au long de mes études de troisième cycle. Son encadrement a été indispensable à la réalisation de ce projet et a par le fait même assuré la productivité de celui-ci. Marie-Claude a toujours traité mes dossiers avec priorité. Je veux donc la remercier pour sa disponibilité et la rapidité avec laquelle elle corrigeait mes travaux. Je veux également remercier Marie-Claude pour la confiance et l'attention qu'elle m'a accordées durant mes années au doctorat.

Je tiens également à remercier le Dr Louis Pérusse pour ses idées et conseils expérimentés. Ses connaissances techniques et théoriques ont été essentielles pour plusieurs parties de ce projet. Sur une base plus personnelle, je veux remercier le Dr Pérusse pour sa disponibilité et l'intérêt qu'il a porté envers mes travaux. De part nos échanges et ses qualités de pédagogue, il a été un mentor important pour moi. Il est clair que le Dr Pérusse, avec le Dr Vohl, ont contribué de façon importante à développer en moi cette passion pour la génétique.

Je désir aussi remercier mon co-directeur de recherche, le Dr Jean-Pierre Després. Dr Després a contribué de façon importante au développement de la recherche à Québec sur les complications métaboliques et cardiovasculaires de l'obésité. Grâce à ses efforts, des ressources impressionnantes sont maintenant à la disposition d'un groupe de recherche qui s'impose au niveau international. Ce milieu de recherche permet ainsi aux jeunes chercheurs comme moi de se réaliser. De plus, les connaissances et l'expérience du Dr Després ont été très utiles dans la totalité des manuscrits présentés dans cette thèse.

Il y a également d'autres personnes que je tiens à remercier. Tout d'abord, les coauteurs pour leur contribution respective mentionnée ci-dessus. Leurs corrections et commentaires pour chacun des manuscrits ont aussi considérablement amélioré le texte et le contenu de ceux-ci. Durant mes trois dernières années, j'ai aussi bénéficié de l'aide et du support de toute l'équipe du Dr Vohl. Je veux mentionner particulièrement l'aide technique de M. Alain Houde qui m'a permis de surmonter les obstacles de laboratoire.

Je souhaite également remercier les membres de ma famille pour leur soutient et les encouragements constants. M'man et P'pa ont toujours été fiers de moi et m'ont supporté dans tout ce que j'ai entrepris. J'ai la chance d'avoir des parents exceptionnels et j'en suis grandement reconnaissant. Mon frère Ynuk et mes deux soeurs Anik et Heidi sont aussi des personnes extrêmement précieuses à mes yeux. La présence et l'amour de ma famille m'ont toujours donné la force de continuer et d'atteindre mes objectifs. Finalement, Mélanie qui m'appuie au quotidien. Je ne sais pas comment la remercier considérant les sacrifices, incluant changements de carrière et de villes, qu'elle a dû subir pour que l'on puisse partager notre vie ensemble. Sa présence auprès de moi est inestimable et je me considère extrêmement chanceux d'avoir une conjointe comme Mélanie.

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Introduction

The Metabolic Syndrome

Definition

In 1988, Reaven noted that several risk fators commonly cluster together under one disorder entity that he originally described as syndrome X[1]. This syndrome has been characterized by the co-occurrence of hypertension, some degree of glucose intolerance, high triglyceride levels and low high-density lipoprotein (HDL) concentrations. The basic abnormalities underlying syndrome X have been presented as the resistance of insulin to mediate glucose disposal. Due to this underlying pathophysiology, many authors have also used the term insulin resistance syndrome to define this aggregation of risk factors. The syndrome has also been given other names, including the metabolic syndrome, the plurimetabolic syndrome and the deadly quartet. More recently, the National Cholesterol Education Program's Adult Treatment Panel III report (NCEP ATP III) has recognized the importance of this syndrome in the prevention of cardiovascular disease (CVD)[2]. However, NCEP ATP III, used the term metabolic syndrome for this clustering of metabolic risk factors. This term avoids the implication that insulin resistance is the primary or the only cause of associated risk factors. Since the description of the syndrome by Reaven[1], several other metabolic abnormalities have been associated with it, including obesity, particularly abdominal obesity, high apolipoprotein (apo) B levels, small dense low-density lipoprotein (LDL), and abnormalities in fibrinolysis and coagulation[3].

At least three health authorities have provided practical tools to identify patients with the metabolic syndrome. However, the clinical criteria differ somewhat between organizations[2,4,5]. Tables 1, 2 and 3 summarize the criteria used by the NCEP ATP III, the World Health Organization (WHO) and the American Association of Clinical Endocrinologists (AACE) to clinically identify the metabolic syndrome. For NCEP ATP III, when 3 out of the 5 characteristics listed in Table 1 are present, a diagnosis of metabolic syndrome can be made. It is the only guideline that considers waist circumference to express the level of adiposity. In the NCEP ATP III an explicit demonstration of insulin resistance is not required. In contrast, the WHO guidelines view insulin resistance as a required component for diagnosis. In addition to insulin resistance, two other risk factors are required for the diagnosis of the metabolic syndrome.

Microalbumineria has been also added to the list as a criterion. The AACE criteria seem to be a compromise between the NCEP ATP III and the WHO guidelines. Although the clinical criteria are listed, the number of risk factors required to claim the existence of the metabolic syndrome is not specified and left to clinical judgment. Both AACE and WHO guidelines included insulin resistance measurements that are beyond routine clinical assessment. Indeed, for these two authorities, values derived from an oral glucose tolerance test are among the risk factors for the metabolic syndrome. Although these measurements give additional information, they add time and cost to clinical practice. Therefore, the NCEP ATP III guidelines may be more suitable on a clinical basis.

Table 1. NCEP ATP III clinical criteria for the metabolic syndrome.

Risk Factors	Defining Level
Abdominal obesity, given as waist circumference	
Men	>102 cm (>40 in)
Women	>88 cm (>35 in)
Triglycerides	≥150 mg/dL (≥1.7 mmol/L)
HDL cholesterol	
Men	<40 mg/dL (<1.0 mmol/L)
Women	<50 mg/dL (<1.3 mmol/L)
Blood pressure	≥130/≥85 mm Hg
Fasting glucose	≥110 mg/dL (≥ 6.1 mmol/L)

Derived from NCEP ATP III[2]

Table 2. WHO clinical criteria for the metabolic syndrome.

Insulin resistance, identified by 1 of the following:
• Type 2 diabetes
• Impaired fasting glucose
• Impaired glucose tolerance
• Or for those with normal fasting glucose levels (<110 mg/dL), glucose uptake below the lowest quartile for background population under investigation under hyperinsulinemic, euglycemic conditions

Plus any 2 of the following:

- Antihypertensive medication and/or high blood pressure (≥140 mm Hg systolic or ≥90 mm Hg diastolic)
- Plasma triglycerides ≥150 mg/dL (≥1.7 mmol/L)
- HDL cholesterol <35 mg/dL (<0.9 mmol/L) in men or <39 mg/dL (1.0 mmol/L) in women
- BMI >30 kg/m^2 and/or waist:hip ratio >0.9 in men, >0.85 in women
- Urinary albumin excretion rate ≥20 μg/min or albumin:creatine ratio ≥30 mg/g

Derived from WHO[4]

Table 3. AACE clinical criteria for the metabolic syndrome.

Risk Factor Components	Cutpoints for abnormality
Overweight/obesity	BMI ≥ 25 kg/m^2
Elevated triglycerides	≥150 mg/dL (1.7 mmol/L)
Low HDL choleterol	
Men	<40 mg/dL (1.0 mmol/L)
Women	<50 mg/dL (1.3 mmol/L)
Elevated blood pressure	≥130/85 mm Hg
2-Hour postglucose challenge	>140 mg/dL
Fasting glucose	Between 110 and 126 mg/dL
Other risk factors	Family history of type 2 diabetes, hypertension, or CVD, polycystic ovary syndrome, sedentary lifestyle, advancing age, ethnic groups having high risk for type 2 diabetes or CVD

Derived from Einhorn et al.[5]

These working definitions of the metabolic syndrome have allowed the prevalence of this condition to be estimated in the population. Based on the NCEP ATP III definition, the prevalence of the metabolic syndrome has been established in the Third National Health and Nutrition Examination Survey (NHANES III). Overall, the unadjusted prevalence of the metabolic syndrome was approximately 22% in this US adult population. However, the prevalence of this condition increases with age both in men and women to reach almost 45% for subjects aged above 60 years (Figure 1). Considering the epidemic of obesity[6], these numbers are

very likely to be higher today compared to the estimates derived from 1988-1994 NHANES III survey. Accordingly, a large proportion of adults are affected by the metabolic syndrome. This condition is thus a rapidly growing threat to public health and a major challenge that physicians and public health agencies must face.

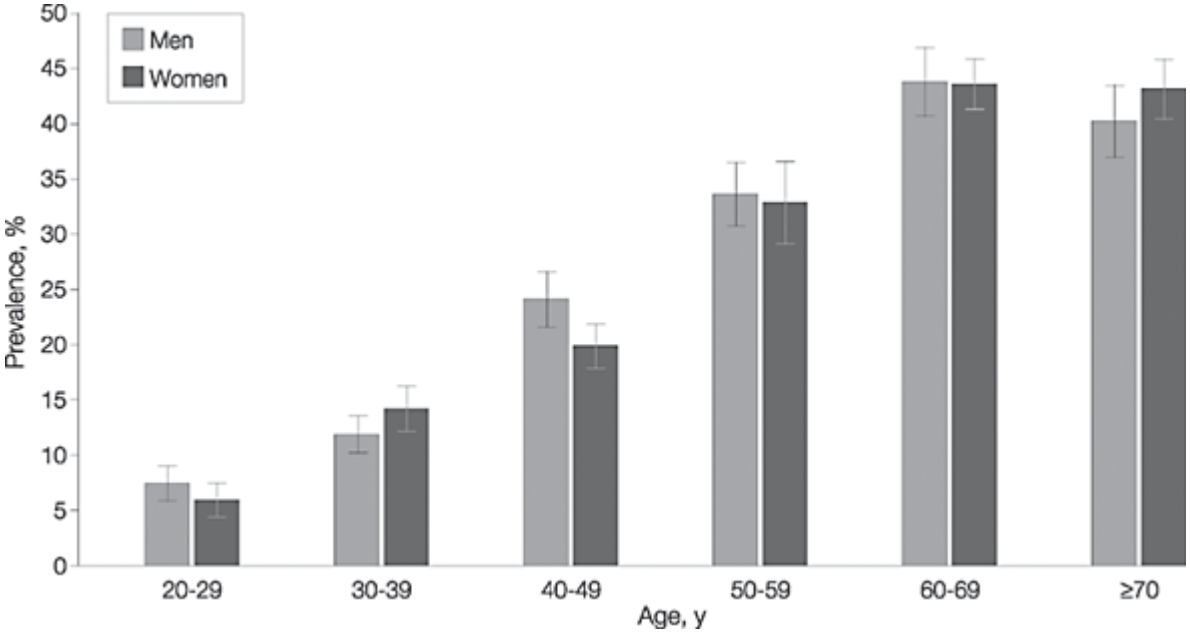


Figure 1. Age-specific prevalence of the metabolic syndrome among 8814 US adults aged at least 20 years, by sex, National Health and Nutrition Examination Survey III, 1988-1994. Taken from Ford et al.[7]

Pathogenesis of the Metabolic Syndrome

The pathogenesis of the metabolic syndrome is complex and is a direct consequence of the interactions between the effects of many susceptibility genes and many environmental exposures. The complexity can be even more appreciated by considering that each component feature of the metabolic syndrome is subjected to its own regulation through both genetic and environmental factors. Changes in lifestyle observed during the last decades are responsible for the rising prevalence of the metabolic syndrome. Indeed, physical inactivity combined with an atherogenic diet (rich in saturated fat, trans fatty acids and refined sugars) constitute the perfect combination giving rise to the metabolic syndrome. These lifestyle variables, acting either through obesity or insulin sensitivity, disturb the metabolic homeostasis and lead to the multiplex risk factors.

The National Heart, Lung, and Blood Institute (NHLBI), in collaboration with the American Heart Association (AHA) identified three potential etiologic categories of the metabolic sydrome: 1- obesity and disorders of adipose tissue, 2- insulin resistance, and 3- a constellation of independent factors (eg, molecules of hepatic, vascular, and immunologic origin) that mediate specific components of the metabolic syndrome[8]. Some investigators place greater priority to obesity, more specifically visceral obesity, to explain the clustering of risk factors. This argument is supported by the fact that obesity is strongly associated with all cardiovascular risk factors[9,10]. In fact, with the strong connection between visceral obesity and risk factors, it is possible to define the metabolic syndrome as a cluster of the metabolic complications associated with obesity. Adipose tissue is now recognized as an endocrine organ that secretes numerous proteins that exert various effects[11]. Indeed, hyperplasia and hypertrophy of adipocytes as seen in obesity leads to an increased production of leptin, tumour necrosis factor α, interleukin-6, resistin, acylation-stimulating protein and many other proteins, and a decreased production of adiponectin. Visceral adipose tissue may be particularly active in producing several of these factors. Through these mechanisms, it is clear that obesity play a central role in the pathogenesis of the metabolic syndrome. The role of obesity in the metabolic syndrome is also accentuated by the benefits observed on all its components following weight loss[12] (Figure 2). Indeed, there is substantial evidence that weight loss, particularly the mobilisation of visceral adipose tissue, leads to simultaneous improvements of the metabolic profile. Taken together, these arguments place obesity, more specifically visceral obesity, at the heart of the metabolic syndrome.

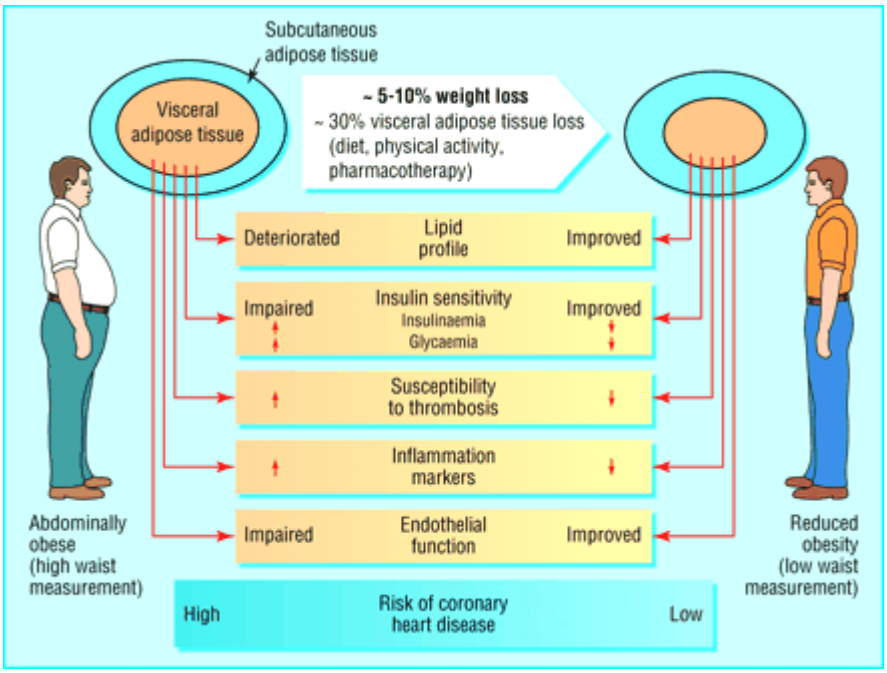


Figure 2. Potential benefits of moderate (5-10%) weight loss in high risk patients with a cluster of atherothrombotic, pro-inflammatory metabolic abnormalities associated with hypertriglyceridaemic waist. Taken from Després et al.[12].

Despite these facts, there are still disagreements as to whether insulin resistance or abdominal obesity is the primary contributor to the metabolic syndrome. It is true that there is a broad range of insulin sensitivity at any given level of body fat and a large spectrum of obesity at any given level of insulin sensitivity[13,14]. This also means that not all insulin-resistant individuals are overweight nor all overweight individuals are insulin resistant. Some investigators place a greater priority on insulin resistance by arguing that insulin resistance/hyperinsulinemic individuals, with or without obesity, are more likely to display the abnormalities of the metabolic syndrome[15]. They believe that insulin resistance or hyperinsulinemia directly causes other metabolic risk factors. One point of agreement is that insulin resistance generally increases with body fat content[14]. On this point, it was proposed that obesity should be viewed as a lifestyle variable that has an adverse effect on insulin-mediated glucose disposal thereby increasing the chance that abnormalities associated with the metabolic syndrome will develop[15]. However, in contrast to weight loss, there is yet little evidence that reduction in insulin resistance will improve components of the metabolic syndrome other than glucose intolerance. Clearly, the dissociation between obesity and insulin resistance is difficult to make because both are associated with the

metabolic abnormalities. It is also obvious that both factors can play an independent role in the syndrome giving their independent effects on cardiovascular risk factors[16] and CVD[13]. One thing is for sure: the rising prevalence of the metabolic syndrome is certainly propeled by the epidemic of obesity, which is driven by changeable factors such as high caloric diets and sedentary lifestyle.

CVD risks associated with the metabolic syndrome

The primary clinical outcome of the metabolic syndrome is CVD. However, it is also worth mentioning that individuals with this syndrome have an increased risk for type 2 diabetes[17], which is also a high-risk condition for CVD. It seems obvious that a condition characterized by multiple risk factors would increase the risk of CVD. However, few studies have examined the relation between the metabolic syndrome and future development of CVD. In the Kuopio Ischemic Heart Disease Risk Factor Study, a population-based prospective study of 1209 Finnish men, it has been demonstrated that men with the metabolic syndrome are at increased risk of CVD and all-cause mortality[18]. Similar results were obtained in the Botnia study including 4483 subjects participating in a family study of type 2 diabetes in Finland and Sweden[19]. In the Framingham study, the metabolic syndrome alone, without diabetes, has been associated with a ten-year risk of CVD that ranged from 10% to 20% in men and that did not exceed 10% in women[8]. In addition, no gain in CVD risk assessment is obtained when the NCEP ATP III metabolic syndrome is added to the usual Framingham risk assessment algorithm[20]. This suggests that the risk associated with the metabolic syndrome is captured by the traditional risk factors including age, blood pressure, total cholesterol, diabetes and HDL cholesterol. However, the metabolic syndrome was highly predictive of new-onset diabetes in the Framingham cohort.

Large prospective studies such as Framingham conducted in the United States[20] and the Prospective Cardiovascular Münster (PROCAM) study conducted in Germany[21] have provided simple algorithms to predict the risk of CVD in originally asymptomatic individuals. These studies have greatly contributed to the identification of the major risk factors of CVD such as age, smoking, diabetes, hypertension and plasma LDL and HDL cholesterol concentrations. In addition, several lipid-lowering trials have clearly demonstrated the importance of targeting LDL cholesterol in order to reduce the risk of CVD[22,23]. For this reason, LDL cholesterol has become the primary target of therapy to reduce the risk of CVD[2]. However, a considerable proportion of patients with CVD have cholesterol levels in the normal range[24,25] and a notable proportion of patients achieving significant LDL reduction with lipid lowering therapy still develop CVD[26]. These results suggest that there is a need to go beyond LDL reduction and traditional risk factors in order to properly identify high risk individuals.

The Quebec Cardiovascular Study has contributed substantially in finding new markers of risk that allow a more refined identification of individuals at high risk for CVD. In the Quebec Cardiovascular Study, more than 2000 men initially free of ischemic heart disease (IHD) have been followed for a period of five years. During that period, 114 of them developed IHD. During the years, investigators of this population-based cohort have taken advantage of this setting to identify non-traditional risk factors involved in IHD. In 1997, Lamarche et al.[27] measured LDL peak particle diameter (LDL-PPD) in 103 case-control pairs to determine whether the LDL size can predict the risk of IHD. Despite the lack of difference in the mean LDL-PPD between case patients and control subjects, a clear shift in the distribution of LDL-PPD was observed between the two groups (Figure 3). In fact, the distribution of LDL-PPD in men who developed IHD during the follow-up tended to be shifted toward lower values compared with the control subjects. It has also been demonstrated that men in the first tertile of LDL-PPD distribution had a 3.6 fold increase in the risk of IHD compared with those in the third tertile. This effect was also independent of lipid variables including LDL cholesterol, triglyceride, HDL cholesterol, and apolipoprotein B concentrations. This important work clearly demonstrated that new cardiovascular risk factors can improve risk evaluation.

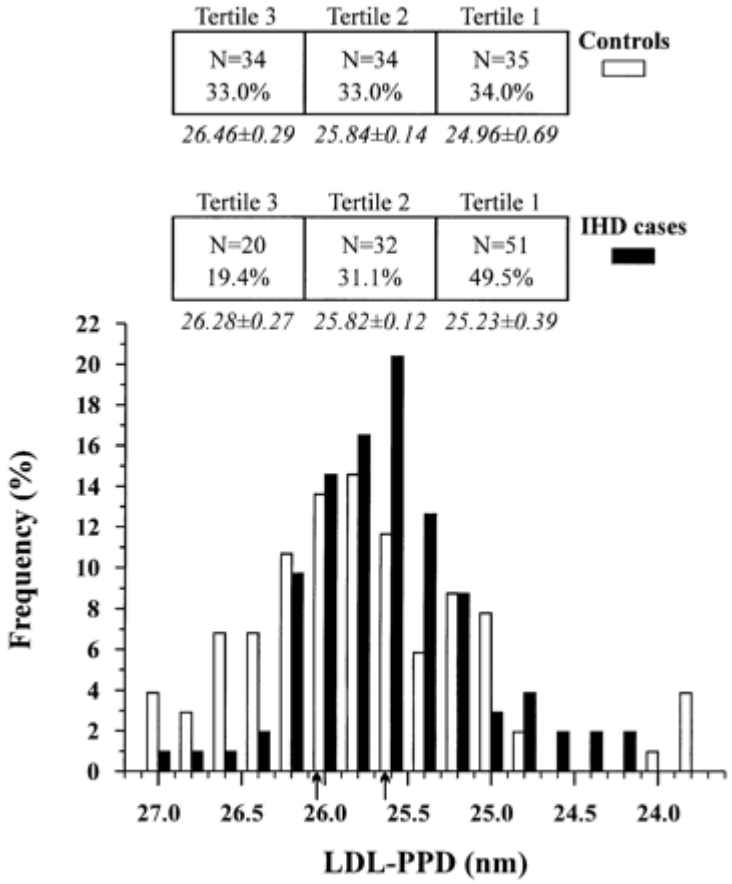


Figure 3. Frequency distributions of LDL-PPD in 103 pairs of case patients (solid bars) and control subjects (open bars). Also presented is the proportion of case patients in each tertile of the LDL-PPD distribution of control subjects and their corresponding mean LDL-PPD (± SD). Arrows on the x axis identify the tertile values of control subjects (25.64 and 26.05 nm). Taken from Lamarche et al.[27].

In the same cohort, it has also been demonstrated that hyperinsulinemia[28] and apoB levels[29] are independent predictors of IHD. Taken together, it suggests that small, dense LDL, insulin and apoB levels can provide substantial information for assessment of IHD. In 1998, Lamarche et al.[30] published another important work demonstrating that these three variables, the so-called metabolic triad of non-traditional risk factors, give substantially more information on the risk of IHD compared with the information provided by conventional lipid variables (Figure 4). Indeed, the risk of IHD was significantly increased in men who had elevated fasting plasma insulin and apoB levels and small, dense LDL particles, compared with men who had normal levels for these three risk factors. It is also worth mentioning that adjustment for traditional lipid variables did not

attenuate this relationship. Figure 4 also highlights the superiority of non-traditional risk variables over traditional variables to discriminate individuals at high risk for IHD.

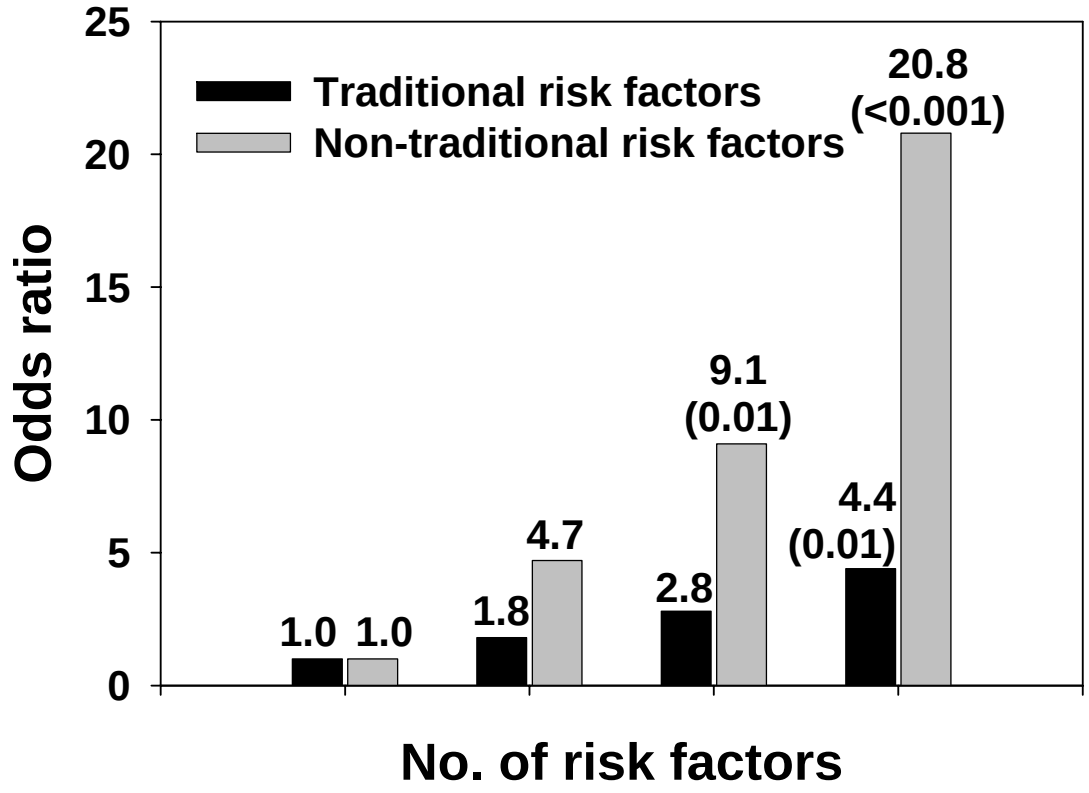


Figure 4. Risk of ischemic heart disease (IHD) according to the cumulative number of traditional and non-traditional risk factors. Traditional factors are LDL cholesterol, triglycerides and HDL cholesterol. Non-traditional factors are insulin, apoB and small, dense LDL particles. Odds ratios are adjusted for systolic blood pressure, family history of IHD and medication use. Taken from Lamarche et al.[30].

Despite being a powerful tool to predict the risk of IHD, the utility of the metabolic triad is somewhat limited due to the costs associated with the measurements of insulin, apoB, and LDL size. Indeed, these new metabolic risk markers are not evaluated in a standard clinical visit and require additional costs. In addition, not all laboratories can perform such measurements. Unfortunately, these factors constitute major barriers for the use of these non-traditional risk

factors in a clinical setting. Therefore, Lemieux et al.[31] have developed a simple screening tool for the identification of men characterized by the metabolic triad (Figure 5). By using simple measurements such as waist circumference and triglyceride levels they have been able to identify the majority of subjects with the metabolic triad. This screening tool has been called the hypertriglyceridemic waist and provides a simple and inexpensive approach to identify high-risk patients.

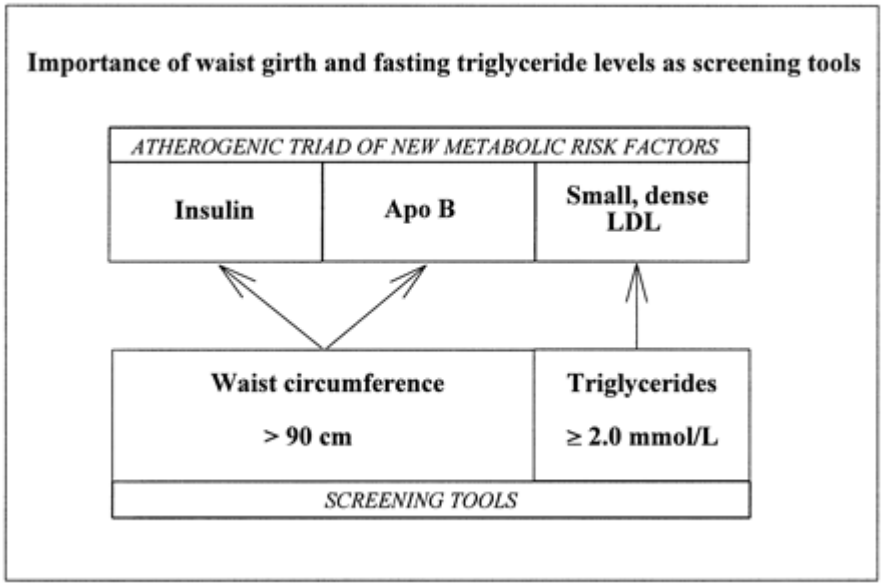


Figure 5. Working hypothetical model providing rationale for use of waist circumference and triglyceride levels as screening tools for the atherogenic triad of new metabolic risk factors. Waist circumference is used as discriminant of elevated fasting insulin and apo B levels, whereas triglyceride concentrations is used as discriminant of small, dense LDL phenotype. Taken from Lemieux et al.[31].

Genetic Dissection of the Metabolic Syndrome

Tools for the Genetic Dissection of Complex Traits

This section provides a brief overview of the methods and strategies for the genetic dissection of complex human traits such as the metabolic syndrome and its individual components. Globally the genetic dissection of complex traits integrates methods from the fields of genetic epidemiology and molecular biology. The methods and strategies employed to identify the genes have greatly changed throughout the years owing to the progress made in statistical analysis and the influence of the Human Genome Project. Heritability studies, complex segregation analyses, candidate gene linkage and association studies, genome-wide linkage scans and animal models are now all part of the arsenal to hunt-down the susceptibility genes.

The complexity of the task

By definition, complex traits are determined by the joint action of multiple genes and environmental factors[32] (Figure 6). For a given phenotype, multiple causes, both genetic and nongenetic, and interactions among them, contribute to its variation. While these factors may make the task of finding genes difficult, their recognition is important and gives a broader perspective of the underlying complexity. The multiple etiologic factors also imply that the effect size of most of these factors is rather modest. In fact, in complex traits, genes with large individual effects are likely to be rare. A more realistic genetic component of many complex traits is oligogenic (a few genes each with a moderate effect) or even polygenic (many genes, each with a small effect). In that spirit, searching for a small effect size calls for a large sample size, which is often not the case in many genetic studies. Indeed multiple studies have generated conflicting findings due to insufficient power. It should also be accepted that a gene with small individual effect may have a substantial contribution to the manifestation of the trait by interacting with a second gene or an environmental factor. Gene-gene and gene-environment interactions are difficult to test with current technologies but are likely to be important in the context of complex traits.

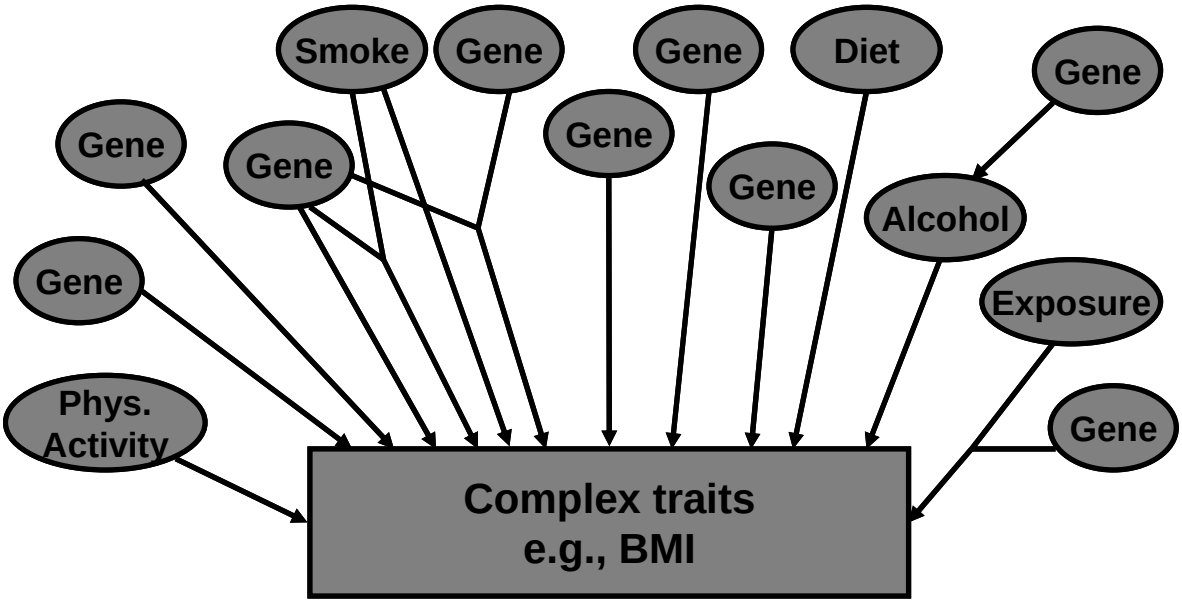


Figure 6. Varying causes of phenotypic variation. Derived from Rao[32].

Genetic and etiological heterogeneity are also expected to be a major challenge in the genetic dissection of complex traits. The multifactorial aetiologies imply that similar phenotypes can be the result of different factors. Accordingly, the phenotype-genotype relationship is not exclusive. On a purely genetic sense, different genes may act for different populations having a distinct genetic background or a manifest characteristic (obesity, type 2 diabetes, etc.). Furthermore, the phenotype is influenced by an unknown number of polygenes and each polygenic effect depends on an unknown number of alleles[33]. With a more global perspective, the connections between the genome and the phenotypes cannot be viewed as a one-way flow of information. In fact, the multigene genotype acts through the primary biochemical and physiological subsystems which subsequently affect the phenotypic measures of health[34]. Between the genotype and the phenotype there is a dynamic and epigenetic network of cellular and organismal dimensions that constantly shapes the phenotype and produces feedback information to influence the expression of participating genes. These complicated networks act throughout the lifetime of an individual and at any moment are influenced by the previous and contemporary environmental exposures. Taken all together it is not surprising that the dissection of complex human traits is the greatest challenge that geneticists have ever faced.

The Global Strategy

Despite the complexity enumerated in the previous paragraph, the genetic basis of complex human phenotypes can be divided into two basic approaches: the unmeasured genotype and the measured genotype approaches[35] (Figure 7). The unmeasured genotype approach is a purely statistical strategy that makes inference about the influence of genes based on the distribution of the phenotype. Because the inference is made from the phenotype to the genes, the approach is also called the top-down approach. A major advantage of the top-down approach is that no prior knowledge about the biology of the phenotypes is required to perform the analysis. Most of the genetic analysis using this approach used relatedness among family members to quantify the contribution of genetic factors and test hypotheses regarding a variety of general and specific models of inheritance. However, a major drawback of the unmeasured genotype is that specific gene(s) involved cannot be identified. Heritability studies and complex segregation analyses fall within this category and have been used in chapters 7 and 9, respectively.

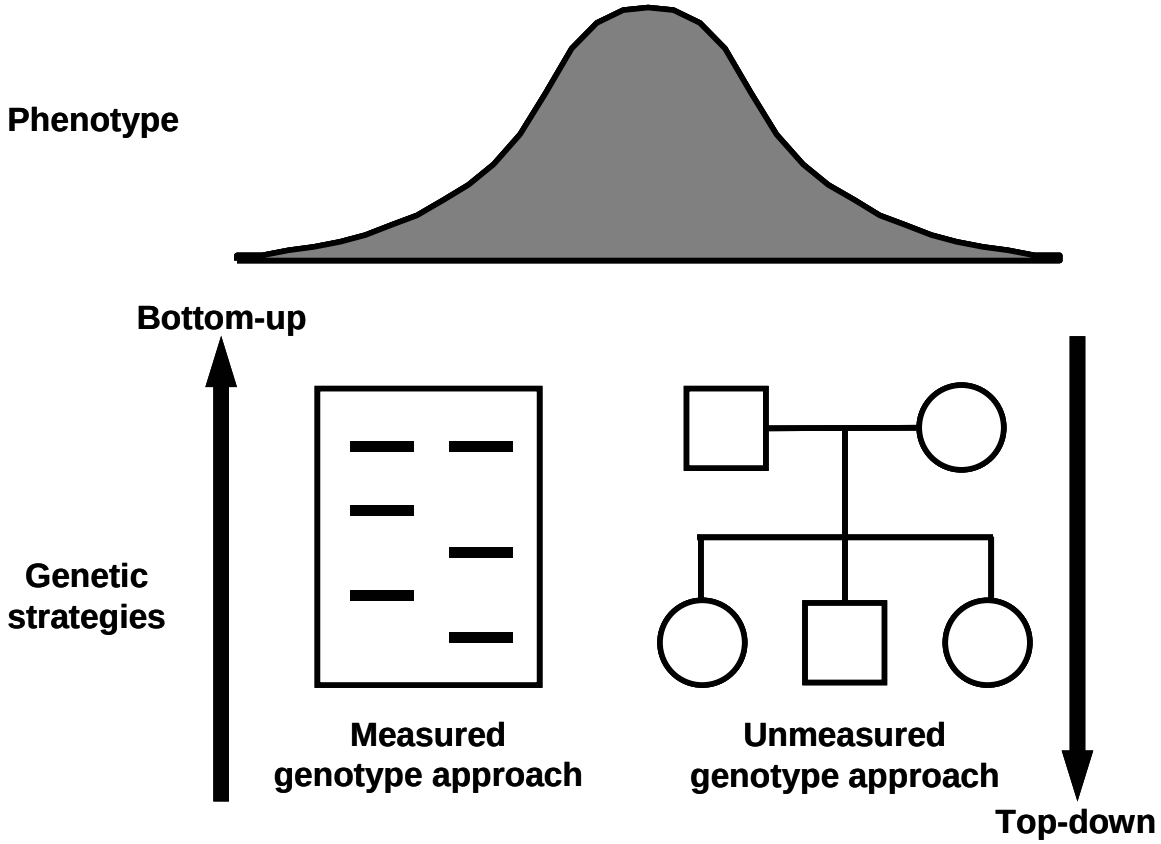


Figure 7. Bottom-up and top-down approaches in the study of continuously distributed phenotypes. Taken from Bouchard et al.[35].

In the measured genotype approach, influences about the roles of genes are made from DNA variations to the phenotype. For this reason, the approach is also referred to as the bottom-up approach. With this approach, genetic variations at the DNA level are genotyped and then tested for association and linkage with the phenotypes. The approach holds for evaluating the impact of a specific gene. DNA variations can be located within a gene believed, based on biological observations, to be involved in the trait (candidate gene approach) or can be random markers distributed across the genome (genome scan approach). Association and linkage studies fall within this category and have been used extensively within this project.

A combination of both bottom-up and top-down approaches is required to have a better understanding of the genetics underlying a complex trait. Throughout the years, the priority has switched from the top-down to the bottom-up approach due to the new technologies and the completion of the Human Genome Project. In the past, the top-down approach was the only

option since genotype measurement was not possible. However, today with the high throughput technologies, most geneticists have favoured the bottom-up approach. Nevertheless, both approaches are required if one wants to appreciate the genetics of a phenotype from its genetic contribution to the specific genes implicated.

The traditional steps to achieve a global appreciation of the genetic basis of a quantitative phenotype are illustrated in Figure 8. These steps are successive and the necessity of moving to the following one is greatly motivated by positive results obtained in the preceding one. These steps also help to understand how specific genetic techniques fit into the larger arsenal of genetic epidemiological methods. The first step is to determine whether or not the phenotype of interest aggregates within families. In the context of family study, familial aggregation can be evaluated using a simple analysis of variance to compare the variance between families to the variance within families. Familial resemblance is claimed if the variance between families is significantly higher than the variance within families. If there is evidence of familial aggregation, the next step consists of evaluating heritability to verify whether or not the familial resemblance is partly or totally attributable to genetic factors. In a family study, heritability can be obtained by estimation of familial correlations[36]. The strength of the correlations between family members (i.e., spouse, offspring, etc) gives an appreciation of the familial resemblance and provides insights about the relative importance of genetic versus nongenetic factors. If there is evidence for a genetic effect, the next step is to determine whether a major gene effect can be detected in the phenotype. The most popular method to detect a major gene effect is segregation analysis. The analytical strategy of segregation analysis relies on fitting a variety of general and specific models of inheritance testing the existence of a major gene effect, the mode of transmission of this major gene effect and its allele frequency. The model providing the best fit to the data is chosen based on specific statistical criteria and inference is made based on the hypothesis tested by this chosen model. It should be noted that up to this step only the unmeasured genotype approach is used. Indeed, familial resemblance, heritability and segregation analysis provide only statistical evidence about the contribution of genes involved in the phenotype of interest but indicate nothing about the specific genes. Thus, the last step consists of performing association and linkage studies to identify the genes or the genomic regions underlying the observed genetic effect. These studies can be performed with DNA variants located in candidate genes or with random genetic markers evenly spaced throughout the genome (see next section).

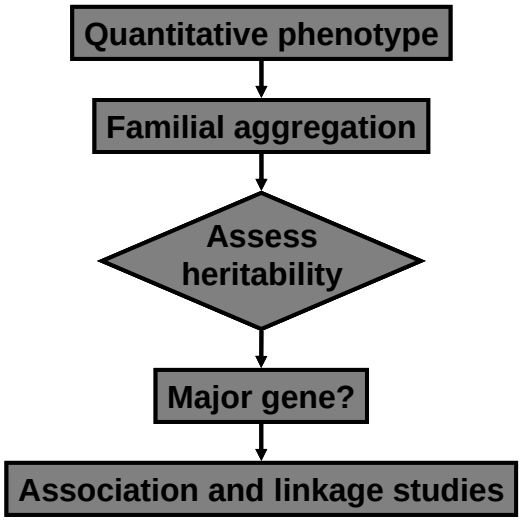


Figure 8. Flow chart describing the different steps in the investigation of the genetic basis of a quantitative phenotype. Derived from Bouchard et al.[35].

Association Studies

The development of rapid throughput genotyping assays and the widespread availability of DNA from large population studies have promoted the use of association studies. The number of association studies published in the literature has steadily increased throughout the years and is now reaching a cadence of 75 to 100 per week[37]. The phenotype under study may be the presence or absence of disease (discrete phenotype) or a quantitative measure[38]. Slightly different analytical techniques are used for discrete and quantitative phenotypes. For a discrete trait, the allele frequency at the polymorphic marker is compared between a case and control groups of unrelated individuals. In the presence of association, allele frequencies differ between cases and controls. For a quantitative phenotype, the test compares mean phenotypic values among individuals of different genotypes at the marker locus. For a rare variant, the analysis may also be done by grouping subjects based on the presence or absence of a particular allele. The mean phenotypic value can then be compared between carriers and noncarriers of that specific allele.

There are three reasons why an association between a marker locus and a trait may be observed[39]. First, it is possible that the relationship is the causal one and the genotyped marker is itself functional. This argument is particularly valid if the different alleles at the genotyped marker result in changes in the amino acid structure of the protein and functional studies have confirmed

the effect on the gene product. A second option is that the genotyped marker is not itself functional, but is in linkage disequilibrium with other polymorphisms that are functional. Linkage disequilibrium is by definition a non-random association of alleles at adjacent loci[40]. If two genetic markers are in close proximity on the same chromosome they tend to be co-inherited. The loci are said to be in disequilibrium when a particular allele at the first locus is found together with a specific allele at a second locus more often than would be expected by chance. Accordingly, a significant association can be the result of a functional variant in linkage disequilibrium with the tested marker. This existing relationship between neighbouring loci has recently justified the launch of the International HapMap Project met to facilitate the discovery of sequence variants that affect common diseases[41]. Finally, the observed association can be spurious and result from population stratification[42]. These spurious associations occur due to ethnic admixture and unintentionally drawn from two or more ethnic groups present in the studied population. Any trait having a higher frequency in a particular ethnic group will have a positive association with any genetic markers having a higher allele frequency in that same ethic group. Population stratification has clearly impaired the credibility of association studies. To prevent these false positive associations, two solutions have been proposed. First, by typing several dozen random markers, one can empirically detect and correct for stratification[43-45]. Secondly, one can use family-based studies such as transmission disequilibrium test (TDT)[46,47]. TDT and other family-based tests of associations are immune to false-positives caused by ethnic admixture and their use has been substantially propelled by the concern driven by population stratification[47].

Other than population stratification, association studies received criticisms due to the lack of replication[48,49]. A recent meta-analysis has demonstrated that among 166 associations (between gene variants and disease) investigated more than three times, only 6 have been consistently replicated[42]. The possible reasons for the inconsistency include false positives due to type 1 error (driven by publication bias for positive associations), false positives due to population stratification, false negatives due to lack of power in potential replication studies, and true differences between study population. Considering these factors, caution is emphasized before drawing conclusions from a single report of an association between a genetic variant and a particular phenotype or disease[42]. However, association studies are widely anticipated to contribute to the understanding of complex traits[50-52]. To achieve this expectation, the association

study needs to be well conducted and some journals have now proposed criteria for acceptance and publication of genetic association studies[53,54]. Ideally, association studies must have a large sample size, small P values, report associations that make biological sense and alleles that affect the gene product in a physiologically meaningful way. In addition, the association gains credibility when the finding is replicated in an independent cohort, the association is observed both in family based and population-based studies, and the odds ratio and/or attributable risk is high. Obviously, very few studies will meet these criteria but they can help judging the credibility of the finding. It is also important to prioritize polymorphisms[50]. We now know that the 3 billion base pairs of the human genome contain more than 10 millions genetic variations and approximately 30 thousand genes[41,55-57]. Accordingly, the prior probabilities that given polymorphisms (located or not in a candidate gene) play a role in disease or any health related phenotype is very low. To increase this likelihood, one should follow genes located in chromosomal regions that co-segregate with the phenotype and/or selected genetic variants that have a demonstrated functional consequence. To take full advantage of association studies, it is important to fit this technique into a larger arsenal of genetic epidemiological methods and molecular studies.

Linkage Studies

Linkage study is another important method to identify genes contributing to diseases or health-related phenotypes. In contrast to association studies, which seek to identify particular variants that are associated with the phenotype at the population level, linkage tests for evidence of cosegregation between a marker locus and a trait within families. Indeed, relatedness among subjects is required in order to perform linkage analyses. Linkage analyses have greatly evolved during the years to exploit the genetic information present in kindreds consisting of sibling pairs to extended pedigrees. Linkage analyses fall into two main categories: parametric (model-based) that strongly models the genetic effect and nonparametric (model-free) that makes few assumptions regarding the etiologic model underlying the phenotypic distribution.

The first linkage method in humans was proposed by Morton in 1955[58]. In this seminal paper, he introduced the lod score method (and simultaneously the concept of lod score) which has been recognized as a major milestone in the genetic dissection of human traits[32]. This method is still in use today and has been remarkably successful in identifying disease genes for Mendelian

disorders[59]. However, before performing the lod score method, prior knowledge is required about the mode of inheritance of the trait. Unfortunately, this information is unknown for complex traits and misspecification of the required parameters may substantially reduce the power[60]. Accordingly, robust nonparametric linkage methods have been developed for complex traits, which rely on patterns of allele sharing between related subjects to infer linkage.

The Haseman and Elston sib-pair linkage method[61] is a classic example and a widely used nonparametric linkage analysis. This method simply regresses the squared phenotypic difference in sibs on the number of alleles shared identical by descent—that is, that are direct copies of the same ancestral alleles. The idea is that sibs that share a greater proportion of alleles identical by descent have a more similar phenotype. In contrast, under the null hypothesis, no relation is observed between the sibs' phenotypes and the degree of allele sharing. Instead of using the squared sib-pairs trait difference, current methods look at the phenotypic covariance or correlation as a function of the identical by descent sharing using the variance components approach[62,63]. Variance component-based linkage analysis has become a widespread statistical tool to identify quantitative trait locus (QTL) involved in complex traits. This increased popularity is mainly explained by its ability to accommodate large pedigrees and to test important biological phenomenon such as epistasis, gene-environment interaction and oligogenic model[64].

Linkage and association studies should not be viewed as distinct genetic statistical tool used independently but more as complementary approaches to find the genes. It has been proposed that genes with small or subtle effects may not be detectable by linkage[65]. However, linkage studies can succeed where association fails and vice-versa (Figure 9). The power to detect the effect of genes depends on the effect size, the allele frequency and the sample size. Obviously, the ability of both association and linkage studies to detect the genes increases when the sample size and the effect size increase. On the other hand, the allele frequency will really dictate whether linkage or association is more powerful. For a more frequent allele, association is favoured and for a rare allele, linkage is prefered. However, these theoretical concepts may be useless since the knowledge about the allele frequency is rarely known in advance. Usually, more practical reasons force the utilities of one to another depending whether a candidate gene or a genome scan strategy is adopted.

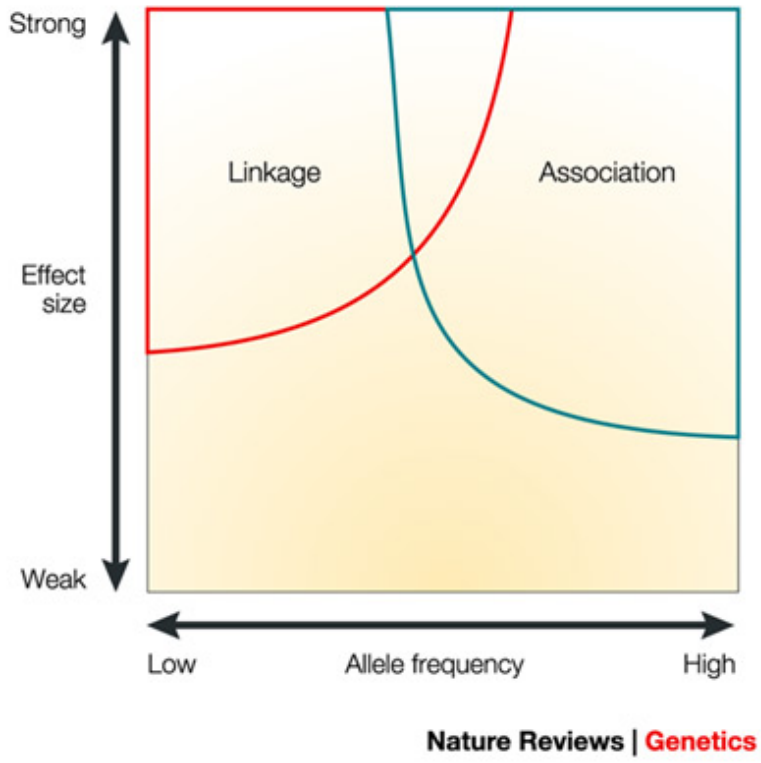


Figure 9. Optimal mapping strategies for different types of loci. Taken from Ardlie et al.[40].

Genome Scan Studies

Genome scans are simply large-scale applications of linkage and association methods. The objective of the approach is to identify the chromosomal regions within which one or more disease-predisposing genes lie. Genes contained within such linked regions become positional candidates and are next examined for mutations potentially causing the signal. Conceptually, genome scan studies can be divided into three steps: 1-scan the entire genome with a dense collection of genetic markers; 2-calculate an appropriate linkage or association statistic at each markers along the genome; and 3-identify the regions in which statistics show a significant deviation from what would be expected by chance[66]. One of the great advantage of the genome scan approach is that it can be applied without prior knowledge of the biological basis of the disease or the phenotypes under study.

For genome-wide linkage scan the usual practice is to test about 300-400 highly polymorphic markers, usually microsatellites, distributed approximately evenly across the genome with an

average spacing between markers in the order of 10 cM. Localization of the locus by linkage analysis can achieve only a certain level of precision. Usually the minimal interval of a QTL in humans range from 10 to 30 Mb which contains approximately 100 to 300 genes[67]. It should also be emphasized that the estimated peak locations are generally not very precise[68]. Accordingly, there is a large gap between QTL and gene finding with genome-wide linkage scan[69]. Nevertheless, this strategy has been highly successful in the identification of genes responsible for simple Mendelian traits[70,71]. However, the general picture derived from genome-wide linkage scan in complex traits is one of the difficulty in locating genes and replicating previous reported linkage signals. This difficult picture is not related to the approach but more to the nature of complex traits characterized by locus heterogeneity, epistatis, low penetrance, variable expressivity, pleiotropy and limited statistical power[39,70].

Genome-wide association studies has been proposed as an alternative to facilitate the mapping of complex disease loci[72]. Similar to genome-wide linkage scan, this approach does not require prior knowledge about the molecular basis of the disease/phenotypes. As it is the case for linkage scan, the objective of association scan is to identify chromosomal regions harbouring susceptibility genes. However, with this new strategy, the resolution attain will be much higher (map the gene to smaller genetic interval), in the order of kilobases. Genome-wide association scan has been driven by empirical studies showing strong associations between nearby SNPs[73-76]. This strong allelic association between variants is known as linkage disequilibrium. The practical implication of linkage disequilibrium is that a few carefully chosen SNPs (tag SNPs) can be genotyped to capture much of the information in a chromosomal region. Therefore the tag SNPs could serve as genetic markers to detect association between a particular chromosomal region and the disease/phenotype, whether or not the tag SNPs themselves have functional effects. The search for causal genetic variants can then be limited to the regions showing association. However, because of the central role of linkage disequilibrium in the concept of genome-wide scan association study and the variable nature of disequilibrium in the human genome[73,74,76,77], the utility of such approach remains to be seen.

Genetics of the metabolic syndrome

Almost a decade ago, Bouchard[78] proposed a multi-layered model of the metabolic syndrome where genes actions, including their interaction with each other and with the environment, can be operative at all levels. This structure recognized that genes can act on the causes of the metabolic syndrome (visceral obesity, insulin resistance and even behaviours affecting healthy lifestyle choices) and on its individual components. In this complicated context, it is essential to properly define the phenotype under study. It is recognized that affection status of clinical diseases based on a discrete binary scale (affected or unaffected) contains considerably less genetic information in comparison to a quantitative disease-related phenotypes[79,80]. Accordingly, in this project we relied mainly on quantitative phenotypes to identify the susceptibility genes of the metabolic syndrome. In the last chapter, we tested the genetics of a quantitative metabolic syndrome variable created by factor analysis. This was an attempt to identify the genes contributing to the underlying cluster of risk factors defining the metabolic syndrome. However, for most of the project, we used quantitative individual components of the metabolic syndrome to identify the metabolic syndrome genes. Many phenotypes were investigated including obesity, lipoprotein/lipid and glucose/insulin variables. However, a major part of the work has focused on the genetics of LDL particle size. Understanding the genetics of LDL size is not only important because it is a component of the metabolic syndrome, but also because it was recently recognized as an independent marker of cardiovascular risk[81].

Genetics of LDL Particle Heterogeneity : From Genetic Epidemiology to DNA-Based Variations.

The following is an attempt to summarize the growing evidence of genetic control on LDL particle heterogeneity.

LDL particles heterogeneity and coronary heart disease

LDL cholesterol is a well-known risk factor for coronary heart disease (CHD) and is now recognized as the primary target of lipid lowering therapy[2]. However, it is known that LDL particles are heterogeneous in terms of size, density, chemical composition and electric charge[82-84]. Data from case-control[85] and prospective[27,81,86,87] studies suggested that small, dense LDL particles are associated with increased risk of CHD. The atherogenicity of these particles is attributed to several possible biological mechanisms including greater susceptibility for oxidation[88-92], decreased affinity for the LDL receptor[93-97], increased binding to the arterial wall[98-101] and greater facility to cross the arterial wall[102,103] as well as having negative effects on the endothelium function[104]. Additional evidence for the relevance of the small, dense LDL on atherosclerotic lesions development and CHD progression are derived from an animal model[105] and lipid lowering trials in human[106,107]. On the other hand, recent findings from the Cholesterol and Recurrent Events (CARE) trial[108] support earlier case-control[109-112] and prospective[113] studies showing that small, dense LDLs are not risk factors for CHD. In fact, some of these studies have shown that larger LDL particles are associated with CHD. While these studies disagree as to which LDL particle size (small or large) is related to CHD risk[114], defining the genetic and environmental factors modulating LDL particle properties may be helpful in understanding its relationship with CHD.

Multiple approaches have been used to hunt down the genes involved in complex human diseases and diseases-related risk factors. Through the years, methods and strategies have evolved following the progress made in genetic epidemiology and the completion of the Human Genome Project. Genetic studies on LDL particles represent a perfect example of this phenomenon. Several studies have investigated the genetics of LDL particle heterogeneity. Heritability studies, complex segregation analyses, linkage and association studies with candidate genes, and genome-wide linkage scans are all part of the arsenal used for dissecting the genetic architecture of this

trait. The following is an attempt to summarize the growing evidence of genetic control on LDL particle heterogeneity.

Several studies have shown that small, dense LDL are associated with a constellation of other well-recognized lipoprotein-related risk factors, including increased plasma triglyceride and apoB levels as well as decreased HDL cholesterol concentrations. Furthermore, small, dense LDL particles coexist in the same subjects as part of multifaceted phenotypes including the metabolic syndrome, the atherogenic lipoprotein phenotype (LDL subclass pattern B) and familial combined hyperlipidemia (FCHL)[115]. Thus, small, dense LDL may be a qualitative trait representing a common atherogenic lipoprotein/metabolic profile and the proposed genetic loci responsible for small, dense LDL may in fact be responsible for a more extensive syndrome. However, throughout the following section a more narrow view of the phenotypes that characterize LDL particle heterogeneity is taken.

A number of analytical techniques are available for characterizing LDL heterogeneity some technicality must be addressed before going through genetic ground. LDL heterogeneity was first described using analytical ultracentrifugation (AnUC)[116]. Over the years, this technique was replaced by others including density gradient ultracentrifugation (DGU), gradient gel electrophoresis (GGE), and more recently by nuclear magnetic resonance (NMR) spectroscopy. The phenotypes derived from these techniques are those used in the genetics studies performed so far. Based on GGE, a continuous variable can be defined as *LDL peak particle diameter* (LDL-PPD), reflecting the size of the major LDL subclass in an individual subject. A dichotomous classification can also be defined based on GGE and referred to as *LDL subclass patterns, or phenotypes, A and B*. LDL subclass phenotype A is characterized by a predominance of large LDL particles and skewing of the densitometric scan toward small particles, while LDL subclass phenotype B is characterized by a predominance of small LDL particles and skewing of the curve toward large particles[117]. Other phenotypes can be constructed using GGE, including *LDL score* which is calculated using the migration distance (mm) of each peak multiplied by its respective relative area[118] and *LDL type* which is a weighed average of seven possible categories of LDL, resulting in a variable ranging from 1 (largest) to 7 (smallest)[119]. A more detailed description of these techniques are found in the following published reviews[85,120,121].

Genetic Epidemiology

Familial aggregation

The first evidence for a genetic determination of LDL properties was reported by Fisher et al. in 1975[122]. Five families, including 11 mating and 16 offspring, were examined for their LDL molecular weight. Only subjects having monodisperse LDL, that is, LDL that is found to be present as a single, essentially homogeneous population of macromolecules, were included in the study. Correlation coefficients between pairs of relatives revealed a significant parent-offspring correlation (0.82, $p < 0.01$) but absence of correlation between fathers' and mothers' (0.32, p = NS). These results provided evidence for the genetic contribution to LDL molecular weight. To further determine the degree of resemblance of the offsprings to their parents, a regression coefficient of the mean molecular weight of the offsprings on the mean parental molecular weight was calculated. The regression coefficient was 0.30 ($p < 0.01$), which made the authors conclude that approximately 30% of the observed LDL molecular weight variance is due to additive gene action. In addition, based on the five families, the authors postulated a model consistent with a single gene (two alleles) locus genetic mode of inheritance without dominance. Although the sample size used in this study was relatively small, it demonstrated for the first time that LDL characteristics segregate within families.

Since this earlier report, accumulating evidence of familial and ethnic aggregation of LDL subclasses have emerged in the literature. Haffner et al.[123,124] demonstrated a significant difference between ethnic groups in LDL size among 1571 subjects from the Insulin Resistance Atherosclerosis Study and 466 subjects from the San Antonio Family Heart Study. These studies cannot disentangle the effect of the genetic background from the effect mediated by the difference in lifestyles between ethnicity, but clearly motivated genetic studies in the field.

Heritability analysis (Table 4)

Twin studies

Studies using identical (monozygotic, MZ) and fraternal (dizygotic, DZ) twins have been used to assess the heritability of LDL size. The first study on this issue was based on 119 MZ and 113 DZ twin pairs participating in the third examination of the National Heart, Lung, and Blood Institute Twin Study[125]. In this study, the LDL subfractions were separated by GGE and the heritability analysis used LDL type. The LDL type intraclass correlation coefficient in MZ twins was significantly higher than the correlation coefficient in DZ twins (0.58 vs 0.32, $p < 0.005$), with an heritability of 0.52 prior to controlling for covariate effects. After adjustment for BMI, alcohol consumption, cigarette smoking, and physical activity, the heritability decreased to 0.39. Despite their great magnitude, these estimates were not statistically significant suggesting the lack of heritability for LDL type. Similar results were obtained when only the major LDL band (LDL-PPD) was used as a variable. Thus, the authors concluded that LDL particle size is not greatly influenced by genetic factors within this population. It is noteworthy that the authors used the more conservative among component[126] estimate of heritability due to some indication of unequal total variance between zygosities. Although, this procedure is considered more suitable in such case, the power to detect significant heritability is substantially reduced.

The heritability estimates were also analyzed based on 203 monozygotic and 145 dizygotic pairs of adult female twins who participated in the second examination of the Kaiser Permanente Women Twins Study[127]. The classical heritability estimate for LDL-PPD was 0.54, and the within-pair estimate was 0.48. These estimates were not changed substantially when the analyses were restricted to postmenopausal, nondiabetic, non β-blocker users or Caucasian pairs with heritability ranging from 0.34 to 0.5. Thus the authors suggested that between one third and one half of the variability in LDL size appears to be attributable to genetic influences in this sample of women twins.

Table 4. Heritability analyses on LDL particle characteristics

References	Study	Subjects characteristics'	Phenotype	Heritability		
				Methods	Covariates or assortment	Results
Lamon-Favas et al.[125]	The third examination of the National Heart, Lung, and Blood Institute Twin Study	119 MZ and 113 DZ male twin pairs aged between 59-70 years.	LDL type	ANOVA (among component		0.52 (p = 0.12)
				ANOVA (among component)	BMI, alcohol consumption, cigarette smoking, and physical activity	0.39 (p = 0.39)
Austin et al.[127]	The second examination of the Kaiser Permanente Women Twins Study	203 MZ and 145 DZ women twin pairs with a median age of 51 years; 90% were white.	LDL-PPD	Classical	All pairs Postmenopausal pairs Nondiabetic pairs Non β-blocker user pairs Caucasian pairs	0.54 (p < 0.001) 0.55 (p < 0.003) 0.35 (p < 0.016) 0.45 (p < 0.002) 0.51 (p < 0.001)
				ANOVA (within-pair)	All pairs Postmenopausal pairs Nondiabetic pairs Non β-blocker user pairs Caucasian pairs	0.48 (p < 0.001) 0.34 (p < 0.021) 0.44 (p < 0.001) 0.52 (p < 0.001) 0.43 (p < 0.001)
Edwards et al.[128]	The Genetic Epidemiology of Hypertriglyceridemia Study	85 high-risk families for CVD including 780 individuals. Primarily white	LDL-PPD	Maximum-likelihood-based approach	Age and sex	0.34 (p < 0.001)
Barzilai et al.[129]	Longevity Genes Project	429 Ashkenazi Jewish with exceptional longevity	LDL size (NMR)	Linear regression	Men Women	0.60 (p = 0.006) 0.46 (p = 0.003)
Rainwater et al.[130]	The San Antonio Heart Study	1157 Mexican Americans	ΔLDL*	Maximum-likelihood-based approach	Sex, age, age^2, diabetes status, contraceptive use, and hypertension medications	0.44 (p < 0.001)
					Sex, age, age^2, diabetes status, contraceptive use, hypertension medications, and triglyceride	0.30
Austin et al.[131]	The Genetic Epidemiology of Hypertriglyceridemia Study	140 subjects member of 26 kindreds. Primarily Caucasians	LDL-PPD	Maximum-likelihood-based approach	Sex, age, oral contraceptive use, menopausal status, and hormone replacement therapy	0.26 (p = 0.025)
					+ triglyceride	0.12 (p = 0.168)
					+ HDL-C	0.15 (p = 0.121)
					+ triglyceride and HDL-C	0.10 (p = 0.213)

*ΔLDL, is a metrics for particle size phenotype to optimally reflect the size correlations between LDL and HDL particles.
LDL-PPD, low-density lipoprotein peak particle size; NMR, nuclear magnetic resonance.

Family studies

Heritability estimates of LDL-PPD was also evaluated using family data. The first family study on this issue was based on 780 individuals members of 85 families participating in the Genetic Epidemiology of Hypertriglyceridemia (GET) study[128]. The GET study is based on 2 family studies one ascertained through hyperlipidemic probands surviving a myocardial infarction and the second through hypertriglyceridemic probands without CHD. After accounting for age and sex effects, results suggested that approximately one third of the residual variance in LDL-PPD (h^2 = 0.34) was attributable to additive genetic effects. Higher heritability coefficients were observed for LDL size in the Ashkenazi Jewish families ascertained for exceptional longevity[129]. In this study, LDL size was characterized by NMR and heritability was estimated at 0.46 in women and at 0.60 in men. These results demonstrated that LDL size is highly heritable irrespective of the analytical methods used to characterize the particles and suggested that the measurement error inherent to each technique does not mask the genetic signal.

By means of a new metric representing coordinated size variation between HDL and LDL size particles, Rainwater et al.[130] conducted an original study to test the hypothesis that there are "lipoprotein size genes". The new metric, named ΔLDL, is a metric for LDL particle size phenotype that optimally reflects the size correlation between LDL and HDL particles. ΔLDL was subjected to quantitative genetic analyses using 1157 Mexican Americans participating in the San Antonio Family Heart Study. Heritability of ΔLDL was highly significant and indicated that nearly half (44%) of the residual variance (after adjustment for sex, age, diabetes status, contraceptive use, and hypertension medications) in ΔLDL was explained by additive gene effects. After including triglyceride levels in the model as a covariate, the heritability estimate decreased from 0.44 to 0.30, indicating gene(s) common for both traits. These data indicate that particle size phenotypes are under substantial genetic control.

Taken together, the above studies suggested that 30% to 60% of the variance in LDL particle size is attributable to genetic factors, with the remainder due to nongenetic influences. Accordingly, these genetic studies also pointed out the importance of nongenetic factors on LDL subclasses since approximately 50% of the variance is attributable to nongenetic factors. A number of environmental influences have been identified, including, among others, dietary factors[132],

physical activity[133], abdominal obesity[134], insulin resistance and hyperinsulinemia[135]. The combination of genetic and environmental influences provides opportunities to develop prevention strategies to reduce CHD risk among genetically susceptible individuals[136].

Inheritance of the LDL heterogeneity: testing for a single-gene effect

Heritability estimates obtained from twin and family studies reinforced the interest in finding gene(s) underlying that genetic effect. The following are the different lines of evidence that provided the existence of a single gene with major effect on the phenotype, including commingling analyses, segregation analyses and complex segregation analyses.

Commingling analyses

Commingling analyses are often used as a preliminary method to screen for the presence of a single gene with major effect[137]. Under the presence of such gene the distribution of a quantitative phenotype is better characterized by a mixture of distributions rather than by a single distribution. An analysis of the LDL-PPD distribution in the Kaiser Permanente Women Twins Study identified three distinct subgroups of subjects[127]. Indeed, a trimodal model provided a better fit to the data compared to a bimodal or an unimodal model. A trimodal model was also reported for the age- and gender-adjusted LDL-PPD distribution in a sample of healthy families from a Mormon community[138]. Finally, in 373 family members of Israeli origin, the mixture of two normal distributions for age- and gender-adjusted LDL-PPD fit the data significantly better than a single distribution[139]. However, the trimodal model could not be rejected over the bimodal model. Taken together, results from commingling analyses are compatible with the presence of a major gene effect affecting LDL-PPD. However, this pattern of distribution may also arise as a result of nongenetic factors. Thus, inference drawn from these studies needs to be interpreted with caution.

Segregation analyses (Table 5)

The first studies that investigated the inheritance of LDL heterogeneity were derived from fitting the data into pedigrees under an hypothetical genetic model. Fisher et al.[122] were the first to provide evidence for a single gene-two allelic system locus affecting LDL heterogeneity. Using pedigrees from five families, they proposed a model of two alleles, one a determinant for high,

the other for low LDL molecular weight. A decade later, Austin et al.[140] evaluated the lipoprotein subclasses (pattern A/B) by GGE in 79 healthy members of sixteen nuclear families living in a local Mormon community. Their data proposed a genetic model consistent with a single-locus, bi-allelic system as well. The estimated frequency of the allele leading to the phenotype characterized by a predominance of small, dense LDL subclasses (pattern B) was approximately 15% under a dominant mode of inheritance. However, in contrast to the observation of Fisher et al[122], expression of the phenotype appears to be age dependent, in that most affected subjects in the population were older than 40 years. Although, different techniques were used to detect LDL properties between these two studies, it is possible that the LDL pattern reported in the later and the molecular weight reported in the former represent the same trait. Although these studies were limited by their sample size, they provided additional evidence in favor of a single gene affecting LDL density and size.

Table 5. Inheritance of LDL particle heterogeneity.

Phenotypes	Family status	n kindreds (n members)	Major gene	Mode of inheritance	Allele frequency	Polygenic component	References
			Segregation analysis				
Pattern A/B	Healthy	8 (79)	Yes	Dominant Recessive	0.15 NA	NA	Austin et al.[140]
Molecular Weight	Normal and hyperlipidaemic	5* (38)	Yes	Codominant	NA	NA	Fisher et al.[122]
			Complex segregation analysis				
Pattern A/B	Healthy	29 (301)	Yes	Dominant	0.25	0%	Austin et al.[141]
Pattern A/B	FCHL	7 (234)	Yes	Dominant Codominant	0.32 0.34	1% 33%	Austin et al.[142]
LDL-PPD	Healthy	29 (301)	Yes	Undetermined	NA	0%	Austin et al.[138]
LDL-PPD	Healthy	80 (373)	Yes	Codominant	0.24	74%	Friedlander et al.[139]
LDL-PPD	FCHL	48 (553)	Women: Yes Men: No	Recessive Polygenic	0.06	0%	Vakkilainen et al.[143]
Parameter K**	Healthy	19* (159)	Yes	Recessive	0.19	12%	de Graaf et al.[144]
Parameter K**	FCHL	40 (623)	Yes	Recessive	0.42	25%	Bredie et al.[145]

*Nuclear family. **A continuous quantitative trait estimating the relative contribution of each LDL subfraction.

FCHL, familial combined hyperlipidemia; LDL-PPD, low-density lipoprotein peak particle size.

Complex segregation analyses (Table 5)

The presence of a major gene effect in addition to its mode of inheritance has been also investigated using complex segregation analyses. The results of these studies are summarized in Table 5. Two years after having proposed a single gene-two allelic system locus affecting LDL patterns, Austin et al.[141] have confirmed their results on an enlarged sample of the same Mormon community containing 61 healthy families including 301 members. The model providing the best fit to the data included a single gene with a dominant mode of inheritance and a frequency of 25% and reduced penetrance for men under age 20 and for premenopausal women. It should be noted, however, that both recessive and additive modes of inheritance could not be rejected. Similar results were observed for 234 individuals of 78 nuclear families with FCHL[142]. In this sample, complex segregation analyses suggested that LDL subclass pattern B is controlled by a single major genetic locus (with either a dominant or an additive mode of inheritance) and a small, but significant, multifactorial inheritance component. The prevalence of LDL subclass pattern B allele was also common in these families (≈ 0.30), suggesting that the proposed allele for pattern B is just as likely to occur in families with FCHL as in healthy families. Again, reduced penetrance for pattern B allele in FCHL families was observed for men under age 20 and for women under age 50.

The two later complex segregation studies were based on the dichotomization of the LDL subfraction into two discrete phenotypes. It is possible that this dichotomous definition oversimplifies the biochemical heterogeneity of LDL particles. de Graaf et al.[144] were concerned by such procedure since much information is lost, i.e. we do not know whether an individual is close to or far from the LDL size threshold for the pattern A / pattern B classification which results in a lost of power[79]. Accordingly, they constructed a continuous variable, named parameter K, that reflect LDL subfraction profile and which is characterized by the relative contribution of the three major LDL subfractions, LDL1, LDL2, and LDL3, determined by DGU. Analysis for this quantitative trait was performed on 19 healthy Dutch families including 159 individuals. Results indicated that the LDL subfraction profile is controlled by a major autosomal, highly penetrant recessive allele with a population frequency of 19% and an additional multifactorial inheritance component. The penetrance of the more dense LDL allele increases with age, for both sexes, and was higher for men than women. Furthermore, it appeared that oral contraceptive use

was associated with a high penetrance of the more dense LDL subfraction profile. Also concerned by the possibility that the dichotomous trait may not provide the best reflection of LDL size distribution, Austin et al.[138] reanalyzed their healthy subjects living in a Mormon community but this time by using LDL-PPD instead of the dichotomous classification reported earlier[141]. The model providing the best fit to the data consisted of a single major gene effect with Mendelian inheritance, and with no additional multifactorial inheritance component. However, the available sample was not sufficient to distinguish dominant versus recessive mode of inheritance. Thus, analysis of the continuous LDL-PPD variable was not superior to the dichotomous LDL subclass pattern classification in determining the mode of inheritance of LDL subclasses in this healthy families sample. The mode of inheritance of parameter K was also investigated in a large sample of Dutch families with FCHL[145]. The genetic basis of LDL subfraction profile in this family was best described by a common, major autosomal gene effect with a population frequency of 42% and a recessive mode of inheritance with a polygenic heritability component of 25%. Subsequently, the mode of inheritance of LDL-PPD was investigated in 373 subjects from 80 kindreds residing in kibbutz settlements in Israel[139]. Complex segregation analyses on sex- and age-adjusted LDL-PPD were inconclusive in this study since both the mixed recessive genetic model and the mixed environmental model could not be rejected. However, when the regression model for sex and age allowed coefficients to be ousiotype (genotype class) specific, the mixed environmental model was rejected while a major Mendelian model was not. Indeed a major additive gene (codominant) model for LDL-PPD with an allele frequency of 24% for small LDL particles could not be rejected. In addition this model contains a large polygenic component (74%). The authors postulated that the ethnic homogeneity and the lifestyle similarity of the sample may explain the high contribution of polygenic factors to LDL-PPD. More recently, the genetic influence of LDL-PPD was modeled in 48 Finnish FCHL families[143]. Complex segregation analyses in these families suggested that the trait is the result of the additive effects of multiple genes where a recessive major gene effect of low frequency (6%) may contribute to large LDL-PPD in women. For men, they could not established that LDL-PPD follows a strictly polygenic model, but the results indicated that LDL size is unlikely to be influenced by a major gene effect in this population.

With the exception of the later study, results from complex segregation analyses support the concept of a major gene effect involved in LDL size and density. However, some dissimilarities

were found between the studies in regard to the mode of inheritance, allele frequency and the presence or not of a multifactorial inheritance component. This discrepancy could be explained by differences in family structures, criteria for proband ascertainment, and the use of different techniques to characterize LDL heterogeneity. Nevertheless, these studies unanimously provided evidence about the contribution of a major gene effect and clearly motivate the race to hunt-it down.

DNA-based variants

Linkage studies

Many investigators have used linkage analyses to identify the genes underlying the genetic contribution of LDL particle characteristics. The early studies have been performed using candidate gene strategies by studying genetic variations located within or in proximity of genes coding protein products known to be involved in lipoprotein/lipid metabolism. On the other hand, recent studies have used a genome-wide scan approach in order to identify chromosomal regions influencing LDL size-related phenotypes. Table 6 presents a summary of the loci and genes, ordered by chromosome number, that have provided evidence of linkage using these two strategies. It should be noted that only positive findings are provided in this table and careful examination of the literature might in fact show significant evidence against linkage for certain loci presented. It is also worth mentioning that opposite results for the same gene may not necessarily imply controversy giving the different study samples.

Table 6. Evidence for the presence of linkage with LDL particle characteristics

Genes and/or markers	Location (Mb)*	Chr. Band*	Samples	Phenotype	p. Z or Lod Values	References
APOB	21.2	2p24.1	119 DZ twin pairs	LDL-PPD	p = 0.014	Austin et al.[146]
D3S2387-D3S2403	**1-13.1**	**3p26.3-p25.2**	470 subjects; 10 pedigrees	LDL-3	Lod = 2.6	Rainwater et al.[147]
D3S1754-D3S1311	**174.4-193**	**3q26.32-q29**	470 subjects; 10 pedigrees	LDL-3	Lod = 4.1	Rainwater et al.[147]
D4S1647-D4S1644	**99.7-142.6**	**4q23-q31.21**	470 subjects; 10 pedigrees	LDL-3	Lod = 4.1	Rainwater et al.[147]
D6S1009-D6S1277	**137.3-164.2**	**6q23.3-q26**	140 subjects; 26 families	LDL-PPD	Lod = 2.1	Austin et al.[131]
D6S1003-D6S1277	**144.3-163.7**	**6q24.2-q27**	470 subjects; 10 pedigrees	LDL-3	Lod = 2.9	Rainwater et al.[147]
SOD2	160	6q25.3	55 sibpairs	LDL-PPD	p = 0.001	Rotter et al.[148]
SOD2 (D6S1008)	163.5	6q26	481 subjects; 18 families	Pattern A/B	p = 0.020	Allayee et al.[149]
LPL	19.8	8p21.3	120 subjects; 5 kindreds	LDL-PPD	Lod = 6.2	Hokanson et al.[150]
APOAI-CIII-AIV	116.2	11q23.3	481 subjects; 18 families	Pattern A/B	p = 0.005	Allayee et al.[149]
			65 sibpairs	LDL-PPD	p = 0.06	Rotter et al.[148]
D15S659	**44.1**	**15q21.1**	240 subjects; 18 families	LDL-PPD	Lod = 2.2	Allayee et al.[151]
LIPC (D15S148)	56.8	15q21.3	498 subjects; 18 families	LDL-PPD	p = 0.008	Allayee et al.[151]
LIPC (D15S643)	57.4	15q22.2	498 subjects; 18 families	Pattern A/B	p = 0.035	Allayee et al.[151]
			498 subjects; 18 families	LDL-PPD	p = 0.019	Allayee et al.[151]
CETP (D16S313)	27.1	16p12.1	87 sibpairs	LDL-PPD	p = 0.03	Rotter et al.[148]
CETP	56.8	16q13	119 DZ twin pairs	LDL-PPD	p = 0.001	Talmud et al.[152]
CETP/LCAT (D16S496)	68.7	16q22.1	481 subjects; 18 families	LDL-PPD	p = 0.035	Allayee et al.[149]
LDLR	11.1	19p13.2	102 sibpairs	LDL-PPD	p = 0.008	Rotter et al.[148]
			51 subjects; 9 families	Pattern A/B	Lod = 4.3	Nishina et al.[153]
D19S714-D19S433	**16.1-31**	**19p13.12-q12**	470 subjects; 10 pedigrees	LDL-1	Lod = 2.3	Rainwater et al.[147]
D19S587-D19S178	**35.8-45.1**	**19q13.1-q13.31**	470 subjects; 10 pedigrees	LDL-2	Lod = 1.9	Rainwater et al.[147]
D19S246	**55.6**	**19q13.33**	240 subjects; 18 families	LDL-PPD	Lod = 1.6	Allayee et al.[151]

Status as of December 2003

Chromosomal locations in bold indicate QTLs from genome-wide linkage scans. When one marker per line is shown, the marker is the one defining the peak. When two markers per line are shown, they indicate a conservative location interval for the QTL and the lod score corresponds to the highest peak observed in the region.

For candidate genes, the location of the gene is provided if the tested marker is located within the gene. However, if the marker is not within the gene but located close to it, the specific tested marker is given in parentheses and the location provided corresponds to the marker.

*The physical and genetic location of markers and genes are from the genome browser of the University of California, Santa Cruz (http://genome.ucsc.edu).

ACE, angiotensin-converting enzyme; APO, apolipoprotein; CART, cocaine- and amphetamine-regulated transcript; CETP, cholesterol ester transfer protein; DZ, dizygote ; LIPC, hepatic lipase ; IRS1, insulin receptor substrate 1; LCAT, lecithin:cholesterol acyl transferase; LDL-1, -2 and -3, cholesterol concentration in LDL fractions size 26.4 to 29.0 nm, 25.5 to 26.4 nm, and 24.2 to 25.5 nm, respectively; LDL-PPD, low-density lipoprotein peak particle diameter; LDLR, low-density lipoprotein receptor; Lod, logarithm of the odds; SOD2, manganese superoxide dismutase.

The APOB gene was of particular interest since it is the principal protein component of LDL particles. With the classic logarithm of the odds (LOD) score-linkage method the first two linkage studies rejected clearly the involvement of this locus with LDL subclass pattern B after obtaining LOD score of -13.3 and -7.5[154,155]. In addition, no evidence of linkage to the APOB locus was observed for LDL-PPD in families ascertained for coronary artery disease (CAD)[148]. However, a subsequent study performed in dizygotic twin pairs indicated, for the first time, positive linkage between LDL-PPD and the APOB locus[146]. Thus, it is possible that the APOB locus has an effect on LDL size in particular subgroups of the population, perhaps in women. Because low-density lipoprotein receptor (LDLR) is responsible for the clearance of apoB-containing lipoproteins, the LDLR locus on chromosome 19p was also a reasonable candidate gene for linkage analyses. Using parametric linkage analyses with reduced penetrance of pattern B, Nishina et al.[153] obtained evidence of linkage to the LDLR locus (LOD = 4.27). This finding was confirmed by a subsequent study using quantitative sib-pair linkage analyses in CAD families[148]. Borderline significant evidence of linkage was also observed between the LDLR locus and LDL-PPD in dizygotic twin pairs from the Kaiser Permanente Women Twins Study (p = 0.082)[146]. On the other hand, results from the Dutch FCHL families[149] and from families identified through hyperlipidemic probands[156] showed no evidence of linkage between the LDLR locus and either the LDL-PPD and the dichotomized pattern A/B phenotype. It is also worth mentioning that a follow-up study of the original families in which linkage to this locus has been demonstrated[153] found no mutation in the coding sequence of the LDLR gene, suggesting that a nearby gene was responsible for the linkage[157]. Using parametric linkage method and adjusting the phenotype for the within-genotype variance, Hokanson et al.[150] found in heterozygous lipoprotein lipase (LPL)-deficient families a highly significant LOD score of 6.24 between LDL-PPD and the LPL gene, which encodes a rate-limiting enzyme in the formation of LDL particles. However, two other studies have been unable to confirm this linkage in different study samples[148,156]. To assess whether the hepatic lipase (HL) gene was linked to LDL size, Allayee et al.[151] conducted sib-pairs analyses among the FCHL Dutch families using two microsatellite markers located near the HL gene (D15S643 and D15S148). In the quantitative analysis (LDL-PPD), both markers yielded evidence of linkage and in the qualitative analysis (pattern A/B) only marker D15S643 reached the level of significance. Finally, two other studies excluded the hypothesis of linkage with the HL locus[146,156]. The cholesteryl ester transfer protein (CETP)

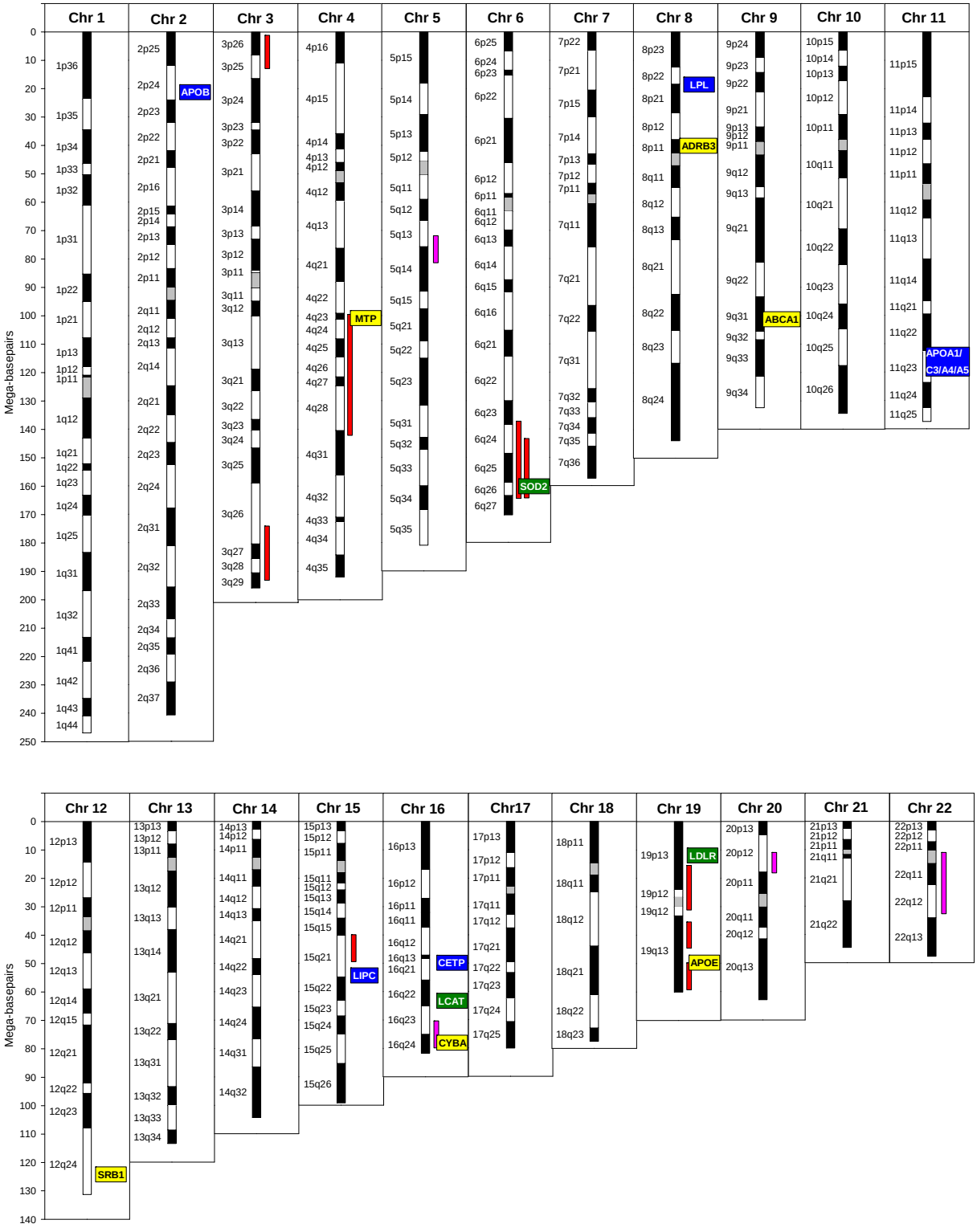
mediates the transfer of cholesterol ester from HDL to apoB-containing lipoproteins in exchange for triglyceride and thus constitutes an excellent candidate gene. Three independent studies using all sib-pairs linkage analysis have shown consistent evidence of linkage for LDL-PPD at this locus[148,149,152]. It should be noted however that the lecithin:cholesterol acyl transferase (LCAT) gene, which is responsible for the esterification of free cholesterol within HDL particles, is located nearby (≈ 10 Mb) the CETP locus and might be responsible for the signal. The APOAI-CIII-AIV gene cluster is also an interesting genetic locus potentially affecting LDL size. Rotter et al.[148] originally suggested (p = 0.06) linkage to this locus with LDL-PPD. A subsequent study was unable to confirm this linkage with the quantitative phenotype, but did so with the qualitative phenotype[149]. However, two other studies rejected the hypothesis of linkage to the APOCIII locus[146,156]. Finally, the manganese superoxide dismutase (SOD2) gene was also linked to LDL size more than once. Although the influence of this candidate gene on plasma lipoproteins is less obvious, it was linked to LDL-PPD[148] and the atherogenic lipoprotein pattern A/B[149]. However, a subsequent study provided significant evidence against linkage (LOD = -4.52) to the SOD2 locus with phenotype A/B[156]. No evidence of linkage was demonstrated for the other candidate genes tested with LDL particle phenotypes, including APOAII[148,156], APOE-CII-CI gene cluster[146,148,156], high-density lipoprotein binding protein (HDLBP)[148], hormone sensitive lipase (HSL)[146], insulin receptor (INSR)[146,156], apo(a) (LPA)[148] and microsomal triglyceride transfer protein (MTP)[146,156].

Taken together, linkage studies based on the candidate gene approach have provided positive but mainly inconsistent results. Based on these observations, Austin et al.[156] emphasized the necessity of finding new genetic loci, other than those harboring known candidate genes, to identify genes potentially involved in determining the small dense LDL phenotype. Genome-wide scans are particularly suited for this purpose. To date, two genome-wide linkage scans have been reported in the literature for LDL-PPD. Results of these genome-wide searches are indicated in bold in Table 6 and are illustrated in the Figure 10. The first whole-genome scan on LDL-PPD was performed on 240 individuals ascertained through 18 unrelated FCHL probands[151]. Results suggested a locus located approximately 12 Mb from the HL gene on chromosome 15 with a LOD score of 2.2. Suggestive linkage (LOD = 1.6) was also observed for a marker located on chromosome 19q13 which contains the APOE-CII-CI gene cluster. The second genome scan on LDL-PPD was based on 140 subjects from 26 familial hypertriglyceridemia families participating

in the Genetic Epidemiology of Hypertriglyceridemia Study[131]. For the whole-genome scan, only one chromosomal region provided possible evidence of linkage on chromosome 6q (LOD = 2.1). When the LDL-PPD was adjusted for other lipoprotein covariates, the LOD score decreased slightly but the location of the peak remained unchanged, suggesting that the signal is independent of other lipoprotein levels. The SOD2 and LPA genes are located within the one-LOD score support interval. An additional genome scan on cholesterol concentrations within LDL size subfractions is also worth mentioning. Rainwater et al.[147], found two QTLs on chromosome 3 and 4 with LOD score above 3 for LDL size 3 (LDL-3) a subfraction that contains small LDL particles. Suggestive linkage was also observed on 3p26-p25 and 6q24-q27 for LDL-3, 19p13-q12 for LDL-1 (a subfraction that contains large particles) and 19q13 for LDL-2 (a subfraction that contains particles with intermediate diameter). This study evaluated LDL size-related phenotypes, but QTLs identified are those affecting the cholesterol concentration within a particular subpopulation of LDL and do not correspond to QTLs affecting the size of the particles.

This genome scan and the two others on LDL-PPD have generated new leads in finding genes involved in LDL particle heterogeneity. Interestingly, these QTLs harbor a good number of candidate genes that have not been tested previously in linkage and association studies. Among these genome scans, only the two suggestive loci observed for LDL-PPD[131] and LDL-3[147] on chromosome 6q have shown replication (Figure 10). This locus contains the SOD2 gene which has been linked to the phenotype before[148,149] (see Linkage studies). However, the number of loci identified by genome-wide scans clearly reveals the complex genetic architecture underlying LDL particle characteristics.

Figure 10. Ideogram of human karyotype showing chromosomal locations of genes and QTLs potentially involved in LDL size/density identified from various lines of evidence. Only positive findings are included in the figure (see text for the complete results). Red and purple lines indicate QTLs identified by genome-wide linkage scans in human and animal, respectively. Genes *linked* (green rectangle), *associated* (yellow rectangle) or both *linked* and *associated* (blue rectangle) to LDL particle characteristics are shown. Genes and QTLs are placed on the hybrid map showing the sequence and the cytogenetic locations. Information to construct the ideogram has been obtained from the UCSC Genome Browser (http://genome.ucsc.edu). The alternated black and white colors on the chromosomes have been used to distinguish a cytogenic band from the adjacent ones and do not correspond to the band colors observed on Giemsa-stained chromosomes. ABCA1, ATP-binding cassette, sub-family A, member 1; ADRB3, β3-adrenergic receptor; APO, apolipoprotein; CYBA, p22 phox; CETP, cholesteryl ester transfer protein; LDLR, low-density lipoprotein receptor; LIPC, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; SOD2, manganese superoxide dismutase; SRB1, scavenger receptor class B type 1.


Chr 1
Chr 2
Chr 3
Chr 4
Chr 5
Chr 6
Chr 7
Chr 8
Chr 9
Chr 10
Chr 11
Chr 12
Chr 13
Chr 14
Chr 15
Chr 16
Chr17
Chr 18
Chr 19
Chr 20
Chr 21
Chr 22
Mega-basepairs
APOB
MTP
SOD2
LPL
ADRB3
ABCA1
APOA1/
C3/A4/A5
SRB1
LIPC
CETP
LCAT
CYBA
LDLR
APOE

Association studies

A considerable number of association studies have been conducted to identify the genes influencing LDL particle characteristics. Table 7 presents a summary of these studies organized by genes and ordered by chromosome number.

APOE.

The gene that encodes apoE lies on chromosome 19, and its three common alleles, ε2, ε3 and ε4 code for the isoforms apoE2, apoE3, and apoE4, respectively. To the best of our knowledge, a total of nine studies have investigated the association between LDL size and apoE genotypes (Table 7). The largest among them was by far the one conducted by Schaefer et al.[158] with 2258 men an women participating in the Framingham Offspring Study. In this study the age-, body mass index-, and plasma triglyceride-adjusted LDL particle type (a larger LDL type reflects smaller LDL particles) was significantly different in men with different apoE genotypes. However, the same trend was observed in men and women for higher LDL type from the ε2 to the ε4 subjects. The lowering effect of the ε4 allele was confirmed in Japanese subjects[159] and in men of North European descent[160] showing that carriers of this allele had smaller LDL particle size than those without the ε4 allele. Consistent with these observations, Haffner et al.[161] demonstrated progressive decrease in LDL size in both men and women from apoE2/3, apoE3/3 and apoE3/4 genotypes. The same study also showed that the risk of having LDL subclass pattern B was higher for subjects carrying the apoE3/4 genotype compared to both apoE2/3 and apoE3/3 genotypes. Consistent with these observations Nikkilä et al.[162] reported that LDL size was the lowest in E4/4 and increased in the order E3/4, E3/3 and E2/3. In contrast, an earlier study conducted in healthy middle aged men[163] and a second one performed in children[164] reported no difference in LDL particle size among the different apoE genotypes. To complicate even more the interpretation, two additional studies, one conducted with 132 subjects from a small 800 individuals island[165], and the other performed in 212 subjects with or without recent onset of angina[166], reported lower particle size among carriers of the ε2 allele compared to noncarriers. In the former, the difference disappeared when data were adjusted for plasma triglyceride levels while the effect of the ε2 allele in the later was still evident after such adjustment.

Table 7. Association studies between candidate genes and LDL particle characteristics.

Genes	Chr. Band	Mutation	Phenotype	n	Study population	Effect	References
APOB	2p24.1	EcoRI	LDL-PPD and LDL score	65	Caucasian men	Carriers = noncarriers	Vohl et al.[167]
		XbaI	Relative charge	104	Hypercholesterolemic men	+/+ < -/+ < -/-	Védie et al.[168]
		EcoRI				-/- and -/+ = +/+	
		MspI				-/- and +/- > +/+	
		BsrDI				-/- and +/- > +/+	
		I/D				II < ID/DD	
		3'VNTR				SS > SB/BB	
MTP	4q23	-493G>T	LDL size (NMR)	2510	Framingham Study	GG = GT = TT	Couture et al.[169]
		-493G>T	LDL-PPD	227	Viscerally obese men	GG = GT/TT	St-Pierre et al.[170]
		-493G>T	LDL-PPD	76	Chinese subjects	TT < GT/GG†	Chen et al.[171]
ADRB3	8p12-p11.2	W64R	LDL-PPD	136	Japanese subjects	RR/RW < WW	Okumura et al.[172]
LPL	8p21.3	G188E	LDL-PPD	16	LPL deficiency families	GE < GG	Miesenböck et al.[173]
		Y302-Ter	LDL-PPD	22	LPL deficiency family	Carriers < noncarriers	Bertolini et al.[174]
		LPL-HTZ	LDL-PPD	120	LPL deficiency families	LPL-HTZ < LPL-HMZ normal	Hokanson et al.[150]
		S447-Ter	LDL-PPD	189	Japanese subjects	SS < SX/XX	Sawano et al.[175]
		S447-Ter	LDL-PPD	377	North European descent men	SS < SX/XX	Skoglund-Andersson et al.[160]
		S447-Ter	LDL size (NMR)	358	Hispanic and non-Hispanic white	SS = SX/XX	Humphries et al.[176]
		D9N and P207L	LDL-PPD	206	LPL deficiency subjects	P207L HTZ < D9N HTZ	Ruel et al.[177]
CYP7	8q12.1	-204A>C	LDL size (NMR)	2330	Framingham Study	AA = AC = CC	Couture et al.[178]
ABCA1	9q31.1	Compound HTZ	LDL size (NMR)	71	One patient with TD	TD patient < controls	Schaefer et al.[179]
		ABCA1-HTZ	LDL size (NMR)	54	ABCA1 deficiency families	ABCA1-HTZ = controls	Kuivenhoven et al.[180]
APOA5	11q23.3	-3A>G	LDL-PPD	558	Japanese American families	Transmission of allele G ↓ LDL-PPD	Austin et al.[181]
APOC3	11q23.3	SstI	LDL size (NMR)	2485	Framingham Study	Carriers < noncarriers*	Russo et al.[182]
		-455T>C	LDL size (NMR)	358	Hispanic and non-Hispanic white	TT = TC + CC	Humphries et al.[176]
		-455T>C and -625T>del	LDL-PPD	320	Residents from Costa Rica	-455TT-625TT = -455CX-625delX	Brown et al.[183]
		C3238G		320		CC = CG/GG	
		SacI	Relative charge	104	Hypercholesterolemic men	-/- and +/- < +/+	Védie et al. [168]
		-625T>del				-/+ and +/+ < -/-	
		-482C>T				-/- and -/+ = +/+	
APOA1	11q23.3	PstI	Relative charge	104	Hypercholesterolemic men	-/- and -/+ = +/+	Védie et al. [168]
		MspI				-/- and -/+ = +/+	
SR-BI	12q24.31	exon 1 G>A	LDL size (NMR)	2650	Framingham Study	GG > GA/AA†	Osgood et al.[184]
		intron 5 C>T				CC = CT/TT	
		exon 8 C>T				CC = CT = TT	
LIPC	15q21.3	-250G>A	LDL-Rf	128	Normolipidemic and CAD subjects	GG < GA/AA	Zambon et al.[185]
		-514C>T	LDL-Rf	120	Premenopausal women	CC < CT/TT	Carr et al.[186]
		-514C>T	LDL type	2667	Framingham Study	CC = CT = TT	Couture et al.[187]
		-514C>T	LDL-PPD	225	Dutch subjects	CC = CT/TT	Allayee et al.[151]
		-514C>T	LDL-PPD	377	North European descent men	CC = CT/TT	Skoglund-Andersson et al.[160]
		-514C>T	LDL size (NMR)	358	Hispanic and non-Hispanic white	CC = CT/TT	Humphries et al.[176]
CETP	16q13	CETP deficiency	LDL-PPD	6	Two CETP deficient patients	CETP deficient < normal	Sakai et al.[188]

		Taq1B	LDL size (NMR)	2916	Framingham Study	B1B1 < B1B2 < B2B2*	Ordovas et al.[189]
		Taq1B	LDL size (NMR)	358	Hispanic and non-Hispanic white	B1B1 + B1B2 < B2B2**	Humphries et al.[176]
		Taq1B	LDL-Rf	120	Premenopausal women	B1B1 = B1B2 = B2B2	Carr et al.[186]
		Taq1B	LDL size (NMR)	852	VA-HIT Study	B1B1 = B1B2 = B2B2	Brousseau et al.[190]
		Taq1B	LDL-PPD	60	Subjects with type 2 diabetes	B1B1 = B2B2	Bernard et al.[191]
		Taq1B I405V	LDL-PPD	136	Japanese subjects	B1B1 = B1B2 = B2B2 VV < VI/II	Okumura et al.[192]
		I405V -629C>A	LDL size (NMR)	1276	Families with high longevity	II < VV CC = CA = AA	Barzilai et al.[129]
		-629C>A	LDL-PPD	377	North European descent men	CC < CA/AA	Skoglund-Andersson et al.[160]
		D442G	LDL-PPD	47	CAD Chinese patients	DD < DG/GG	Wang et al.[193]
CYBA	16q24.2	C242T	LDL-PPD	260	Japanese subjects	CC = CT/TT	Hayaishi-Okano et al.[194]
ACE	17q23.3	I/D	LDL-PPD	136	Japanese subjects	II = ID/DD and DD = DI/II	Okumura et al.[159]
FATP1	19p13.11	intron 8 G>A	LDL-PPD	373	Swedish men	GG = GA = AA	Gertow et al.[195]
APOE	19q13.32	ε2/3/4	LDL type	2258	Framingham Study	E4 < E3 < E2*	Schaefer et al.[158]
		ε2/3/4	LDL-PPD	136	Japanese subjects	E4 < no E4	Okumura et al.[159]
		ε2/3/4	LDL-PPD	361	North European descent men	E4 < E3 = E2	Skoglund-Andersson et al.[160]
		ε2/3/4	LDL-PPD	337	San Antonio Heart Study	E4 < E3 < E2	Haffner et al.[161]
		ε2/3/4	LDL-PPD	321	Men and women of 40 and 70 years of age	E4 < E3 < E2	Nikkilä et al.[162]
		ε2/3/4	LDL-PPD	196	Healthy men	E2 = E3 = E4	Zhao et al.[163]
		ε2/3/4	LDL size (NMR)	505	Children	E2 = E3 = E4	Isasi et al.[164]
		ε2/3/4	LDL-PPD	132	Subjects from the Ustica island	E2 < E3/E4	Barbagallo et al.[165]
		ε2/3/4	LDL-PPD	212	Subjects with or without angina	E2 < no E2	Dart et al.[166]

*Effect seen only in men. **Effect seen only in women. †Effect seen only in diabetic.

ACE, angiotensin-converting enzyme; ADRB3, β3-adrenergic receptor; APO, apolipoprotein; CETP, cholesteryl ester transfer protein; CYBA, p22 phox; CYP7, cholesterol 7α-hydroxylase; FATP1, fatty acid transport protein-1; HMZ, homozygotes; HTZ, heterozygotes; LIPC, hepatic lipase; LDL-PPD, low-density lipoprotein peak particle diameter; LDL-Rf, low-density lipoprotein flotation rate; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; NMR, nuclear magnetic resonance; SR-BI, scavenger receptor class B type 1; TD, Tangier disease.

LIPC.

The human hepatic lipase (HL) gene is located on chromosome 15q and encodes for a protein that play an important role in lipoprotein metabolism. Two polymorphisms, namely -250G>A and -514C>T (also referred to as -480C>T), have been tested with LDL particle size/density. These two polymorphisms are in linkage disequilibrium[196] and the rare allele is associated with lower HL activity[185,186]. Zambon et al.[185] were the first to describe an association between the -250G>A polymorphism and LDL particle flotation rate (LDL-Rf) measured by DGU. They have shown that the less common A allele was associated with more buoyant LDL particles among normolipidemic subjects and men with CAD. This finding was then replicated in a group of premenopausal women showing more buoyant LDL particles among carriers of the T-514 allele[186]. However, a larger study, conducted in 2667 subjects participating in the Framingham Offspring Study, found no relationship between HL polymorphism at position -514 and the LDL particle size measured by GGE[187]. This lack of association between this variant and LDL size measured by GGE was also observed in a subgroup of unrelated subjects from FCHL Ducth families[151] and in a cohort of healthy, middle-aged men[160]. The different methods used to characterize LDL particles might explain the inconsistency. However, an additional study rejected the hypothesis of association between -514C>T polymorphism and LDL particle size measured by NMR[176].

CETP.

The cholesteryl ester transfer protein (CETP) gene lies on chromosome 16q an encodes a protein that facilitates the exchange of triglyceride and cholesterol between lipoproteins. The LDL particles of CETP deficient patients comprised a group of abnormal heterogeneous particles which show polydispersity on GGE with a smaller mean particle size[188]. The most studied RFLP in this gene, Taq1B in intron 1, was associated with CETP activity and mass. In fact, the B2 allele (absence of the Taq1 restriction site) was associated with decreased CETP activity and mass, which mimics a mild form of CETP deficiency[186,189]. The Framingham Offspring Study has again provided the largest population-based cohort (n = 2916) investigating this polymorphic site with LDL size[189]. This study reported that the B2 allele in men was associated with increased levels of large LDL subfraction whereas B1B1 homozygous subjects had increased levels of

small LDL subfraction. Therefore, the B2 allele was associated with increased LDL particle size, an effect seen only in men. The effect of the B2 allele was also observed in the Columbia University BioMarker Study, but this time only in women[176]. In this study, women homozygous for the B2 allele had larger particles compared to carriers of the B1 allele. This difference was also observed in men, but the small number of men tested might have lacked the power to detect a significant effect. In contrast, absence of association between the Taq1B polymorphism and LDL-Rf was reported in a cohort of normolipidemic premenopausal women[186]. A trend toward greater LDL size with increasing number of B2 allele was observed in the VA-HIT group, but the effect did not reach statistical significance[190]. This lack of association was also observed in a cohort of patients with type 2 diabetes[191] and in Japanese subjects[192]. However, a second polymorphism in the CETP gene, namely I405V, showed a significant association in this Japanese study. They demonstrated lower LDL size in patients with the VV genotype compared to carriers of the I allele. A significant effect of this polymorphism was also observed in families ascertained for exceptional longevity[129]. However, in this cohort subjects carrying the VV genotype had larger LDL particles. The later study also reported no association between LDL size and a third gene variant in linkage disequilibrium with the Taq1B polymorphism named -629C>A. In contrast, carriers of the -629C>A polymorphism had increased LDL-PPD compared to CETP-629C homozygotes in a cohort of healthy, middle-aged men[160]. Finally, a less frequent missense mutation, D442G in exon 15, in the CETP gene was investigated in patients with CAD. The presence of this mutation was associated with lower CETP concentrations and larger LDL size[193].

MTP.

The microsomal triglyceride transfer protein (MTP) gene, located on chromosome 4q, encodes for a protein essential in the assembly and secretion of apoB-containing lipoproteins in hepatocytes and enterocytes. A common functional polymorphism in the promotor of the MTP gene, -493G>T, was investigated in relation to LDL particle size. Couture et al.[169], showed no association between the -493G>T genotype and LDL size in 2510 subjects participating in the Framingham Offspring Study. This absence of association was also observed in a group of viscerally obese men[170]. The only positive association between the -493G>T variant and LDL

size was observed in a small sample of type 2 diabetes Chinese[171]. They reported smaller LDL particle size among TT homozygotes compared to the other genotypes.

LPL.

The lipoprotein lipase (LPL) gene is located on chromosome 8p and encodes a protein that is responsible for the hydrolysis of triglyceride within apoB-containing lipoproteins. Several mutations have been identified in the LPL gene and some of them have been associated with LDL particle size. First, a missense mutation at codon 188 cause a clear reduction in LPL activity. Carriers of this defective mutation had smaller LDL size compared with noncarrier family members[173]. Similarly, lower LDL size was observed among carriers of the LPL Tyr302-Ter mutation in an Italian family[174]. Hokanson et al.[150] confirmed the LDL reducing size effect of LPL deficiency in five families with structural mutations in the LPL gene. Subsequently, the Ser447-Ter mutation in exon 9 was associated with larger LDL size[160,175]. This mutation cause a premature termination codon which surprisingly increases the enzymatic activity of LPL[160,197]. These observations suggested that the mutation that decreases LPL activity cause a reduction in LDL size and the mutations that increase LPL activity increase LDL size. This hypothesis was confirmed in a cohort of 206 heterozygote subjects carrying of either the null P207L or the defective D9N mutations[177]. In this study, LDL particle size was smaller in the P207L carriers than in the D9N subjects, suggesting that a greater reduction in LPL activity results in smaller LDL particle size. However, this relation between LPL genetic variants, LPL activity and LDL size is not without controversy given that the greater LDL particle size observed among carriers of the Ser447-Ter mutation was not confirmed in the Columbia University BioMarkers Study[176].

APOA1/C3/A4/A5 cluster.

The APOA1/C3/A4/A5 cluster lies on chromosome 11q and encodes four proteins involved in lipoprotein metabolism. Genetic variations within individual gene have been associated with LDL particle characteristics. Russo et al.[182] tested the SstI polymorphism on the 3' untranslated region of the APOC3 gene and showed that male carriers of the S2 allele had significantly lower concentrations of large LDL and a significant reduction in LDL size. In women, there was no significant effect on LDL size. The other polymorphisms tested in the APOC3 gene (-455T>C,

-625T>del and C3238G) showed no association with LDL particle size[176,183]. However, the SacI and the -625T>del polymorphisms located in the 3' untranslated and the promotor regions of the APOC3 gene, respectively, were significantly associated with LDL charge[168]. In contrast, the same study reported no association between APOA1 restriction sites (PstI and MspI) and LDL charge. Recently, Austin et al.[181] demonstrated, with several analytic approaches, that common SNP variants in the APOA5 gene are associated with LDL particle size in a community-based sample of Japanese American families. This study particularly pinpoints the -3A>G variant to decreased LDL size. However, considering the close proximity of the four genes in the cluster, it is difficult to infer with certainty that the effect observed with one of them is mediated by the gene tested. Indeed, a positive finding in one gene might be due to linkage disequilibrium of the tested marker with a polymorphism in a second gene within the cluster. Accordingly, further studies in the APOA1/C3/A4/A5 gene cluster will be required to identify the functional site.

Other candidate genes: APOB, CYP7, ACE, ADRB3, CYBA, FATP1, SR-BI, LDLR and ABCA1.

One study verified the effect of the APOB EcoRI polymorphism in a group of Caucasian men and showed no effect on either LDL-PPD or LDL score[167]. However, five polymorphisms in the APOB gene were found to influence LDL charge heterogeneity evaluated by relative electrophoretic mobility[168]. A common A to C substitution at position -204 in the promoter of the cholesterol 7α-hydrolase (CYP7) gene showed no association with LDL particle size[178]. The hypothesis that the angiotensin-converting enzyme (ACE) gene insertion/deletion polymorphism was associated with LDL size was also rejected in a small Japanese cohort[159]. However, a recent paper suggested a positive association between the Trp64Arg variant in the β_3-adrenergic receptor (ADRB3) and LDL-PPD[172]. The authors reported that the LDL particle size was smaller in the subjects with the Arg64 allele than those without the Arg64 allele. The effect remained significant after triglyceride adjustment, but disappeared after adjustment for body mass index or parameters of insulin resistance. The phox 22 gene (CYBA), which is a small subunit of vascular NAD(P)H oxidase playing an important role in superoxide production, was also investigated in a group of healthy Japanese subjects[194]. A trend ($p = 0.08$) toward larger LDL-PPD was observed among carriers of the C242T polymorphism compared to noncarriers. In addition, the proportion of subjects with pattern B was significantly larger in the CC group than CT/TT group. No

association was observed between a functional intronic variation in the fatty acid transport protein-1 (FATP1) gene and LDL-PPD in a cohort of healthy Swedish men[195]. However, the cholesterol concentration ratio of the largest and smallest LDL subfractions (LDL-I/LDL-III ratio) were found to be different between FATP1 intron 8 genotypes. No clear association was observed between three SNPs located within the scavenger receptor class B type 1 (SR-B1) gene and LDL size in the Framingham Study[184]. However, this study showed reduced LDL particle size in carriers of the A allele at the SR-BI exon 1 gene in the subgroup of diabetic subjects. Finally, no study investigated the effect of common polymorphisms in the LDLR gene on LDL particle characteristics. However, earlier studies showed that the LDL particles of patients with familial hypercholesterolemia are characterised by higher peak flotation rate and lower density[198,199]. Similarly, one patient with the Tangier disease was shown to have smaller particle size compared to control subjects highlighting the possible implication of the ATP-binding cassette 1 (ABCA1) gene[179]. However, the reducing LDL size effect of a defective mutation in the ABCA1 gene was not reported in a group of heterozygous subjects[180].

Complementary genetic approaches

LDL size: a component of the metabolic syndrome and a trait representing a common atherogenic lipoprotein profile

The metabolic syndrome is characterized by a cluster of CAD risk factors including hypertension, upper-body obesity, glucose intolerance and the atherogenic lipoprotein phenotype which consists of elevated plasma triglyceride levels, low plasma levels of HDL-C and a predominance of small, dense LDL[200]. The strong association between the small, dense LDL phenotype and the atherogenic lipoprotein profile raises the question whether the gene proposed by complex segregation analyses is also responsible for the associated lipid and lipoprotein levels. Using factor analysis, Edwards et al.[201,202] investigated the clustering of risk factors in the Kaiser Permanente Women Twins Study by examining the correlation structure among the components of the metabolic syndrome. Factor analysis reduced 10 correlated risk factors to 3 uncorrelated factors, each reflecting a different aspect of the metabolic syndrome. One of the factor was considered the *lipid factor* due to the strong factor loading for the lipid variables including triglyceride, HDL-C and LDL-PPD. Heritability estimates for the lipid factor was calculated using various approaches and ranges from 0.25 to 0.32. Thus the authors suggested that approximately a quarter to a third of the variance in this composite lipid factor may be attributable to genetic influences. Using a candidate gene strategy, the same research group subsequently found a strong evidence of linkage between the lipid factor and the CETP gene[203]. The authors proposed that the CETP gene variations influence the covariation in LDL size, triglyceride and HDL-C levels, and may account for a portion of the phenotypic correlation between these risk factors.

To investigate the interrelationship between LDL particle size, triglyceride and HDL-C levels, Edwards et al.[128] reported genetic correlations between pairs of traits. The genetic correlation between LDL-PPD and triglyceride was -0.87, suggesting that 76% [$\rho_G^2 = (-0.87)^2 = 0.76$] of the additive genetic variance in LDL size is shared with triglyceride. The genetic correlation between LDL-PPD and HDL-C was more modest (0.65) but suggested that nearly 50% of the additive genetic variance in each of these traits is due to shared genes. However, based on the likelihood-ratio test, the hypothesis of complete pleiotropy was rejected for the two genetic correlations, suggesting the existence of unique genes for each trait. These results demonstrated that the

observed phenotypic associations between these three traits are largely under genetic control and indicated that searching for genes implicated in LDL size may actually mean searching for genes also involved in triglyceride and HDL-C. A similar study conducted by Rainwater et al.[130] reported a genetic correlation between lipoprotein size traits (ΔLDL and ΔHDL) and triglyceride. Triglyceride and ΔHDL were strongly correlated with ΔLDL, with genetic correlations of -0.76 and 0.56, respectively. Thus, shared genes accounted for 58% and 31% of the genetic variance in each pair of traits.

Small, dense LDL is also metabolically associated with elevated plasma apoB levels and both features are found in patients with FCHL[115]. Thus, some investigators searched for a common genetic mechanism between these two traits in families characterized by FCHL. Using bivariate segregation analysis, Juo et al.[204] reported the evidence of a common genetic mechanism controlling both apoB levels and the distribution of LDL subfraction (parameter K) in FCHL families. The best-fitting model proposed a common gene with codominant allele for both traits, plus distinct polygenic component for each trait. This major gene explained 37% and 23% of the variance in parameter K and in apoB levels. On the other hand, Jarvik et al.[205] have shown that LDL subclass phenotype B and apoB levels are two traits influenced by two mendelian locus independent of each other and modulating the risk of FCHL. This conclusion was drawn by showing: 1- that the major gene effect seen in segregation analysis for apoB levels remained after adjustment for LDL subclass phenotypes and 2- by showing lack of association between LDL subclass phenotype and the apoB level predicted genotypes in contingency analysis. Finally, using commingling analysis, Austin et al.[206] reported bimodality of apoB levels in individuals with LDL subclass phenotype B. This finding suggested distinct genetic mechanisms for LDL subclass phenotype and apoB levels in FCHL families. The conflicting results between these studies may due to the different statistical strategies employed or may simply reflect the complexity of the genetic mechanisms for these traits.

Taken all together, it appears that distinct sets of genes influence LDL size: those that influence LDL size independent of triglyceride and other lipid parameters and those that affect several components of the lipid profile. Thus, in addition to the genes uniquely influencing LDL size, there appear to be genetic factors that are responsible for covariation in lipoprotein/lipid traits, which demonstrate the complexity of characterizing genetic influences on LDL size.

Animal model

Few, but relevant studies on animal models have confirmed the presence of genetic factors influencing LDL size. First, LDL size vary substantially between different strains of mice, showing the effect of the genetic background. Jiao et al.[207] characterized LDL size by liquid chromatography in 10 inbred strains and observed a LDL size range starting at 24.16 nm in BALB/c strain to 29.39 nm in SWR strain, with the whole spectrum of size within this interval for the other strains. In an attempt to test whether LDL size was an inherited trait in mice, three sets of recombinant inbred strains were produced by crossing strains with different LDL size. By this mean, authors have shown that LDL size of recombinant inbred strains segregated to one or another progenitor 88% of the time, implying that LDL size may be controlled by the product of a major gene. Attempts to identify the major LDL-size determining gene yielded only marginal significant results for a RFLP analysis in the APOB gene.

An attempt was also made to establish whether genes control variation in LDL size in baboons[208]. A 150 baboons members of 19 sire groups were investigated. Baboons were fed three diets contrasting in levels of fat and cholesterol. A multifactor ANOVA revealed that 18.3% of the variation in LDL size was explained by the sire groups. In addition, there was a significant sire×diet interaction on the phenotype, indicating that members of different sire groups responded differently to various dietary compositions. Taken together, these results suggested that genes influence LDL size and the patterns of LDL response to different diets in baboons. Recently, a genome-wide linkage scan was performed among an enlarged group of these baboons to localize the genes that control LDL size fractions[209]. Using GGE, four LDL size-related phenotypes were constructed based on fractional absorbance in four intervals of LDL (LDL4, 24-26 nm; LDL3, 26-27 nm; LDL2, 27-28 nm; and LDL1, 28-30 nm). The LDL median diameter was also estimated, which is a diameter where half the LDL absorbance is on larger and half is on smaller particles. Genome scans were performed on LDL size-related phenotypes taken from blood samples collected at the end of each experimental diets. On a high-cholesterol high-fat diet, a significant evidence of linkage (LOD = 4.22) for LDL2 was observed on the baboon homologue of human chromosome 20 and 22 (Figure). Two additional QTLs were suggested, one on the baboon homologue of human chromosome 16 for LDL3 when exposed to a low-cholesterol low-

fat diet (LOD = 2.15), and one on the baboon homologue of human chromosome 5 for LDL3 when exposed to a low-cholesterol high-fat diet (LOD = 2.67). The later QTL is particularly relevant since the signal was also observed for the LDL median diameter (LOD = 2.21).

These results have clearly shown the usefulness of animal studies to identify the LDL size genes. Due to our ability of controlling tightly the animals environment, these studies might prove to be even more relevant in the future for testing gene-environment interactions.

Gene-gene and gene-environment interactions

Relatively little is known about gene-gene and gene-environment interactions in LDL particle characteristics, but it would be surprising if they were not important. A preliminary study has shown that the LDL bands of monozygotic twins were more concordant than dizygotic twins before but not after a 22 weeks exercise program, suggesting that the genetic contribution of LDL subfractions decreases with exercise[210]. It was also demonstrated that the LDL size response to a low-fat diet in children was predicted by the parental LDL subclass pattern[211]. Tentative evidence of interactions with LDL size phenotypes were also reported for specific loci. A significant interaction was observed between SR-BI exon 1 genotypes and type 2 diabetes on LDL size, indicating that diabetes status modifies the effect of this polymorphism on LDL particle size[184]. St-Pierre et al.[170], for example, have shown an inverse effect of the MTP -493G>T genotypes according to visceral adipose tissue and fasting insulin. It is also apparent from association studies (Table 4) that the effect of some loci are sex-specific or reserved to subgroup of the population (diabetic for example). Zambon et al. [212] also reported an interesting pharmacogenetic interaction on LDL density. They showed that the -514C>T polymorphism in the HL gene promoter strongly influences the LDL flotation rate response in middle-aged men undergoing intensive lipid-lowering therapy. Although these studies are interesting examples, they demonstrate the high number of interactions that could be tested and the difficulty to do so in humans. Clearly, when the loci controlling small LDL will be mapped, there will be a greater potential for determining the gene-gene and gene-environment interaction effects.

Plan and strategy

As seen in the previous sections, the metabolic syndrome and its individual components are under genetic influences. However, the progress made in the search for single genes and QTLs associated with phenotypes related to the metabolic syndrome has been slow and difficult so far. Although a great deal of literature exists in the field, the overall picture is ambiguous and more research is clearly needed.

In the following chapters, we used a combination of measured (bottom-up) and unmeasured (top-down) genotype approaches to uncover the genetic architecture underlying the metabolic syndrome and its individual components. We believe that genetic dissection of complex traits requires multiple approaches in order to achieve our goals. In the first four chapters, we used a candidate gene approach. The genes were chosen based on their biological relevance with the metabolic syndrome. It is also well known that complex traits arise from interactions between multiple genes and environments, but not much has been done to date. Accordingly, in chapters 1 and 3 we also integrated the concept of pharmacogenetics and gene-gene interactions. In the next two chapters (5 and 6) we used a genome-wide search approach to identify novels or replicate previous QTLs acting on the variability of serum lipid, lipoprotein and apolipoprotein levels. This attempt is made to generate useful leads of positional candidate lipoprotein/lipid genes that will need to be tested in future studies. Chapters 7 to 9 focus on the genetic of LDL peak particle size. As shown in the previous sections, this component of the metabolic syndrome is an independent cardiovascular risk factor for which the genetic basis has just begun to be uncovered. From the preceding reports, its becoming clear that the small, dense LDL phenotype is under genetic influences. However, the specific genes remained to be identified. In this series of chapters, we used the traditional steps to understand the genetic basis of a quantitative phenotype (LDL-PPD) as presented in Figure 8. These steps include familial aggregation, heritability and segregation analyses as well as genome-wide linkage scan and association studies on positional candidate genes. Finally, the last chapter deals with the metabolic syndrome as a whole entity. The goal is to find the genetic loci contributing to the cluster of the metabolic syndrome-related phenotypes. The approach used might identify pleiotropic genes acting on several features of the metabolic syndrome or genes explaining the common variance of these clustering risk factors.

General hypothesis

Genetics factors are involved in the development of the metabolic syndrome and its individual components.

Specific hypotheses

1. The lipoprotein/lipid response to gemfibrozil therapy is modulated by the PPARα L162V polymorphism;

2. PPARα, PPARγ_2 and PLTP are candidate genes containing genetic variants influencing features of the metabolic syndrome;

3. Lipid, lipoprotein and apolipoprotein levels variations are influenced by quantitative trait loci.

4. LDL size is under genetic control and is influenced by positional candidate genes;

5. The common variance among components of the metabolic syndrome can be used to construct a single quantitative variable influenced by quantitative trait loci.

General objectives

1. To assess candidate genes potentially involved in the metabolic syndrome and its individual components;

2. To identify genomic regions harboring genes influencing features of the metabolic syndrome;

3. To determine the genetic contribution and the genetic determinants of LDL size.

Specific objectives

1. To determine whether the lipid response to gemfibrozil therapy is influenced by the PPARα L162V polymorphism;

2. To evaluate the effect of the PPARα L162V polymorphism on obesity-related phenotypes;

3. To verify the independent effect of PPARα L162V and PPARγ_2 P12A polymorphisms as well as their interactive impact on indices of plasma glucose and insulin homeostasis;

4. To evaluate the effect of PLTP variants on obesity-related phenotypes;

5. To identify the genomic regions influencing total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, apoB and apoAI levels;

6. To verify whether familial factors influence LDL-PPD and assess the heritability of this phenotype;

7. To performe a genome scan to identify genomic regions containing genes influencing LDL-PPD;

8. To sequence, genotype and test positional candidate genes potentially implicated in LDL-PPD;

9. To performed a genome scan on a quantitative metabolic syndrome factor derived by factor analysis.

Chapter 1.

Influences of the PPARα-L162V Polymorphism on Plasma HDL_2-Cholesterol Response of Abdominally-Obese Men Treated with Gemfibrozil.

Yohan Bossé, Agnès Pascot, Martine Dumont, Martin Brochu, Denis Prud'homme, Jean Bergeron, Jean-Pierre Després, Marie-Claude Vohl.

L'effet hypolipidémiant du gemfibrozil est assuré par l'activation des récepteurs activés par les proliférateurs de peroxisomes alpha (PPARα). L'objectif de cette étude était de déterminer si le polymorphisme L162V du gène PPARα influençait la réponse lipidique suite à un traitement au gemfibrozil. À cet effet, soixante-trois hommes caractérisés par de l'obésité abdominale ont été randomisés dans un groupe placebo (n=31) et un groupe gemfibrozil (n=32) pour une durée de six mois. En réponse au gemfibrozil, les sujets homozygotes pour l'allèle L162 ont subit une augmentation de leur niveau plasmatique de cholestérol-HDL_2 de 5,5% comparativement a une augmentation de 50% pour les hommes porteurs de l'allèle V162. Ces résultats suggèrent que le changement des concentrations plasmatiques de cholestérol-HDL_2 est influencé par le polymorphisme PPARα L162V suivant un traitement au gemfibrozil.

Influences of the PPARα-L162V Polymorphism on Plasma HDL_2-Cholesterol Response of Abdominally-Obese Men Treated with Gemfibrozil

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Short title: PPARα-L162V Polymorphism and Response to Fibrate

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Abbreviations: PPARα, peroxisome proliferator-activated receptor alpha; HMZ, homozygotes; NCEP, National Cholesterol Education Program; Apo, apolipoprotein; PCR, polymerase chain reaction; ABC-A1, ATP-binding cassette transporter 1; CLA-1, CD36- and LIMPII-analogous 1; SR-B1, scavenger receptor class B type 1.

Abstract

Purpose: The effect of gemfibrozil is mediated by the activation of PPARα. The objective of this study was to determine whether the lipid response to gemfibrozil therapy is influenced by the PPARα-L162V polymorphism.

Methods: Sixty-three abdominally-obese men were randomly assigned to a 6-month-intervention program with either receiving a placebo (n=31) or gemfibrozil (n=32).

Results: In response to gemfibrozil therapy, L162-homozygotes exhibited a 5.5% increase in HDL_2-C levels compared to a 50.0% increase among carriers of the V162 allele (p=0.03).

Conclusion: These results suggest that the HDL_2-C response to gemfibrozil is modulated by the PPARα-L162V polymorphism.

Key words : genetics, fibrates, lipid response, lipoproteins, HDL-cholesterol.

Introduction

Alterations of the lipoprotein-lipid profile are associated with an increased risk to develop coronary heart disease, the leading cause of death in westernized societies. Many lipid-lowering agents have been developed for the treatment of dyslipidemia. In this regard, the clinical benefits of gemfibrozil, a fibrate agent, have been reported in both primary[1] and secondary[2] prevention trials. Although fibrates have been used in clinical practice for more then three decades, their molecular mechanism of action has just recently been elucidated[3] and it is now recognized that their effects are mediated by the activation of a specific nuclear receptor termed peroxisome proliferator-activated receptor alpha (PPARα). The activation of PPARα by fibrates causes the transactivation of PPARα-responsive genes which include those encoding proteins that control lipid metabolism[3].

Recently a molecular scanning of the human PPARα has revealed a L162V polymorphism associated with alterations of the lipoprotein-lipid profile[4,5]. The frequency for the rare allele is established to be 0.062 in the healthy European population[5] and reaches 0.128 in French-Canadians[4]. In order to demonstrate the functional consequences of this polymorphism, transient transfection assays in Hepa-1 and HepG2 cell lines have been performed[5,6]. Both studies have shown enhanced transactivation activity in cells containing vectors expressing PPARα-V162 allele, compared to L162 allele, when treated with the PPARα ligand WY-14,643. In this context, it was interesting to verify whether the L162V polymorphism in the PPARα gene can modulate the plasma lipoprotein/lipid response to gemfibrozil.

Materials and methods

Subjects

Subjects were asymptomatic, non diabetic volunteers who had to fulfill the following criteria. Men had to be between 25 and 55 years of age willing to participate to a 6-month-intervention program in which they were asked to follow the NCEP Phase 1 dietary guidelines with or without gemfibrozil. Experience from the Helsinki Heart Study revealed that the effect of gemfibrozil was largely confined to overweight subjects[7]. Men in the present study were weight stable obese (27 and 40 kg/m^2) and characterized by a dyslipidemic state (1.7 mmol/L ≥ TG ≤ 5.7 mmol/L; HDL-C ≤1.2 mmol/L and total plasma cholesterol < 6.7 mmol/L). The study was approved by the Medical Ethics Committee of Laval University. All subjects gave their informed written consent to participate in this study.

Study design

After having completed their baseline measurements, 71 subjects were selected to participate to the study and were randomly assigned to either receiving a placebo or gemfibrozil 600 mg bid. Dietary recommendations were given by a registered dietician on a voluntary basis for the duration (6 months) of the study. Drug safety was assessed every four weeks by the physician in charge of the project. Subjects were tested at baseline and at the end of the 6-month intervention protocol. During that period, there were 6 dropouts and genotype information was not available for two subjects. We thus ended-up with a total of 63 subjects who completed the trial.

Lipids and lipoproteins

Fasting blood samples were collected and plasma lipid, lipoprotein and apolipoprotein levels were measured as previously described[8]. HDL_2 was precipitated from the HDL fraction with dextran sulfate[9]. The cholesterol content of the supernatant fraction (HDL_3) was determined, and HDL_2-C levels were derived by subtracting HDL_3-C from total HDL-C concentrations. Nondenaturing 4-30% polyacrylamide gel electrophoresis was performed for the measurement of the average HDL size using whole plasma kept at -80°C as recently described[10]. The same

plasma were also used to determine the LDL peak particle size measured by electrophoresis on a 2-16% polyacrylamide gradient gel as previously described[11,12].

DNA analysis

The L162V polymorphism does not alter any restriction site. A mismatch PCR method previously described was used to genotype all the subjects participating to the study[4].

Statistical analyses

Variables not normally distributed were $\log_{10}$ transformed prior to analysis. The difference in response between genotype groups was assessed by a two-tailed unpaired Student's *t*-test. To evaluate whether the PPARα-L162V genotype may interact with gemfibrozil treatment, we performed an ANOVA in which the interaction term was included (two-way factorial ANOVA). The source of variation in lipoprotein-lipid profile was computed using the type III sum of squares. This sum of squares applies to unbalanced study designs and quantifies the effects of an independent variable after adjustment for all other variables included in the model. All statistical analyses were performed using the SAS package (SAS Institute, Cary, NC) and a statistically significant difference was defined as $p < 0.05$.

Results

Among the 63 men who completed the study, 31 were assigned to the placebo group and 32 to the gemfibrozil group. The effects of the 6-month-intervention program on plasma lipoprotein/lipid profile have been published elsewhere[8]. In the present study, the placebo and gemfibrozil groups were further subdivided on the basis of the PPARα-L162V genotype (Figure 1).

The baseline lipoprotein-lipid profile according to the PPARα-L162V genotype is presented in Table 1. For these analyses, subjects in the placebo and gemfibrozil groups were combined since medication had not started. No statistically significant difference was observed between the two genotype groups for baseline lipid and lipoprotein concentrations. However there was a tendency toward higher LDL-C levels among carriers of the V162 allele compared to L162-HMZ ($p = 0.08$).

The plasma lipoprotein-lipid changes of the six-month gemfibrozil therapy was compared between carriers and non-carriers of the PPARα-L162V polymorphism (Table 2). There was no statistically significant difference between the two genotype groups for all lipoprotein-lipid changes except for HDL_2-C levels. Indeed, V162 carriers exhibited a 50% increase in HDL_2-C concentrations compared to a 5.5% increase among L162-HMZ (Figure 2). The same trend was observed with changes in HDL-C levels although it did not reach statistical significance ($p=0.08$). These results were the same after adjustment for either baseline measurements or changes observed in body weight (data not shown). To corroborate the difference observed between the two genotype groups on HDL_2-C changes with gemfibrozil, we analysed the average HDL size change with the therapy. Although not statistically significant, carriers of the V162 allele have a more favorable HDL size response compared to L162-HMZ (Table 2). There was also a tendency toward a greater decrease in LDL-apo B for those carrying the V162 allele ($p=0.09$). This trend might be explained by a greater reduction in LDL-C and a greater increase in LDL size observed in V162 allele carriers although the difference did not reach statistical significance.

When individual HDL-C responses to gemfibrozil were plotted (Figure 3), two phenomena were noted. First, there was a large interindividual variation in HDL-C changes with gemfibrozil therapy. Second, there was a cluster of carriers of the V162-allele on the right side of the graph

indicating a greater increase of HDL-C levels among these individuals. In these individuals, the apparent difference in plasma HDL-C levels was mainly explained by changes in the HDL_2-C subfraction since no difference was observed between the genotype groups regarding the change in the HDL_3-C subfraction (Figure 2). Subsequent analyses performed in men treated with gemfibrozil revealed that after the six-month-intervention program, V162 carriers were characterized by higher levels of HDL_2-C compared to L162-HMZ (Figure 4).

To test the potential interaction between the PPARα-L162V polymorphism and gemfibrozil treatment on plasma lipoprotein-lipid concentrations, an analysis of variance was performed for each lipoprotein-lipid variable. The effects of the genotype, the treatment, as well as the potential interaction between these two independent variables are presented in Table 3. As expected, the treatment (placebo vs gemfibrozil) had a statistically significant impact on plasma lipoprotein-lipid changes. On the other hand, the PPARα-L162V genotype by itself did not have a significant impact on plasma lipoprotein-lipid responses. However, a significant genotype-by-treatment interaction was observed for changes in plasma HDL_2-C levels. This interaction explained 7.0% of the total variance of the change in plasma HDL_2-C concentrations. This finding suggests that the PPARα-L162V polymorphism may influence plasma HDL_2-C responsiveness to gemfibrozil therapy. Additionally, it also implies that men carrying the PPARα-V162 allele will experience higher increase in HDL_2-C levels when treated with fibrates.

Discussion

The documented inter-individual variation in the response to fibrate therapy is clinically relevant. In this report, we demonstrated that a naturally occurring variation, L162V, in the PPARα gene is associated with greater responsiveness of HDL_2-C levels following a 6-month-therapy with gemfibrozil. Prior to this study, Flavell et al.[5] had reported a greater lowering effect of bezafibrate on total cholesterol and non-HDL-C in V162 carriers. In the present study, there was a trend toward higher increase in HDL-C levels following the administration of gemfibrozil but it did not reach statistical significance. Taken together these results suggest potentially greater benefits of fibrate treatment among individuals carrying the PPARα-V162 allele. This could be of great interest since previous work suggest that the PPARα-L162V polymorphism seems to have a deleterious impact on plasma lipoprotein-lipid levels[4,5,13].

PPARα regulates the expression of gene encoding proteins that control lipoprotein metabolism. *In vitro* studies performed on hepatic cells (HepG2 and Hepa-1) have demonstrated higher transactivation activity in PPARα carrying the V162 allele when treated with a PPARα agonist[5,6]. Differences observed in HDL_2-C responsiveness in the present study, could then be explained by a greater transcriptional regulation of PPARα target genes in subjects carrying the V162 allele. Several proteins controlling HDL metabolism are regulated at the gene level by PPARα activators such as fibrates. In fact, fibrate therapy has been shown to induce overexpression of apo A-I and apo A-II genes leading to an increase in plasma HDL-C levels[14,15]. Recently, other proteins such as ABC-A1 and CLA-1/SR-B1 involved in the reverse-cholesterol-transport pathway have been shown to be upregulated by PPARα activators in macrophages[16,17]. Furthermore, it has been demonstrated in mice that fibrate treatment increases phospholipid transfer protein gene expression through a PPARα-dependent mechanism which accounts for a marked enlargement of HDL particles[18]. The functional consequence of the PPARα-L162V polymorphism could result in higher transcriptional regulation of genes controlling HDL metabolism and then explain the relationship between the PPARα-L162V polymorphism and the HDL_2-C response to gemfibrozil observed in the present study.

This study reported associations that make biological sense and study genetic variations that affect the gene product in a physiologically meaningful way. However, certain limitations may give some uncertainty about our results. First, dealing with the low frequency of the polymorphism and with a small sample size impaired our ability to recognize association with smaller effect. Second, considering the multiple comparisons made in the study, some of the significant findings may have occurred by chance. Consequently, further studies with larger sample size are warranted to determine the interest of the PPARα L162V polymorphism in the management of dyslipidemia.

In summary, we have demonstrated that subjects carrying the PPARα-V162 allele showed higher increases in HDL_2-C levels in response to gemfibrozil therapy. This finding could be of clinical relevance for these individuals since a low concentration of the HDL_2 subfraction seems to be more closely related to the incidence of ischemic heart disease[19,20]. We speculate that the greater HDL_2-C increase among gemfibrozil-treated-men carrying the PPARα-L162V polymorphism is mediated by a more pronounced transcriptional regulation of genes controlling HDL metabolism.

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Table 1. Baseline Lipoprotein-Lipid Concentrations Between Carriers and Non-Carriers of the PPARα-L162V Polymorphism.

	PPARα-L162V Genotype		
Variable (mmol/L)	L162 HMZ n = 52	V162 Carriers n = 11	p
Triglycerides	2.66 ± 0.80	2.34 ± 0.59	0.20
LDL-TG	0.39 ± 0.10	0.34 ± 0.04	0.23
HDL-TG	0.23 ± 0.05	0.21 ± 0.03	0.15
Cholesterol	5.57 ± 0.61	5.75 ± 0.37	0.35
LDL-C	3.71 ± 0.60	4.03 ± 0.35	0.08
HDL-C	0.84 ± 0.12	0.90 ± 0.14	0.16
HDL_2-C	0.17 ± 0.06	0.19 ± 0.09	0.43
HDL_3-C	0.67 ± 0.10	0.71 ± 0.07	0.18
Plasma apo B (g/L)	1.22 ± 0.15	1.24 ± 0.12	0.63
LDL-apo B (g/L)	1.04 ± 0.16	1.10 ± 0.09	0.23
HDL-apo A1 (g/L)	1.16 ± 0.12	1.20 ± 0.09	0.28
Average HDL size (Å)	82.08 ± 2.03	82.18 ± 2.49	0.90
LDL size (Å)	248.99 ± 3.81	251.04 ± 3.63	0.15

Values are means ± SD. All values are mmol/L except when indicated. HMZ : homozygotes.

*10 L162/V162-heterozygotes and 1 V162-homozygote.

Table 2. Changes in Plasma Lipid and Lipoprotein Levels in Gemfibrozil-Treated Participants According to PPARα-L162V Genotypes.

	PPARα-L162V Genotype		
Variables (mmol/L)	L162 HMZ n = 26	V162 Carriers n = 6	p
Triglycerides	-1.14 ± 0.84	-0.76 ± 1.09	0.35
LDL-TG	-0.07 ± 0.09	-0.10 ± 0.05	0.41
HDL-TG	-0.02 ± 0.06	-0.00 ± 0.06	0.67
Cholesterol	-0.51 ± 0.52	-0.67 ± 0.49	0.49
LDL-C	-0.05 ± 0.69	-0.48 ± 0.33	0.15
HDL-C	0.06 ± 0.09	0.14 ± 0.10	0.08
HDL_2-C	0.01 ± 0.07	0.09 ± 0.11	0.03
HDL_3-C	0.06 ± 0.11	0.05 ± 0.11	0.91
Plasma apo B (g/L)	-0.13 ± 0.21	-0.21 ± 0.08	0.38
LDL-apo B (g/L)	-0.04 ± 0.19	-0.18 ± 0.08	0.09
HDL-apo A1 (g/L)	0.03 ± 0.10	0.09 ± 0.12	0.27
Average HDL size (Å)	-0.43 ± 1.44	0.59 ± 1.12	0.13
LDL size (Å)	1.07 ± 3.67	1.28 ± 2.86	0.91

Values are means ± SD. All values are mmol/L except when indicated. HMZ : homozygotes.

*6 L162/V162-heterozygotes.

Table 3. Effects of PPARα-L162V Polymorphism, the Treatment (Placebo vs Gemfibrozil) and their Interaction on Plasma Lipid-Lipoprotein Response to the Intervention Program.

		p		
Phenotypes (mmol/L)	Mean Changes n = 63	Genotypes	Treatment	Interaction
Triglycerides	-0.55 ± 0.94	0.52	0.0009	0.42
LDL-TG	-0.04 ± 0.08	0.83	0.001	0.35
HDL-TG	0.00 ± 0.06	0.70	0.18	0.80
Cholesterol	-0.36 ± 0.47	0.66	0.005	0.50
LDL-C	-0.13 ± 0.52	0.96	0.86	0.62
HDL-C	0.03 ± 0.10	0.21	0.0001	0.14
HDL_2-C	0.02 ± 0.08	0.32	0.15	0.04
HDL_3-C	0.01 ± 0.11	0.88	0.01	0.76
Plasma apo B (g/L)	-0.10 ± 0.16	0.69	0.02	0.29
LDL-apo B (g/L)	-0.05 ± 0.15	0.31	0.08	0.06
HDL-apo A1 (g/L)	0.03 ± 0.10	0.74	0.06	0.20
Average HDL size (Å)	0.03 ± 1.56	0.19	0.56	0.55
LDL size (Å)	0.15 ± 2.91	0.12	0.51	0.18

Values are means ± SD. All values are mmol/L except when indicated.

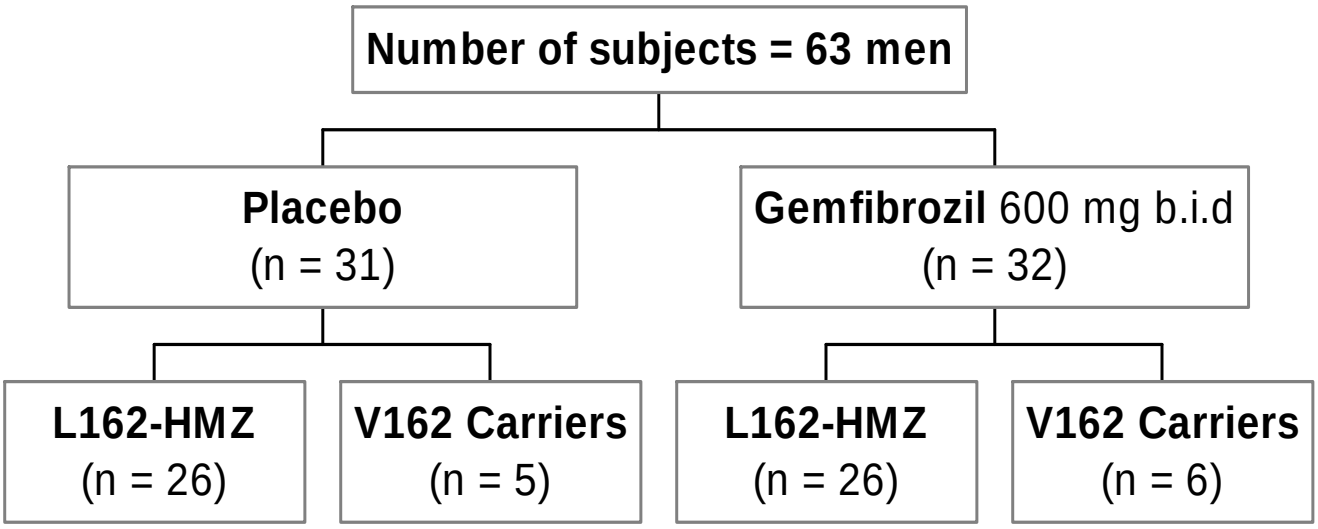

Figure 1. Distribution of subjects into the intervention program. Men were randomly assigned to either receiving a placebo or gemfibrozil and then divided according to PPARα-L162V genotype. HMZ : homozygotes.

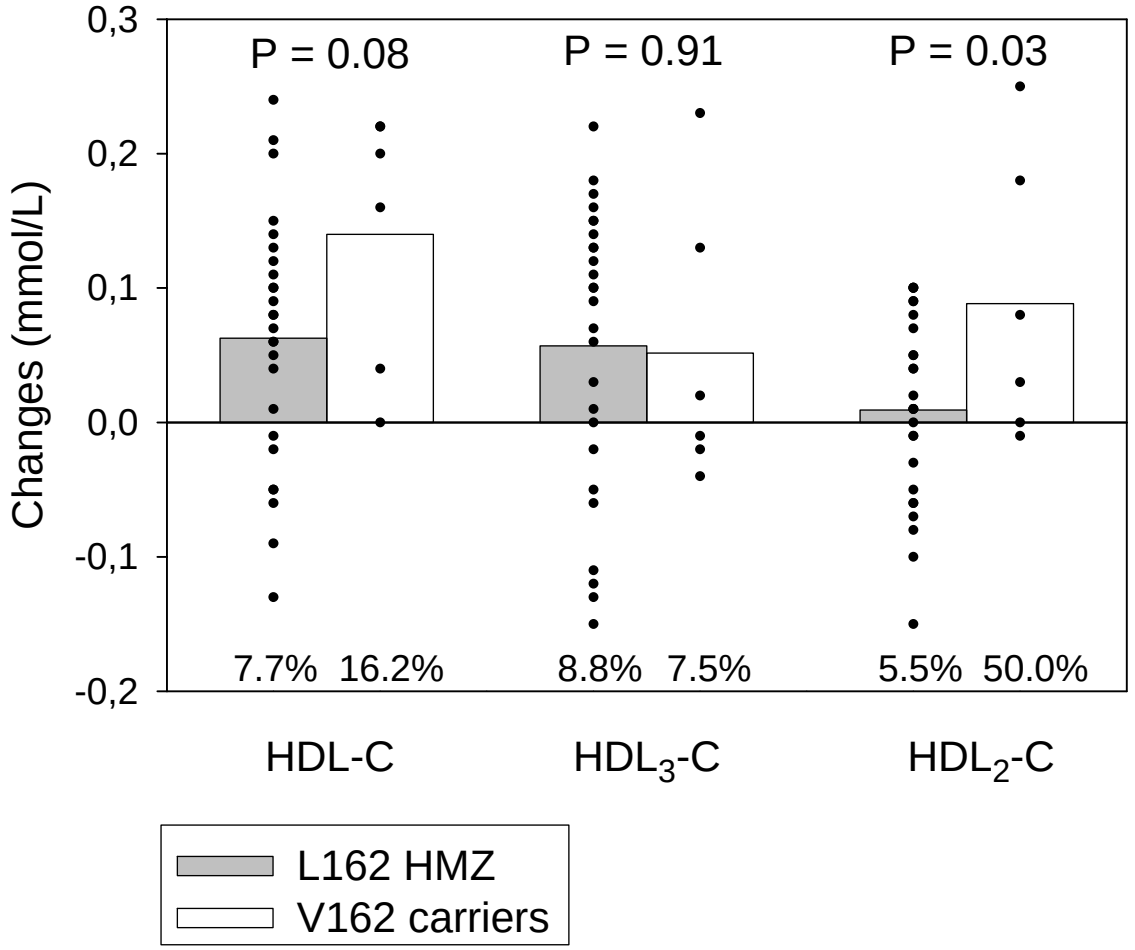

Figure 2. Changes in HDL-cholesterol and HDL-cholesterol subfractions in gemfibrozil-treated participants according to PPARα-L162V genotype (n = 26 L162-HMZ and 6 V162 carriers). Each dot represent the change observed for one individual within his respective group. Relative change is indicated at the bottom of the graph. The bar chart illustrates mean HDL changes in the different groups.

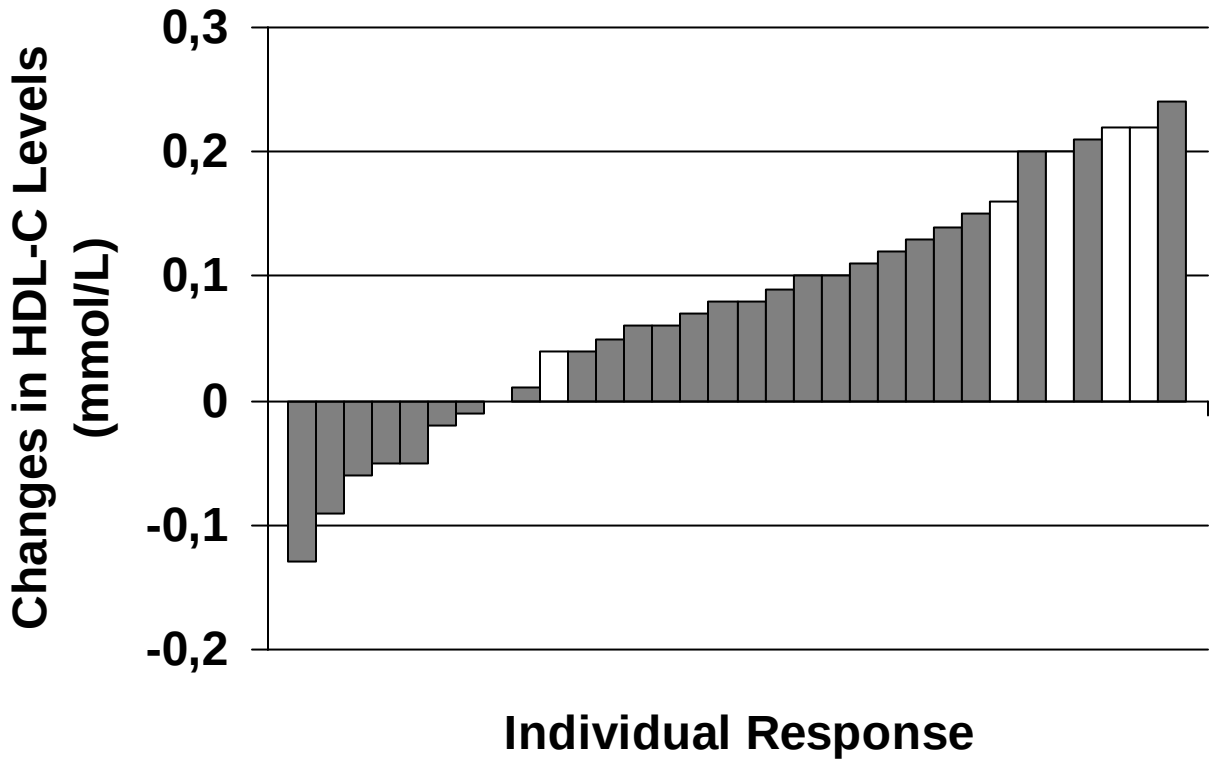

Figure 3. Individual changes in HDL-cholesterol levels among gemfibrozil-treated subjects (n = 32). Each bar represents the after-before difference in HDL-cholesterol levels observed in response to gemfibrozil therapy. Individual responses of L162-homozygotes are illustrated by the grey bars whereas the white bars show the responses of carriers of the V162 allele.

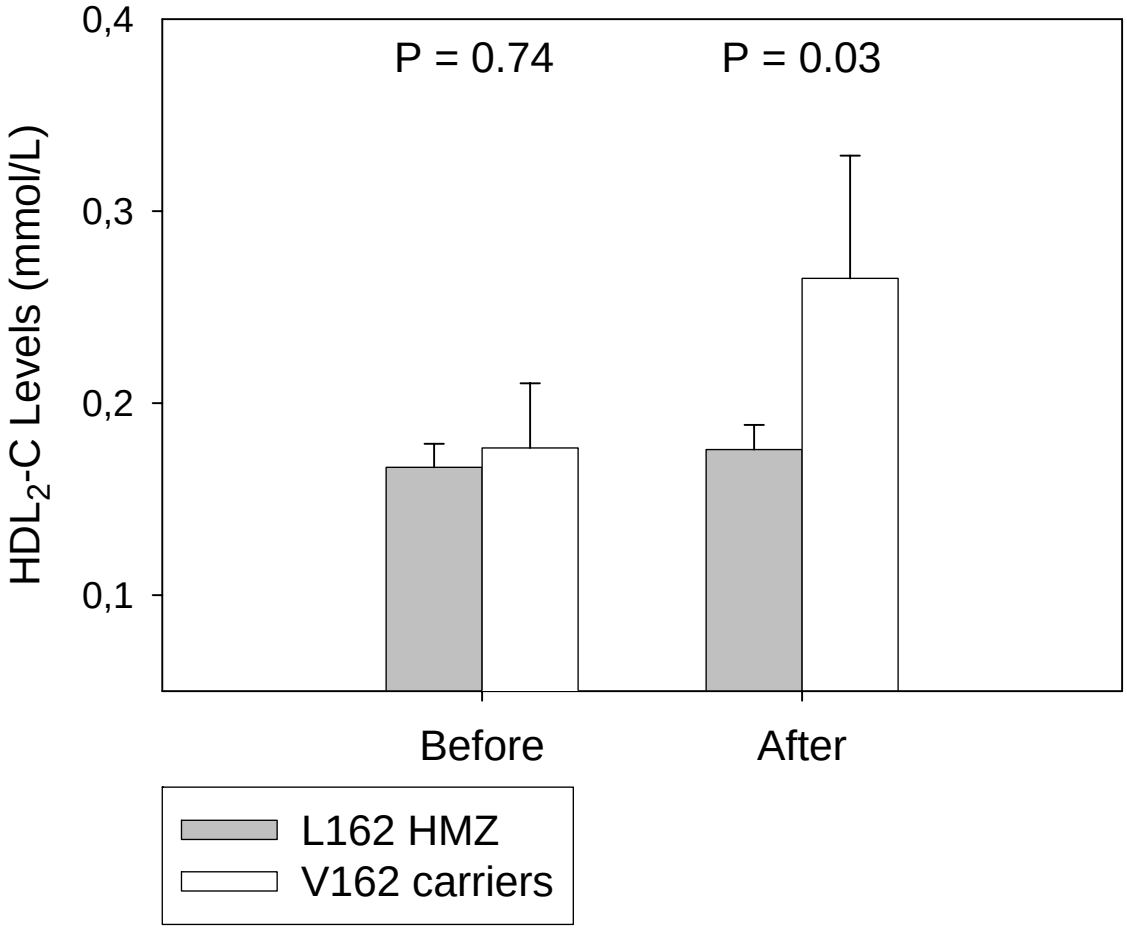

Figure 4. Mean HDL2-C values before and after gemfibrozil treatment according to PPARα-L162V genotype (n = 26 L162-HMZ and 6 V162 carriers). Values are mean ± SE. HMZ : homozygotes.

Chapter 2.

The Peroxisome Proliferator-Activated Receptor α L162V Mutation Is Associated with Reduced Adiposity.

Yohan Bossé, Jean-Pierre Després, Claude Bouchard, Louis Pérusse, Marie-Claude Vohl.

L'objectif de cette étude était de déterminer l'importance du polymorphisme PPARα L162V sur les variations de plusieurs indices d'adiposité mesurés chez des adultes participant à l'Étude des familles de Québec. Les phénotypes d'adiposité ont été obtenus par des mesures anthropométriques standards, pesé hydrostatique et tomographie axiale. Pour tous les phénotypes d'adiposité, les sujets porteurs de l'allèle V162 avaient de plus faibles valeurs comparativement aux sujets homozygotes L162. Le rapport de cote désignant le risque d'avoir un indice de masse corporelle supérieur à 30 kg/m^2 était de 1.77 (1.02 ; 3.07, IC à 95%) pour ces derniers. Sur une base individuelle ce risque peut être considéré modeste. Par contre, étant donné que 85% des sujets sont affectés par ce petit risque, l'impact populationnel est important. Ces résultats suggèrent que le polymorphisme L162V du gène PPARα est associé avec les indices d'adiposité et un risque populationnel substantiel.

The Peroxisome Proliferator-Activated Receptor α L162V Mutation Is Associated with Reduced Adiposity.

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Short title: Reduced body fatness among carriers of PPARα L162V mutation.

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Abstract

PPARα is highly expressed in tissues with elevated fatty acid catabolic rates. In rodents, PPARα activation by specific ligands has been shown to prevent high fat diet-induced obesity and to reduce body weight in genetic models of obesity.

Objective: Determine the contribution of the PPARα L162V mutation to the variation of several indices of body fatness obtained from healthy adults who participated in the Quebec Family Study.

Research Methods and Procedures: The presence of the PPARα L162V mutation was determined by a PCR-RFLP based method and subjects were classified into L162 homozygote (HMZ) or V162 carriers. Adiposity phenotypes were obtained by standardized anthropometric measurements, underwater weighing technique and computed tomography (CT) and compared among the two groups after adjustment for age and gender effects.

Results: For all adiposity phenotypes, subjects carrying the V162 allele had lower values compared to L162 HMZ [BMI (kg/m^2): 27.8 ± 7.6 vs 26.0 ± 5.6, $p < 0.05$; percent body fat: 28.5 ± 10.7 vs 25.7 ± 10.1, $p < 0.05$; waist circumference (cm): 89.0 ± 18.1 vs 85.7 ± 15.8 , $p = 0.07$; total CT abdominal fat areas (cm^2): 406 ± 221 vs 359 ± 192, $p = 0.15$; means ± SD for L162 HMZ vs V162 carriers respectively]. Differences in cross-sectional abdominal adipose tissue areas and waist circumference were abolished after adjustment for total body fat mass. Similar trends were observed when results were analyzed by gender although associations seemed stronger in women. The odds ratio of having a BMI above 30 kg/m^2 reached 1.77 (1.02 ; 3.07, 95% confidence intervals) for L162 HMZ. This risk could be considered marginal on an individual basis, but, since 85% of the subjects are affected by this small risk, the impact on the population is important.

Discussion: The PPARα V162 allele is associated with reduced adiposity and has a substantial population attributable risk.

Key words : nuclear receptor, missense mutation, fat mass, population risk

Introduction

Obesity has reached epidemic proportions that have generated a progressive economic burden on medical health care (1-3). However, obesity is a heterogeneous condition which is attenuated or exacerbated by genetic and nongenetic factors and is referred to as a complex multifactorial trait. Understanding the genetic contribution of such trait is of great interest since a large spectrum of susceptibility genes play a role in the development of obesity (4). In order to detect the modest effect of each gene, association studies performed on large population samples are required (5). These additive modest contributions can then be used to predict the risk to become obese or could also be used as molecular targets for pharmacological treatment of this condition. Recently, the peroxisome proliferator-activated receptor alpha (PPARα) gene has emerged as one of these potential genes which could be involved in the etiology of obesity.

PPARα is a nuclear hormone receptor member of the superfamily of nuclear receptors (6) activated by endogenous and xenobiotics ligands (7). This ligand-activated transcription factor is expressed in several tissues but predominantly among those with elevated rates of fatty acid catabolism (8, 9). Since the identification of PPARα a decade ago (10), several ligands for this nuclear receptor have been identified, including fatty acids, particularly polyunsaturated fatty acids, eicosanoids, and hypolipidemic drugs such as fibrates (11-13). After activation by its ligand, the activated PPARα heterodimerizes with the retinoid X receptor (RXR) and this complex then binds to peroxisome proliferator response elements (PPRE) of genes to regulate their expression. PPARα-responsive genes include those encoding crucial enzymes involved in the regulation of intra- and extracellular lipid metabolism (14, 15). Non-exhaustively, PPARα upregulates genes involved in fatty acids uptake and transport, in the β- and ω-oxidation pathways and in ketone body synthesis (16, 17). Thus, PPARα regulates the intracellular fate of fatty acids by increasing fatty acid oxidation, and prevent fat storage into adipocytes which is the long term process leading to obesity. The potential preventing effect of PPARα against obesity development is represented schematically in Figure 1.

Two lines of evidence confirm this theoretical model. Firstly, in rodents PPARα activators were shown to reduce body weight and adiposity in diet-induced obesity (18-20). These results suggest that the activation of PPARα in rodents prevents and reduces obesity. Secondly, PPARα

deficient mice were founded to develop late onset obesity despite a stable caloric intake (21). These mice have been proposed as a model of monogenic obesity with a marked sexual dimorphism. In fact, females PPARα deficient mice develop a more pronounced obesity than their male counterpart. Thus, variation in the PPARα gene may play a role in the development of obesity.

Recently, a missense mutation has been identified in the DNA binding domain of the human PPARα gene (22, 23). This mutation is located in exon 5 and results in the substitution of a leucine for a valine at codon 162. In vitro transfection studies revealed that the rarer V162 allele has greater transactivation on the reporter gene construct (23, 24). It was thus of great interest to verify whether the L162V mutation would be associated with adiposity in human. Thus, the objective of the present study was to investigate the contribution of the PPARα L162V mutation on several phenotypes of body fatness obtained from healthy adults who participated in the Quebec Family Study (QFS). accordingly

Methods

Population

Subjects were participants in phases 2 and 3 of the QFS (25). Briefly, the QFS is a population-based study of French-Canadian families living in and around Quebec City area. Subjects were recruited through the media. Only adults (305 men and 393 women), 20 years and older, were considered for the present analyses. The 698 subjects included in this study are members of 253 nuclear families. The mean number of subjects by family is 3.00 ± 1.34 (range 1 to 8). Characteristics of the subjects are presented in Table 1 which indicates that the QFS cohort covered a wide range of body fatness values. The Medical Ethics Committee of Laval University approved the protocol and a written consent was obtained from all the subjects.

Body fatness measurements

Body weight, height and waist circumference were measured following standardized procedures (26). Body density was measured by the hydrostatic weighing technique (27). Pulmonary residual volume was assessed before immersion in the hydrostatic tank, using the helium dilution technique of Meneely and Kaltreider (28). Percentage of body fat, fat mass and fat free mass were derived from body density using the Siri equation (29). Finally, a cross-sectional abdominal scan was performed by computed tomography using a Siemens Somatom DRH scanner (Erlanger, Germany) to quantify the adipose tissue areas between L4 and L5 vertebra as described in details elsewhere (30).

DNA analysis

The L162V mutation, caused by a C→G transversion at nucleotide 484 in exon 5, does not alter any restriction site. A mismatch PCR method previously described was then used to genotype individuals of the QFS cohort (22). Briefly, the mismatch PCR was performed with the following primers 5'-GACTCAAGCTGGTGTATGACAAGT-3' and 5'-CGTTGTGTGACATCCCGACAGAAT-3' (note the mismatch nucleotide in the reverse primer is underlined). PCR conditions were as follows: reaction volume was 50 μl, 1.25 unit AmpliTaq

Gold polymerase (Perkin-Elmer Cetus) in the buffer recommended by the manufacturer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, primers at a final concentration of 0.5 μM and 100 ng of template genomic DNA. This products were then digested with *Hinf*I, electrophoresed through either 12% acrylamide or 4% agarose gel, and stained with ethidium bromide.

Statistical methods

Variables with a skewed distribution were $\log_{10}$-transformed. Means of these variables are given in tables and illustrations with their raw scores (scores before transformation) instead of their geometric means, but the p-values are given from the $\log_{10}$-transformed distributions. Differences between genotypic groups were assessed using the MIXED model procedure for association studies, which takes the nonindependence of family members into account. In this model, age as well as gender, when males and females are considered together, were used as covariates. Logistic regression analyses were used to assess the association between PPARα L162V mutation and obesity. This association was investigated by classifying subjects into two groups using a 30 kg/m^2 as the cutoff point. The risk of being obese for subjects that did not carry the mutation was estimated as the relative risk to have a BMI > 30 kg/m^2 compared to subjects who had the mutation. Odds were adjusted for the potential confounding effects of age, gender, smoking status and alcohol consumption. Covariates that did not significantly influence BMI were removed from the model. All statistical analyses were performed using the SAS package (SAS Institute, Cary, NC) and statistical significance was set at $p < 0.05$.

Results

Body fatness and body fat distribution variables according to the PPARα L162V genotypes are presented in Figure 2. In the total group, carriers of the V162 allele had lower BMI compared to L162 HMZ. As for BMI, carriers of the V162 allele tend to have lower body weight. Results from underwater weighing derived phenotypes revealed that the relative amount of adipose tissue was lower among carriers of the V162 allele. However, no difference between the genotypic groups was observed for fat free mass in the overall sample. This finding suggests that the mutation is specifically related to the adipose tissue compartment of the body.

We verified the effect of the mutation on body fat distribution. Carriers of the V162 allele tended to have lower waist circumference compared to L162 HMZ (Figure 2). However, this trend was no longer observed after adjustment for body fat mass suggesting that the mutation was more closely associated with the amount of total fat in the body rather than with its distribution (not shown). Comparison of abdominal adipose tissue distribution indices assessed by computed tomography revealed lower values of total, visceral and subcutaneous cross-sectional areas of adipose tissue for carriers of the V162 allele but the difference did not reach statistical significance (Figure 2). Finally, no difference in the visceral/total adipose tissue areas ratio was observed between carriers and non-carriers of the V162 allele ($p = 0.844$) which demonstrates again that the mutation is not associated with fat distribution.

Since fat mass and fat distribution differed for men and women, data were also analyzed separately by sex (Table 2). Independently of the gender, carriers of the V162 allele seem to have lower values of adiposity compared to L162 HMZ. Although the genotype difference was not significant, the trends appeared to be stronger in women. Taken all together, subjects carrying the V162 allele had lower obesity indices compared to L162 HMZ. When data were analyzed by gender, similar trends were observed but associations appeared to be stronger in women than in men.

Further analyses were also performed with the three genotype groups instead of combining V162 HMZ together with L162/V162 HTZ (L162/L162, L162/V162 and V162/V162). Percent body fat, fat mass and total and subcutaneous abdominal fat areas seemed to be lower in V162 HMZ compared to the other groups (data not shown). However results must be interpreted with caution

due to the low number of subjects included in the V162 HMZ group (n = between 6 and 12). Since ethanol has been shown to inhibit PPARα activity (31), analyses were also performed after adjustment for alcohol consumption. However, such an adjustment did not influence the results (data not shown). Additional adjustment for smoking status did not modify the results as well.

The risk of having a BMI above 30 kg/m^2 for L162 HMZ genotype is shown in Figure 3. The odds ratio is estimated to be approximately 1.46 ($p = 0.150$) without adjustment for confounding factors. After adjustment for factors that are known to affect obesity such as age, gender and alcohol consumption, the odds ratio reached 1.77 ($p = 0.041$). Thus, according to these data, the PPARα L162V mutation may appear as having only a modest impact on the adiposity. However, since the prevalence of the elevated risk genotype (L162 HMZ) was found to be very high (a frequency of 85% in our sample), an adjusted genotype relative risk of 1.77 could correspond to a high population attributable risk.

Discussion

Results of the present study suggest that the PPARα L162V mutation may be involved in the pathogenesis of obesity. Indeed, for all adiposity phenotypes, subjects carrying the PPARα V162 allele had lower values compared to L162 HMZ. Similar trends were observed when results were analyzed by gender although associations were stronger in women. On the other hand, the risk of becoming obese in the absence of the mutation was relatively small (OR = 1.77, p = 0.041), suggesting that the PPARα L162V mutation only had a modest impact on an individual basis. However, since the vast majority (85% of individuals in our sample) of whites subjects are exposed to this moderate risk, the effect could translate into a large population-attributable risk.

Given the central role of PPARα in the intra and extracellular lipid metabolism, the L162V mutation has been investigated in the development of several pathologies including type 2 diabetes and obesity. Three independent studies reported no significant difference in the allele frequency between diabetics and nondiabetics, suggesting that this missense mutation does not seem to play a major role in the development of type 2 diabetes (22, 32, 33). However, Evans et al. (33), reported in a group of type 2 diabetes patients (BMI = 29 ± 7) and in a second group of morbid obese subjects (BMI = 51 ± 8) having a fasting glucose ≥ 7 mmol/L, that the frequency of the V162 allele in patients with BMI below the median (28 and 49 kg/m^2 in type 2 diabetes and in morbidly obese patients, respectively) was higher compared to subjects above those respective cutoffs. However, the missense mutation was not associated with BMI in subjects without type 2 diabetes. There results suggested that the BMI lowering effect of the V162 allele could be present only among patients with type 2 diabetes. Results of the present study were derived from a relatively "healthy" population. Difference between results of Evan et al. and those of the study could be explained by a greater contribution of the mutation among people in whom an elevated PPARα activation is expected, such as type 2 diabetes. In such case, a smaller study sample may be sufficient to detect an effect. On the other hand, a larger sample size might be required when dealing with subjects free of chronic metabolic diseases.

The "protective" effects of PPARα against the development of obesity have been highlighted. Fibrates are a widely used class of hypolipidemic agents which act through PPARα activation (34). In rodent models, fibrate treatment prevents weight gain and reduces adipose

tissue in high fat diet-induced obesity and genetic models of obesity, respectively (18-20). Thus, PPARα activation seems to decrease body weight. In addition the lack of PPARα appears to favor the development of obesity, since PPARα-deficient mice (under the C57BL/6N background) become progressively obese on a regular chow diet (21). The latter conclusion was based on a long term experiment (8 months) demonstrating that the lack of this nuclear receptor in mice caused a progressive onset of obesity. Taken into account the key regulatory enzymes controlled by PPARα, the authors suggested that the onset of obesity in this mouse model may depend upon the impairment of pathways regulating lipid metabolism since they were not hyperphagic. These mice were also characterized by a sexual dimorphism. Indeed, females developed a more pronounced obesity than males, a finding consistent with the greater associations between the PPARα L162V mutation and the body fatness phenotypes observed in the present study. However, in a similar experimental set up (9 months on a chow diet), Akiyama et al. (35) concluded that the weight gain and the average body weight in wild-type and PPARα-null mice were not markedly different between genotypes. Nonetheless, a trend was observed for higher body weight throughout the protocol among PPARα male and female null mice bred on a C57BL/6N background. In addition, adipose tissue stores were significantly greater in PPARα-null mice than in controls. Furthermore, adding WY-14643, a potent PPARα activator, in the diet of the wild-type mice significantly reduced their body weight as well as adipose tissue stores, an effect not observed in PPARα-null mice. Greater weight gain in PPARα-null mice (C57BL/6N) following a chow diet was also reported during a shorter experimental period (four weeks) (36). Taken together these *in vivo* studies indicate that deactivation of PPARα increases body fatness and its activation does the opposite.

The observation that the PPARα L162V mutation may play a causal role in body fat gain is strengthened by studies on functional differences between the leucine-containing and valine-containing protein products (23, 24). In fact, co-transfection assays have demonstrated that the PPARα V162 allele has an enhanced transactivation activity on the reporter gene construct compared with the PPARα L162 allele in presence of a PPARα ligand. Thus, it is tempting to speculate that the reduced adiposity values observed in subjects carrying the PPARα V162 allele are explained by a greater activity of the valine-containing protein. Therefore, individuals with

the PPARα V162 allele would have an enhanced fatty acid oxidation, limiting body fat accumulation.

Obesity is a complex multifactorial trait which involves the additive effect of several gene polymorphisms (4). Association studies are widely anticipated to contribute to the understanding of complex traits (5). However, to be credible an association study must fill several criteria (37). Ideally, such studies require large sample size that allow sufficient power to detect genetic variation having modest effects. Studies with small sample size can often fail to detect true associations. Association studies can be useful if their findings make biological sense and if the allelic variation upon which they are based result in functional biological differences, which is the case in the present study.

In conclusion, the present study reports that the PPARα L162V mutation is associated with several phenotypes of body fatness obtained from healthy adults who participated in the QFS. In addition, the greater risk of being obese associated with the absence of the PPARα V162 allele appears to have a modest impact on an individual basis, but could have a major effect from a general population point of view because of the high frequency of the L162 wild-type allele. The PPARα L162V mutation highlights the potential importance of common alleles with a rather weak effect. We speculate that this effect is mediated by a greater transactivation of the PPARα V162 allele on fatty acid metabolizing enzymes. Further studies will be necessary in order to replicate our results in populations with different genetic backgrounds.

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Table 1. Characteristics of the Subjects.

			Range	
	n	**Mean ± SD**	**Minimum**	**Maximum**
Age (years)	698	44.2 ± 16.4	20.0	93.5
Weight (kg)	698	75.0 ± 20.7	35.0	181.1
BMI (kg/m^2)	698	27.5 ± 7.4	16.8	64.9
Waist circumference (cm)	670	88.5 ± 17.8	57.9	164.5
Percent body fat	570	28.2 ± 10.7	2.9	59.8
Fat mass (kg)	570	22.3 ± 13.7	1.7	108.3
Fat free mass (kg)	570	52.6 ± 10.6	30.7	87.6
Abdominal adipose tissue areas (cm^2)	482			
Total		400 ± 218	46	1066
Visceral		116 ± 76	15	443
Subcutaneous		284 ± 169	16	872

Table 2. Body Fatness and Body Fat Distribution Phenotypes by PPARα L162V Genotype for Men and Women Separately.

Phenotypes	Men			Women		
	L162 HMZ (n)	V162 Carriers (n)	p*	L162 HMZ (n)	V162 Carriers (n)	p*
Antropometry measurements						
Weight (kg)	81.3 ± 19.1 (261)	78.1 ± 13.6 (44)	0.447	71.4 ± 22.1 (330)	65.6 ± 15.3 (63)	0.104
BMI (kg/m^2)	27.4 ± 6.3 (261)	26.1 ± 4.6 (44)	0.156	28.1 ± 8.6 (330)	25.9 ± 6.3 (63)	0.075
Waist circumference (cm)	94.2 ± 16.4 (257)	91.1 ± 12.3 (43)	0.088	84.7 ± 18.3 (312)	81.7 ± 17.0 (58)	0.300
Underwater weighing measurements						
Percent body fat	23.5 ± 9.1 (229)	20.8 ± 7.7 (33)	0.134	32.8 ± 10.1 (267)	29.7 ± 10.1 (41)	0.101
Fat mass (kg)	20.4 ± 12.8 (229)	17.0 ± 8.6 (33)	0.399	24.7 ± 14.7 (267)	20.9 ± 11.6 (41)	0.110
Fat free mass (kg)	60.8 ± 8.1 (229)	61.6 ± 7.7 (33)	0.231	45.7 ± 7.0 (267)	45.2 ± 5.8 (41)	0.704
Abdominal fat areas by CT (cm^2)						
Total	355 ± 196 (186)	331 ± 174 (23)	0.605	446 ± 231 (234)	376 ± 202 (39)	0.173
Visceral	131 ± 83 (186)	133 ± 80 (23)	0.531	105 ± 67 (234)	94 ± 76 (39)	0.126
Subcutaneous	224 ± 134 (186)	198 ± 116 (23)	0.501	341 ± 182 (234)	282 ± 141 (39)	0.184
Visceral/Total ratio	0.38 ± 0.10 (186)	0.40 ± 0.12 (23)	0.720	0.24 ± 0.08 (234)	0.24 ± 0.08 (39)	0.853

Values are means ± SD.

*adjusted for age.

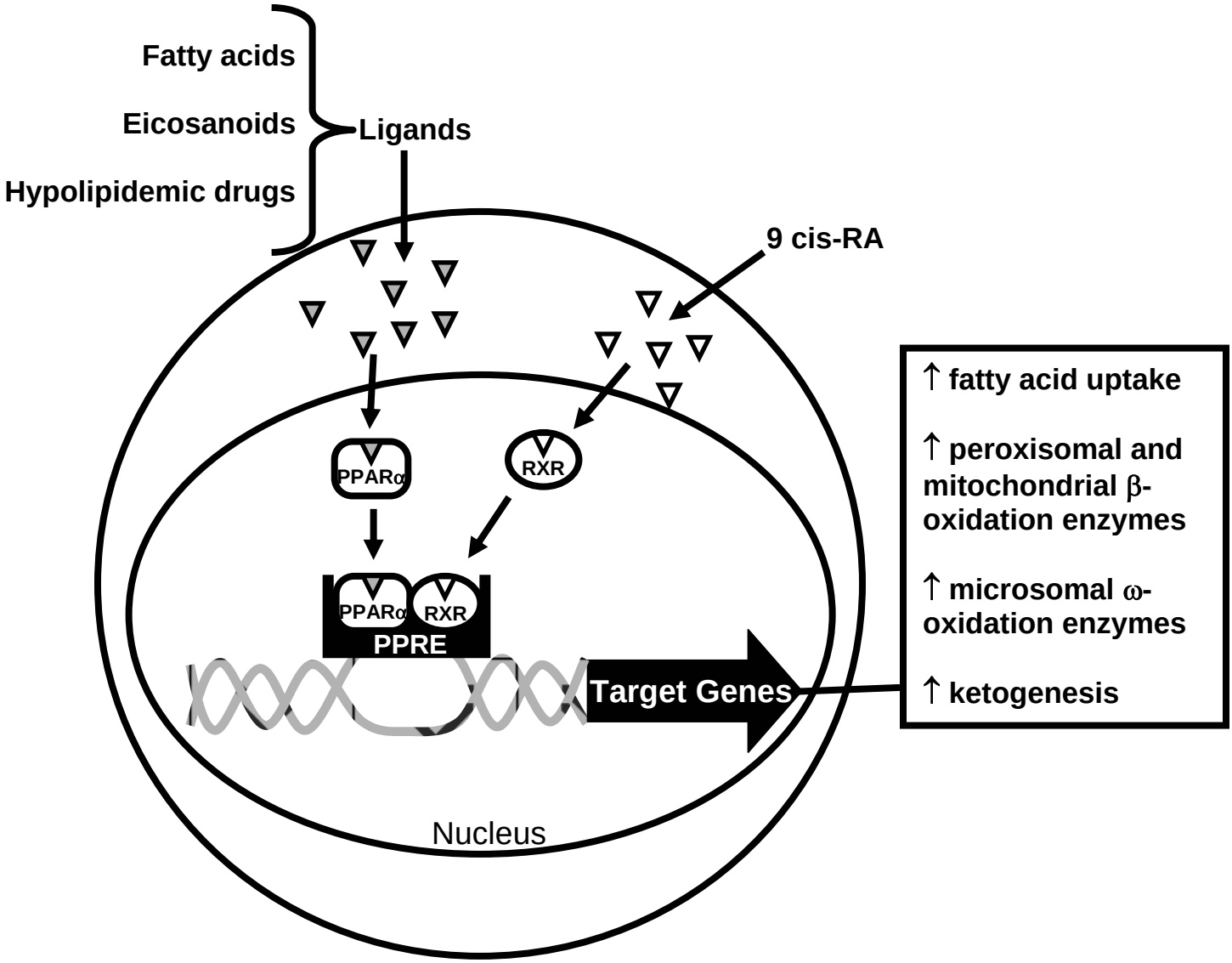

Figure 1. The potential mechanism by which PPARα may reduce body fat accumulation.

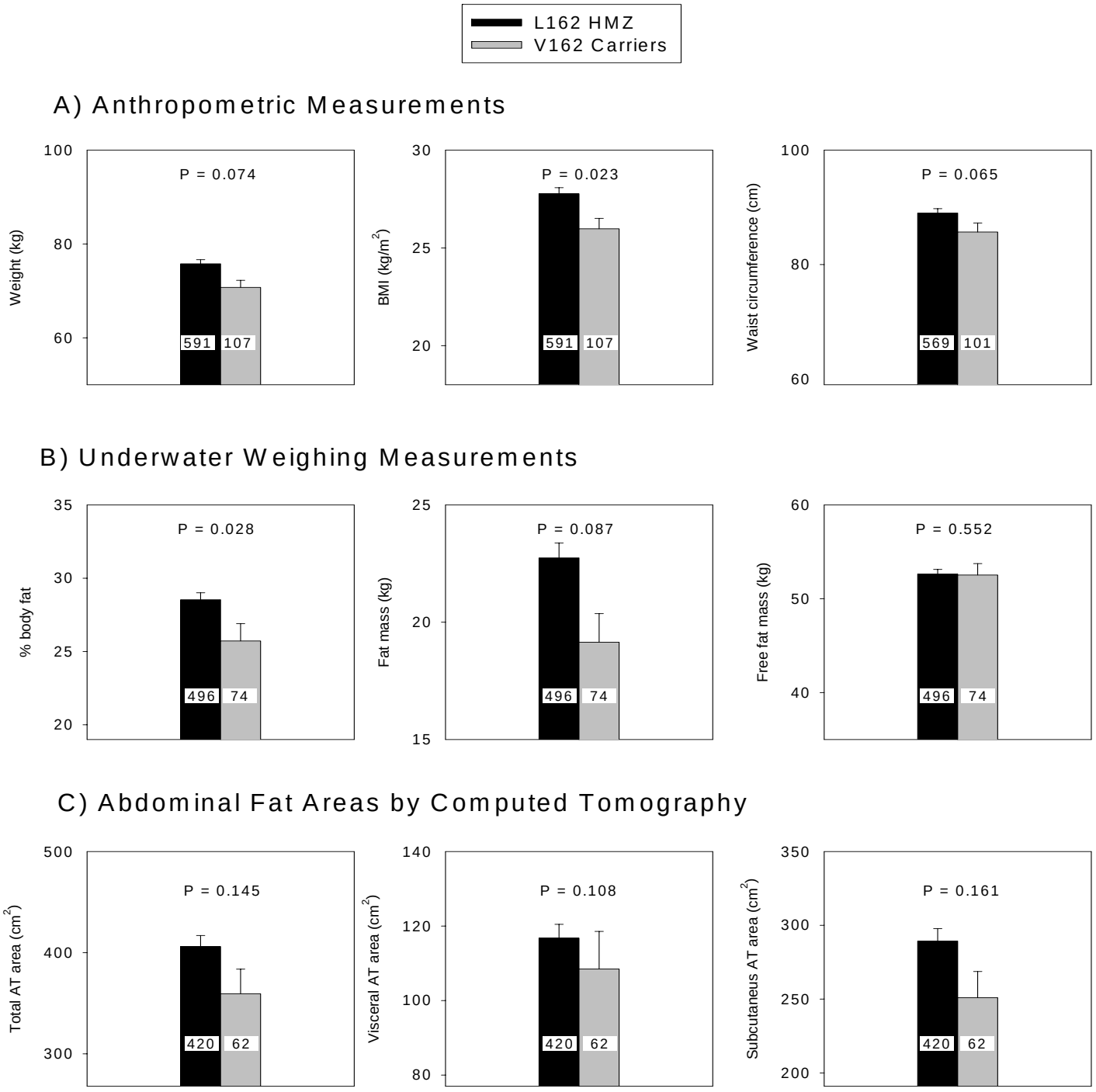

Figure 2. Body fatness and body fat distribution phenotypes by PPARα L162V genotypes. Number of subjects is indicated within each bar. P values are adjusted for age and gender. AT, indicates adipose tissue.

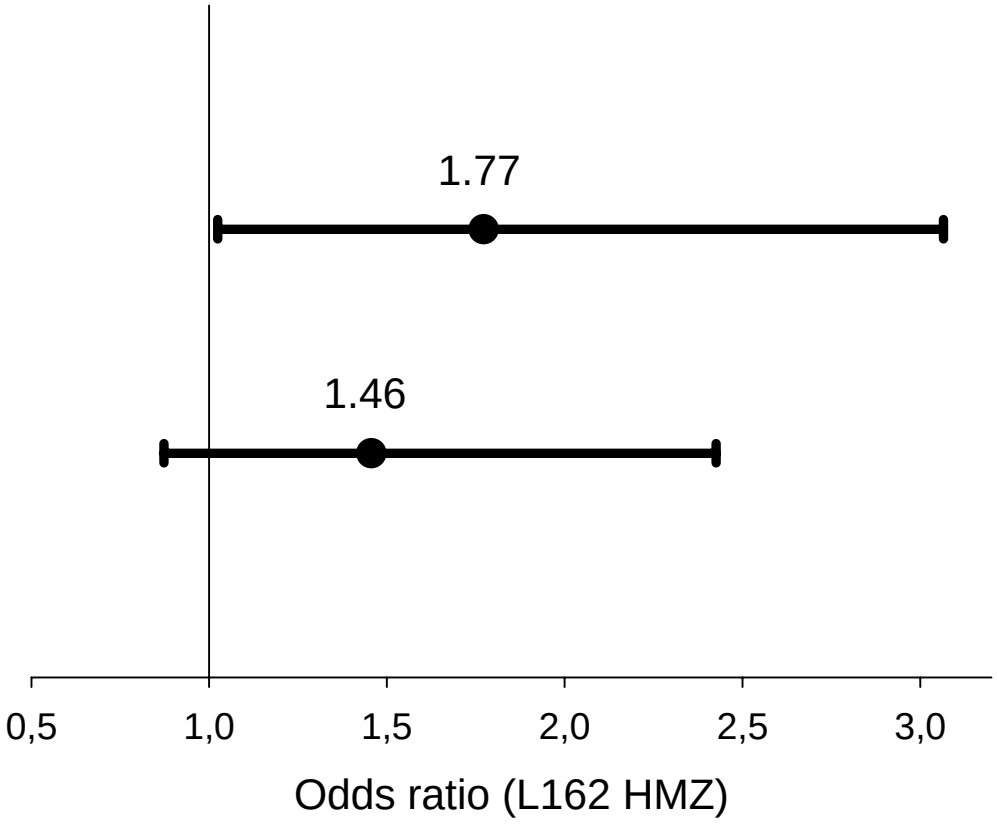

Figure 3. Odds ratio, with the 95% confidence intervals, of having a BMI > 30 kg/m2 for L162 HMZ individuals. The risk is shown without adjustment for confounding factors (bottom, p = 0.150) and after adjustment for age, gender and alcohol consumption (top, p = 0.041).

Chapter 3.

Combined Effects of PPARγ_2 P12A and PPARα L162V Polymorphisms on Glucose and Insulin Homeostasis: the Québec Family Study.

Yohan Bossé, S. John Weisnagel, Claude Bouchard, Jean-Pierre Després, Louis Pérusse, Marie-Claude Vohl

PPARγ_2 et α sont des facteurs nucléaires jouant un rôle important dans la régulation du métabolisme du glucose. L'objectif de cette étude était de vérifier l'influence des mutations PPARγ_2 P12A et PPARα L162V ainsi que leur interaction sur l'homéostasie du glucose et de l'insuline. Les génotypes ont été déterminés chez 663 adultes nondiabétiques participant à l'Étude des familles de Québec ayant subi une hyperglycémie orale provoquée (HGOP). Les niveaux d'insuline et de peptide-C suivant l'HGOP étaient plus élevés chez les sujets porteurs de l'allèle PPARα V162 comparativement aux homozygotes L162. Par contre, cet effet délétère de l'allèle PPARα V162 disparaissait lorsque les sujets étaient aussi porteurs de l'allèle PPARγ_2 A12. De plus, un effet d'interaction gène-gène significatif a été observé pour la réponse aigu (0-30 min) et total du peptide-C suivant l'HGOP. Ces résultats démontrent l'existence d'interaction gène-gène dans la régulation de l'homéostasie du glucose et de l'insuline plasmatique.

Combined Effects of PPARγ$_2$ P12A and PPARα L162V Polymorphisms on Glucose and Insulin Homeostasis: the Québec Family Study.

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Running title: PPARγ$_2$ and PPARα interaction on OGTT parameters

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Abstract

Peroxisome proliferator-activated receptors γ_2 and α are nuclear factors known to be important regulators of lipid and glucose metabolism. Two polymorphisms, namely PPARγ_2 P12A and PPARα L162V, were investigated for their individual and interaction effects on glucose and insulin homeostasis. Genotypes were determined in 663 non-diabetic adults participating in the Québec Family Study and who underwent an oral glucose tolerance test (OGTT). The insulin and C-peptide areas under the curve (AUC) following the OGTT were higher in subjects carrying the PPARα V162 allele compared to homozygous for the L162 allele. When subjects were grouped according to both polymorphisms, higher levels of insulin and C-peptide during the OGTT were observed for those carrying the PPARα V162 allele except when they carry at the same time the PPARγ_2 A12 allele. Thus, the PPARγ_2 A12 allele seems protective against the deleterious effect of the PPARα V162 allele. Furthermore, a significant gene-gene interaction was observed for the acute (0-30 min) ($p<0.001$) and the total ($p=0.05$) C-peptide AUC following the OGTT. These results provide evidence of a gene-gene interaction in the regulation of plasma glucose-insulin homeostasis, and emphasize that these interactions need to be taken into account when dissecting the genetic etiology of complex disorders.

Keywords Peroxisome proliferator-activated receptors, OGTT, epistasis, type 2 diabetes, C-peptide, insulin homeostasis, PPARγ_2 P12A mutation, PPARα L162V mutation.

Introduction

Understanding the genetic aetiology of type 2 diabetes is recognized as an urgent priority (King et al. 1998). The complexity of such a task is related to the multifactorial aspect of the disease. Considerable efforts have been devoted during the past few years on the identification of genes contributing to type 2 diabetes and related phenotypes. However, despite the evidence that there is a strong genetic component to the disease, only few "diabetogenes" have been identified (Busch and Hegele 2001; Newman et al. 1987). Genetic heterogeneity, variable penetrance, gene-gene (epistasis) and gene-environment interactions are among the factors that contribute to the difficulty of identifying the relevant genes.

Epistasis in type 2 diabetes and related phenotypes is likely to occur for genes encoding proteins involved in the regulation of lipid and glucose metabolism. In this regard, genes encoding peroxisome proliferator-activated receptors (PPARs) are relevant candidates. PPARs are members of the superfamily of nuclear hormone receptors (Mangelsdorf et al. 1995). They heterodimerize with the retinoid X receptor and bind to direct repeats within the promoter region of many genes to regulate their transcription (Berger and Moller 2002). Three subtypes have been identified, namely PPARα, γ, and δ. PPARs were first discovered as mediators of peroxisome proliferators (Issemann and Green 1990) but are now considered as key messengers responsible for the translation of nutritional and metabolic stimuli into changes in gene expression (Schoonjans et al. 1997). In addition, PPARα and γ mediate the effect of fibrates (Staels et al. 1998) and thiazolidinediones (Hauner 2002), respectively, two classes of drugs recognized to regulate lipid and glucose metabolism.

Two common polymorphisms affecting the amino acid sequence of the PPARα and PPARγ_2 gene have been identified, PPARα L162V (Sapone et al. 2000) and PPARγ P12A (Yen et al. 1997). Three independent studies reported no significant difference in the V162 allele frequency between subjects with and without type 2 diabetes, thus suggesting that the L162V polymorphism in the PPARα gene does not play a major role in the development of type 2 diabetes (Evans et al. 2001; Lacquemant et al. 2000; Vohl et al. 2000). On the other hand, there is controversy concerning the effect of PPARγ_2 P12A polymorphism on type 2 diabetes and intermediate phenotypes (Altshuler et al. 2000; Hegele et al. 2000; Mancini et al. 1999). The conflicting

results may potentially indicate that the effect of the polymorphism is modulated by environmental and/or other genetic factors. Recently, it has been shown that the effect of PPARγ_2 P12A polymorphism on insulin sensitivity and insulin levels is dependent on the background of the Gly972Arg polymorphism in the insulin receptor substrate-1 (IRS-1) gene (Stumvoll et al. 2002) and on the background of the Trp64Arg β_3-adrenergic receptor gene (Hsueh et al. 2001), respectively. Such examples reinforce the idea that allelic effects at some loci may be attenuated or amplified in the presence of variants at other loci. These interactions may explain the conflicting results observed in some association studies and highlight the importance of investigating gene-gene effects. In addition, incorporating epistasis in analytical models is necessary and important when searching for genes involved in complex traits. The objective of the present study was therefore to study the independent effect of PPARα L162V and PPARγ_2 P12A variants as well as their interactive impact on indices of plasma glucose and insulin homeostasis in 663 subjects enrolled in the Québec Family Study (QFS).

Materials and Methods

Subjects

The QFS is composed of French-Canadian families living in and around the Quebec City area, representing a mixture of random sampling and ascertainment through obese (BMI>32 kg/m^2) probands (Bouchard 1996). Only adults aged above 18 years old and non-diabetics were included in the present study. A total of 663 subjects (291 men et 372 women) included in 241 nuclear families were genotyped for the PPARγ_2 P12A and the PPARα L162V polymorphisms (age: 42.2 ± 17.0 years; BMI: 26.8 ± 6.9 kg/m^2). The Medical Ethics Committee of Laval University approved the protocol and a written consent was obtained from all the subjects.

Glucose, insulin and C-peptide measurements.

Fasting blood samples were collected and plasma glucose, insulin and C-peptide levels were measured by standard procedures as previously described (Desbuquois and Aurbach 1971; Heding 1975; Richterich and Dauwalder 1971). Subjects also underwent a 75-g oral glucose tolerance test (OGTT) after an overnight fast. Blood glucose, insulin and C-peptide levels were measured at –15, 0, 15, 30, 45, 60, 90, 120, 150 et 180 minutes after the glucose load. OGTT areas under the curve (AUC) were calculated using the trapezoid method. The area over the first 30 min defined the acute response, whereas the complete area (0-180 min) was the total response.

DNA analysis.

The PPARγ_2 P12A variant is caused by a C→G substitution at nucleotide 34 producing a BstU-I restriction site. Genotypes were obtained by digestion of PCR products as described in details elsewhere (Yen et al. 1997). The PPARα L162V polymorphism is caused by a C→G substitution at nucleotide 484 in exon 5 and does not alter any restriction site. A mismatch PCR method previously described was therefore used to genotype individuals of the QFS cohort (Vohl et al. 2000).

Statistical analysis.

Beside age and glycemia at 30, 45 and 60 minutes all variables under study were abnormally distributed. These variables were normally distributed after a $\log_{10}$ transformation. Antilogarithms of the transformed means were used to obtain geometric means. For statistical comparisons, heterozygous and homozygous carriers of the polymorphic allele were grouped together for both genes and are referred to as PPARγ_2 X/Ala and PPARα X/Val. The independent effect of both polymorphisms was tested by comparing the mean phenotype values between carriers and non carrriers using the MIXED procedure implemented in SAS (version 8.2), which takes the nonindependence of family members into account. This procedure is used when the experimental units (in this case the subjects) can be grouped into clusters (family), and the data from a common cluster are possibly correlated. For all metabolic parameters, age, sex and logBMI was included in the model. The interaction between the two polymorphisms was tested using two different statistical approaches: first, we constructed a dummy variable that divided the subjects into four genotype combinations, depending upon whether subjects had a variant in neither of the two genes (PPARγ_2 Pro/Pro + PPARα Leu/Leu, n=456), in PPARα only (PPARγ_2 Pro/Pro + PPARα X/Val, n=75), in PPARγ_2 only (PPARγ_2 X/Ala + PPARα Leu/Leu, n=107), or in both genes (PPARγ_2 X/Ala + PPARα X/Val, n=25). Differences between the four groups were assessed using the MIXED procedure. Secondly, the effect of the PPARγ_2 P12A, PPARα L162V, and their interaction were quantified in the MIXED model by contrasting the dummy variable for the two main effects (PPARγ_2 P12A and PPARα L162V) and the interaction.

Results

The independent effect of each polymorphism on glucose and insulin related variables is presented in Table 1. In the fasting state, both polymorphisms (P12A and L162V) were not associated with glucose, insulin and C-peptide levels. However, carriers of the PPARα V162 allele had higher total insulin AUC following the oral glucose load than non carriers. There was also a trend toward higher acute insulin and C-peptide AUC as well as higher total C-peptide AUC among carriers of the V162 allele.

In order to evaluate the interaction between P12A and L162V polymorphisms, subjects were divided into four genotype groups based on the presence or absence of the two variants. No difference was observed between the four genotype groups for the fasting glucose, insulin and C-peptide levels (not shown). Figure 1 illustrates glucose, insulin and C-peptide responses to the OGTT for each genotype group. Glucose and insulin levels during the OGTT as well as the glucose and insulin acute and total AUC were not different between the four groups (Figure 1A and 1B). However, compared to the other genotypes, a trend was observed for higher insulin levels among subjects bearing the PPARα V162 allele who were non carriers of the PPARγ_2 A12 allele. These results suggest that carriers of the PPARα V162 allele have higher insulin levels after a glucose load except when they also carry the PPARγ_2 A12 allele. In contrast, C-peptide levels at some time points and the acute response to the glucose challenge were different between the genotypes (Figure 1C). Indeed, similar C-peptide responses were observed for both genotypes that are non carriers of the PPARα V162 allele. However, distinct C-peptide responses were observed between PPARγ_2 P12A genotypes under a PPARα V162 background. These results suggest that the PPARγ_2 P12A polymorphism has an effect on C-peptide levels but only when the PPARα V162 allele is present.

To evaluate whether the interaction between PPARγ_2 P12A and PPARα L162V polymorphisms was significant we contrasted the four genotype groups for an interaction effect. The independent effect of each polymorphism and their interaction are summarized in Table 2. In this model, PPARα L162V was not independently associated with plasma glucose, insulin and C-peptide concentrations in the fasting state and in response to the OGTT. In contrast, PPARγ_2 A12 allele was associated with lower acute insulin and C-peptide levels. In addition, the interaction between

the two genes was statistically significant for the acute and total C-peptide AUC during the OGTT. Figure 2 presents the interaction effect for the acute C-peptide AUC. The PPARγ_2 A12 allele was associated with lower C-peptide levels but only in the presence of the PPARα V162 allele. Thus, the effect of one allele in one gene seems to depend upon the presence of another allele in a second gene.

Discussion

The present study investigated the independent effect of PPARγ_2 P12A and PPARα L162V polymorphisms as well as their interaction on glucose, insulin and C-peptide levels in the fasting state and following an OGTT. We showed that carriers of the PPARα V162 allele had higher insulin and C-peptide levels in response to a glucose challenge compared to those homozygous for the L162 allele. In contrast, subjects classified on the basis of the PPARγ_2 P12A polymorphism did not differ in terms of glucose and insulin phenotypes. Dividing subjects into four genotype combinations defined by the absence or presence of both variant alleles revealed significant differences between groups. Carriers of the PPARα V162 allele had higher levels of insulin and C-peptide during the OGTT except when they carry the PPARγ_2 A12 allele. Furthermore, the PPARγ_2 A12 allele decreased C-peptide concentrations but only on a PPARα V162 genetic background.

Although the effect of the PPARγ_2 P12A polymorphism on glucose and insulin homeostasis has been extensively studied (Stumvoll and Haring 2002), this study is the first to demonstrate that the PPARα V162 allele increases insulin and C-peptide levels during a glucose challenge. *In vivo* experiments using mice models have clearly confirmed the participation of PPARα to maintain blood glucose during an acute metabolic stress (Kersten et al. 1999; Leone et al. 1999). Indeed, PPARα-null mice developed severe hypoglycemia when fasted. In addition, PPARα-null mice are protected from a high fat diet-induced insulin resistance (Guerre-Millo et al. 2001). This observation is consistent with present findings suggesting higher insulin and C-peptide levels among carriers of the PPARα gene product with the greater transcriptional activity (V162 allele) (Flavell et al. 2000; Sapone et al. 2000). On the other hand, PPARα activation with fibrates in rodent models of high fat diet-induced insulin resistance and in genetic models of insulin resistance markedly improved their condition (Guerre-Millo et al. 2000). The genetic advantage of PPARα deficiency on insulin resistance and the favorable effect of its activation by fibrates therapy is analogous to the paradox observed with PPARγ, with both heterozygous PPARγ deficiency and PPARγ agonist treatment improve insulin sensitivity (Walczak and Tontonoz 2002).

With similar ligands and overlapping functions, gene-gene interactions between PPAR isotypes should not be surprising. In fact, evidences are starting to emerge in the literature suggesting that epistasis is probably more common than previously thought for glucose- and insulin-related phenotypes (Bruning et al. 1997; Hsueh et al. 2001; Savage et al. 2002; Stumvoll et al. 2002). However, providing functional data in support of our results is difficult. The PPARγ_2 P12A and PPARα L162V polymorphisms have opposite effects on the transcriptional activity of their respective receptor. Indeed, the A12 allele results in a less active form of PPARγ_2 (Deeb et al. 1998) while the V162 allele results in a more active form of PPARα (Flavell et al. 2000; Sapone et al. 2000). The observation that the PPARγ_2 A12 allele is associated with lower insulin and C-peptide levels corroborates the phenotype observed in PPARγ deficient mice (heterozygous PPARγ -/+ mice) (Miles et al. 2000). However, the observation that the PPARγ_2 A12 allele mediates its lowering effect only on a PPARα genetic background complicates the explanation. One possible explanation may come from results obtained by Stumvoll et al. (Stumvoll et al. 2002) who demonstrated that the PPARγ_2 A12 allele was associated with greater insulin sensitivity but only in subjects carrying a polymorphism in the gene encoding IRS-1 which had been associated with diabetes. They suggested that the A12 allele of PPARγ_2 becomes particularly advantageous on the background of a disadvantageous genetic polymorphism in a second gene. Similarly, V162 allele in PPARα impacts negatively on glucose and insulin homeostasis in our study and the A12 allele in PPARγ_2 attenuates the effect of the PPARα V162 allele. This hypothesis is also consistent with the greater insulin sensitivity, measured by euglycaemic-hyperinsulinaemic glucose-clamp, reported only in a subgroup of obese subjects carrying the A12 allele (Koch et al. 1999). Taken together these observations suggest that the A12 allele increase insulin sensitivity but only in a metabolically challenging *milieu*. On the other hand, evidences in the literature suggested that the A12 allele may impaired insulin secretion under particular metabolic challenges such as lipid infusion (Stefan et al. 2001) and type 2 diabetes (Mori et al. 2001). Accordingly, the metabolic stress caused by the L162V polymorphism may reduce insulin secretion in subjects carrying the A12 allele and explained the decrease in C-peptide levels observed in subjects carrying both variants. However, similar levels of glucose among genotype groups make this last mechanism unlikely and suggest a greater

insulin sensitivity among carriers of the A12 allele, rather than an impairment in insulin secretion.

The effect of the PPARγ_2 P12A polymorphism on type 2 diabetes and related phenotypes is controversial. For example, while the PPARγ_2 A12 allele was associated with a decreased risk of type 2 diabetes in Caucasians (Altshuler et al. 2000), the same allele was associated with an increased susceptibility to type 2 diabetes in Oji-Cree population (Hegele et al. 2000). The effect of this polymorphism is also inconsistent regarding obesity and related phenotypes. Some studies suggested that the A12 allele is associated with lower adiposity caused by decreased adipogenesis (Deeb et al. 1998), while others claimed that this allele increases adiposity as a result of reduced lipolysis (Stumvoll and Haring 2002). The discrepancy between studies may reflect the complex relationships between the PPARγ_2 P12A polymorphism and metabolic traits. Gene-gene interactions may explain the discrepancy among association studies (Hirschhorn et al. 2002). Thus, results of the present study may partly explain the controversy surrounding the PPARγ_2 P12A polymorphism and type 2 diabetes related phenotypes.

Evaluating the effect of two polymorphisms in addition to their interactions on several variables related to glucose and insulin homeostasis will inevitably lead to multiple testing. We did not adjust p-values for the number of tests reported because even if numerous variables were used, all were related to the same phenotype. However, it worth mentioning that the interaction observed between the two variants for the acute C-peptide levels remained largely significant even after Bonferroni correction ($p = 0.0072$). Nevertheless, because of the multiple testing nature of this study, the results should be interpreted with caution and require replication. The study should therefore be considered as exploratory generating hypotheses rather than testing hypotheses.

In conclusion, we observed a deleterious effect of the PPARα V162 allele on glucose and insulin levels during a glucose challenge. In addition, results of the present study suggest that PPARγ_2 P12A and PPARα L162V polymorphisms interact with each other to modulate some features of glucose and insulin homeostasis. A replication of this study is, however, required before a firm conclusion can be reached. The present study demonstrated that genetic polymorphisms in candidate genes encoding proteins with overlapping functions can interact and make a substantial

contribution to the final manifestation of the trait. It also confirmed the importance to take into consideration gene-gene interactions in the genetic dissection of complex metabolic phenotypes.

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Table 1. Independent Effects of the PPARγ_2 P12A and PPARα L162V Polymorphisms.

	PPARγ_2 P12A			**PPARα L162V**		
Variables	**Pro/Pro (n)**	**X/Ala (n)**	**p**	**Leu/Leu (n)**	**X/Val (n)**	**p**
Fasting glucose (mmol/L)	4.95 [4.86 ; 5.05] (464)	4.94 [4.83 ; 5.05] (118)	0.764	5.19 [5.06 ; 5.31] (509)	5.06 [4.86 ; 5.26] (86)	0.155
Fasting insulin (pmol/L)	70.1 [62.4 ; 78.7] (457)	68.8 [58.9 ; 80.5] (118)	0.826	63.1 [56.2 ; 70.9] (504)	61.9 [50.2 ; 76.3] (85)	0.854
Fasting C-peptide (pmol/L)	656 [614 ; 702] (435)	657 [602 ; 716] (108)	0.991	679 [630 ; 731] (478)	695 [614 ; 787] (79)	0.617
Acute glucose AUC (mmol/L·30min)	206 [203 ; 209] (412)	204 [199 ; 210] (101)	0.560	213 [210 ; 215] (464)	217 [212 ; 223] (67)	0.123
Acute insulin AUC (nmol/L·30min)	8.24 [7.61 ; 8.92] (405)	7.70 [6.89 ; 8.60] (101)	0.216	8.50 [7.86 ; 9.20] (459)	9.75 [8.38 ; 11.35] (66)	0.062
Acute C-peptide AUC (nmol/L·30min)	44.2 [42.0 ; 46.6] (401)	44.3 [41.6 ; 47.3] (100)	0.942	46.7 [44.7 ; 48.8] (453)	49.6 [46.1 ; 53.3] (66)	0.076
Total glucose AUC (mmol/L·180min)	1188 [1127 ; 1252] (410)	1180 [1111 ; 1253] (101)	0.722	1231 [1177 ; 1287] (461)	1259 [1184 ; 1338] (67)	0.361
Total insulin AUC (nmol/L·180min)	65.8 [61.2 ; 70.7] (403)	61.8 [55.6 ; 68.6] (101)	0.168	62.3 [57.8 ; 67.2] (456)	71.4 [62.7 ; 81.2] (66)	**0.022**
Total C-peptide AUC (nmol/L·180min)	439 [424 ; 455] (392)	434 [410 ; 460] (99)	0.705	442 [426 ; 459] (444)	464 [437 ; 493] (65)	0.094

Values are geometric means [95% CI] (adjusted for age, sex and logBMI). AUC indicates area under the curve.

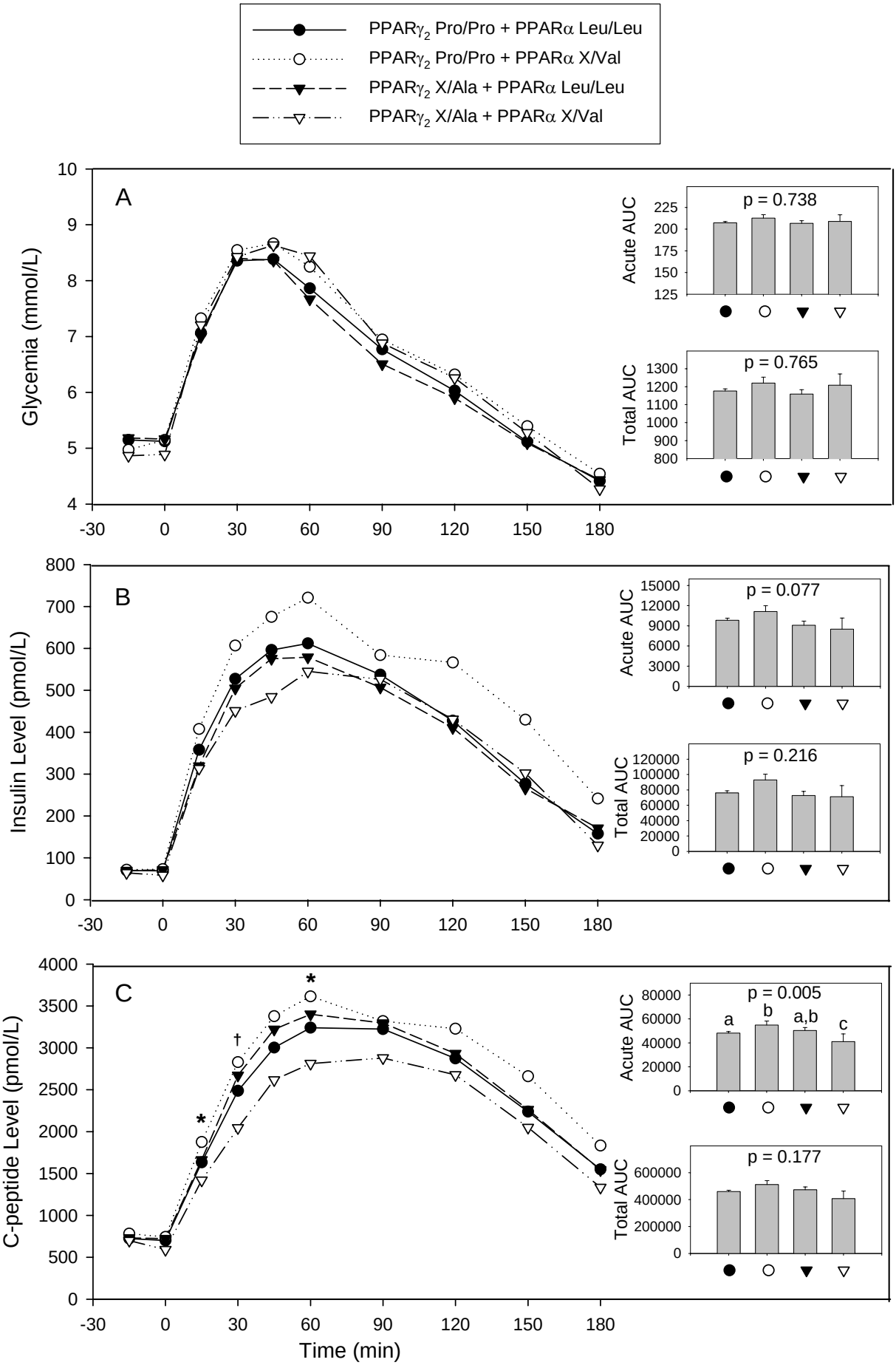
Table 2. Effects of the PPARγ$_2$ P12A, PPARα L162V, and their Interaction.

	PPARγ$_2$ P12A / PPARα L162V				p value[a]		
	PPARγ$_2$ Pro/Pro PPARα Leu/Leu (n)	**PPARγ$_2$ Pro/Pro PPARα X/Val (n)**	**PPARγ$_2$ X/Ala PPARα Leu/Leu (n)**	**PPARγ$_2$ X/Ala PPARα X/Val (n)**	**PPARγ$_2$ P12A**	**PPARα L162V**	**Interaction**
Fasting glucose (mmol/L)	4.96 [4.85 ; 5.08] (379)	4.89 [4.68 ; 5.11] (61)	4.97 [4.84 ; 5.11] (96)	4.77 [4.52 ; 5.02] (17)	0.465	0.114	0.379
Fasting insulin (pmol/L)	69.5 [61.2 ; 78.9] (374)	66.4 [52.1 ; 84.5] (60)	68.0 [56.4 ; 81.8] (96)	64.7 [47.5 ; 88.0] (17)	0.827	0.689	0.987
Fasting C-peptide (pmol/L)	637 [590 ; 688] (356)	665 [603 ; 732] (56)	653 [589 ; 723] (88)	596 [498 ; 712] (15)	0.413	0.685	0.203
Acute glucose AUC (mmol/L·30min)	208 [202 ; 213] (346)	212 [204; 220] (47)	206 [198 ; 214] (86)	207 [189 ; 226] (12)	0.496	0.636	0.731
Acute insulin AUC (nmol/L·30min)	8.27 [7.20 ; 9.50] (341)	9.55 [7.98 ; 11.43] (46)	7.87 [6.65 ; 9.30] (86)	6.82 [4.94 ; 9.42] (12)	**0.024**	0.991	0.101
Acute C-peptide AUC (nmol/L·30min)	44.3 [40.1 ; 48.9] (336)	52.0 [44.6 ; 60.7] (46)	46.6 [41.5 ; 52.2] (85)	38.3 [32.6 ; 44.9] (12)	**0.011**	0.776	**0.0008**
Total glucose AUC (mmol/L·180min)	1199 [1127 ; 1275] (344)	1214 [1127 ; 1309] (47)	1182 [1098 ; 1271] (86)	1234 [1124 ; 1355] (12)	0.969	0.315	0.524
Total insulin AUC (nmol/L·180min)	65.5 [57.3 ; 75.0] (339)	74.0 [61.5 ; 89.1] (46)	61.9 [52.2 ; 73.5] (86)	60.0 [45.4 ; 79.4] (12)	0.079	0.591	0.323
Total C-peptide AUC (nmol/L·180min)	442 [405 ; 482] (329)	504 [439 ; 578] (45)	447 [400 ; 499] (84)	412 [355 ; 478] (12)	0.071	0.635	**0.050**

Values are geometric means [95% CI] (adjusted for age, sex and BMI). AUC indicates area under the curve.

[a]p values are from the MIXED procedure in which the three independent variables are PPARγ$_2$ P12A (Pro/Pro or X/Ala), PPARα L162V (Leu/Leu or X/Val) and the interaction (PPARγ$_2$ P12A X PPARα L162V). Age, sex and logBMI were also included in the model.

Figure 1. OGTT glucose (A), insulin (B) and C-peptide (C) levels. Subjects have been divided into four groups according to the presence or absence of PPARγ_2 P12A and PPARα L162V mutations. Heterozygous and homozygous carriers of the variant allele were grouped together for both genes and are referred to as PPARγ_2 X/Ala and PPARα X/Val. Values are least-squared means adjusted for age, sex and logBMI. Error bars on vertical histograms are standard errors. Differences between groups were tested using the MIXED procedure for each time point during the OGTT and for the acute and the total areas under the curve (AUC). Groups with similar letters above the bar do not differ significantly. *Group (○) differ from group (●) and (∇); † group (∇) differ from group (●), (○) and (▼) and group (○) differ from group (●).


PPARγ2 Pro/Pro + PPARα Leu/Leu
PPARγ2 Pro/Pro + PPARα X/Val
PPARγ2 X/Ala + PPARα Leu/Leu
PPARγ2 X/Ala + PPARα X/Val
A
Glycemia (mmol/L)
Acute AUC
p = 0.738
Total AUC
p = 0.765
B
Insulin Level (pmol/L)
Acute AUC
p = 0.077
Total AUC
p = 0.216
C
C-peptide Level (pmol/L)
Acute AUC
p = 0.005
a
b
a,b
c
Total AUC
p = 0.177
Time (min)

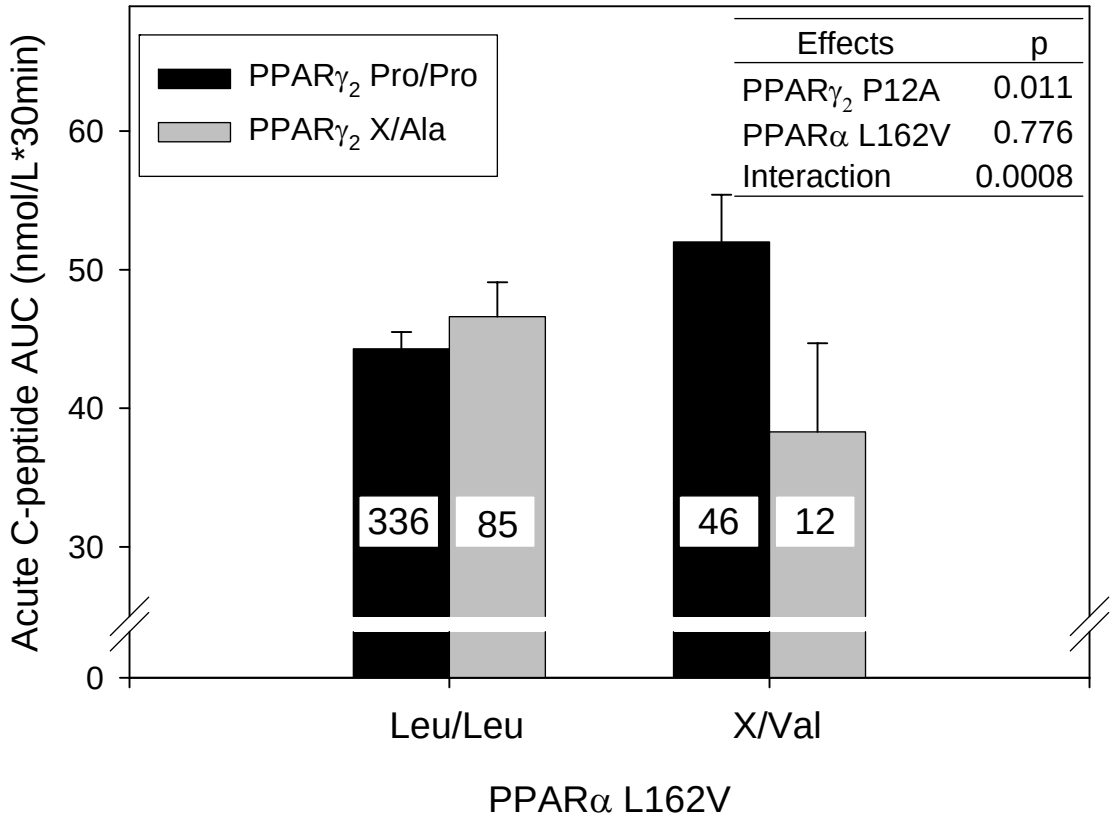


Figure 2. Interactions between PPARγ2 P12A and PPARα L162V polymorphisms for the acute C-peptide area under the curve (AUC) following the OGTT. Bars represent the adjusted geometric mean ± SE for each of the four genotype groups divided according to the presence or absence of PPARγ2 P12A and PPARα L162V variants.

Chapter 4.

Haplotypes in the Phospholipid Transfer Protein Gene are Associated with Obesity-Related Phenotypes: The Québec Family Study

Yohan Bossé, Luigi Bouchard, Jean-Pierre Després, Claude Bouchard, Louis Pérusse, Marie-Claude Vohl

La protéine de transfert des phospholipides (PLTP) pourrait jouer un rôle dans la régulation du poids corporel. L'objectif était d'étudié l'association entre des polymorphismes du gène de la PLTP et des phénotypes d'adiposité. Deux variations introniques, localisées dans les intron 1 (-c.-87G>A) et 12 (c.1175+68T>G), ont été génotypées chez 811 sujets participant à l'Étude des familles de Québec. Des tests d'association familiale ont été réalisés pour chacun des polymorphismes ainsi que pour les haplotypes. L'allèle A de l'intron 1 était associé avec des moyennes phénotypiques plus élevées pour le poids, l'indice de masse corporelle, la circonférence de taille et la masse maigre. Pour les analyses d'haplotype, la transmission de l'haplotype AT était associée positivement avec les phénotypes d'adiposité, alors que l'haplotype GT semblait protecteur contre l'obésité. Le séquençage du promoteur et des parties codantes du gène n'a révélé aucune mutation pouvant expliquer ces résultats.

Haplotypes in the Phospholipid Transfer Protein Gene are Associated with Obesity-Related Phenotypes: The Québec Family Study

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Running title: PLTP and obesity

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Abstract

Some line of evidence suggested that the phospholipid transfer protein (PLTP) may play a role in body fat regulation. We thus investigated the association between PLTP genetic variants and obesity-related phenotypes. Two intronic variants, one in intron 1 (c.-87G>A) and the other in intron 12 (c.1175+68T>G), were genotyped in 811 participants of the Québec Family Study. Ten obesity-related phenotypes were under study, including body-mass index (BMI), obesity (a dichotomous trait with a threshold of BMI ≥ 30 kg/m^2), weight and waist circumference as well as percentage of fat, fat mass and fat-free mass assessed by the hydrostatic weighing technique and total, visceral and subcutaneous abdominal adipose tissue areas assessed by computed tomography. Single marker and haplotype tests of association in family-based studies were performed using the FBAT program. The SNP located in intron 1 showed significant association with obesity, weight, BMI, waist circumference and fat-free mass ($p < 0.05$). The low frequency allele (A allele) was associated with greater trait values suggesting that the transmission of this allele is associated with an increased risk of being obese. For haplotype analyses, significant associations were observed with obesity, waist circumference, percentage of fat and fat-free mass ($p < 0.05$). The transmission of the AT haplotype (frequency = 0.180) was positively associated with obesity-related phenotypes whereas the GT haplotype (frequency = 0.468) seemed to be protective against obesity. By sequencing the promotor and the coding regions of the PLTP gene, we were unable to identify a mutation that could provide functional meaning to the results. Considering the number and the relevance of candidate genes surrounding the PLPT locus, it is unclear whether PLTP itself is responsible for the association or the effect is mediated by a second gene allele in linkage disequilibrium with the marker locus.

Key words: PLTP, FBAT, haplotype, tests of association, obesity.

Introduction

The phospholipid transfer protein (PLTP), also referred to lipid transfer protein 2, belongs to the lipopolysaccharide binding/lipid transfer protein family, together with cholesteryl ester transfer protein (CETP), lipopolysaccharide-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI). The PLTP gene was mapped to chromosome 20q12-q13.1[1] and encoded a mature protein of 476 residues[2]. The PLPT mRNA transcipt is detectable in a wide variety of tissues including pancreas, lung, kidney, heart, liver, skeletal muscle and brain[3]. The messenger is also highly expressed in subcutaneous and visceral adipose tissues with a depot-specific difference[4,5]. The predicted model structure of PLTP consists of two lipid-binding pockets characterized by apolar residues, with a N-terminal pocket critical for PLTP transfer activity and a C-terminal pocket involved in lipid binding[6,7].

The initial physiological function ascribed to plasma PLTP was one of transfer of phospholipids from triglyceride-rich lipoproteins to high-density lipoproteins (HDL) during lipolysis[8]. Since then, animal and human studies have suggested that plasma PLTP level is an important factor in lipoprotein/lipid metabolism and atherosclerosis development[9,10]. More recently, PLTP activity has been shown to be positively and independently related to coronary artery disease[11]. Despite these great physiological insights, the role of PLTP in human metabolism and particularly the function of peripheral PLTP is still limited. Recently, some lines of evidences suggested that PLTP might play a role in the regulation of body fat content. First, the mRNA levels and the activity of PLTP have been consistently associated with obesity[4,12-15]. This tight relationship between PLTP and obesity is not fully understood. Kaser et al.[12] have proposed that the increased synthesis of the protein may be the result of the enlarged mass of adipose tissue. This has been supported by Murdoch et al.[16] who reported that PLTP activity is decreased following a diet-induced weight loss. Secondly, the inactivation of the PLTP gene in *Caenorhabditis elegans* by RNA interference (RNAi) cause increase in fat storage, suggesting that loss-of-function mutations in mammalian homologue could underlie obesity[17]. Third, two independent genome-wide scans provided significant evidence of linkage with obesity-related phenotypes within the PLTP region[18,19]. In addition, several mouse studies have suggested that genes influencing body fatness in mice reside on chromosome 2, a region homologous to human chromosome 20q[20-22]. Finally, it has been shown that PLPT facilitates the production of triglyceride-rich apoB-

containing lipoproteins[23,24] and facilitates as well the transport of lipids from cells[25], which are central functions of lipid homeostasis. Based on these observations, we suspect that PLTP itself could modulate the level of adiposity. To test this hypothesis, we investigated the association between PLTP genetic variants and obesity-related phenotypes.

Materials and Methods

Subjects

Subjects were from the Québec Family Study (QFS) which is an ongoing project of French-Canadian families representing a mixture of random sampling and assortment through obese proband. This project was specifically design to understand the genetic basis of obesity and its comorbidities. Details of recruitment procedures have been published[26]. Only adults, 18 years and older, were considered for the present study. Table 1 presents the characteristics of the subjects. The Laval University Medical Ethics Committee approved the study, and all subjects provided written informed consent.

Anthropometry , body composition and fat distribution measurements

Body weight, height, and waist circumference were measured following standardized procedures[27]. Body density was measured by the hydrostatic weighing technique[28]. Pulmonary residual volume was assessed before immersion in the hydrostatic tank, using the helium dilution technique of Meneely and Kaltreider[29]. Percentage of body fat, fat mass and fat-free mass were derived from body density using the Siri equation[30]. Finally, a cross-sectional abdominal scan was performed by computed tomography using a Somatom DRH scanner (Siemens, Erlanger, Germany) to quantify the adipose tissue areas between L4 and L5 vertebra as described in detail elsewhere[31].

SNPs selections and genotyping

To explore the possible involvement of PLTP in obesity-related phenotypes, we selected and genotyped two variants previously reported in dbSNP (rs394643 and rs553359) (Figure 1). The dbSNPs were chosen based on the number of chomosomes tested and their average estimated heterozygosity. They were then defined as c.-87G>A (denotes an intronic G to A substitution located 87 nucleotides downstream of the start codon) and c.1175+68T>G (denotes an intronic T to G substitution located 68 nucleotides upstream of the last nucleotide of exon 12) according to the nomenclature recommendations[32]. A total of 898 and 893 subjects were genotyped for the c.-

87G>A and c.1175+68T>G polymorphisms, respectively. Genotyping was performed using a mini-sequencing assay[33]. PCR primers [forward (f), reverse (r)] and minisequencing (ms) primers were as follow: rs553359 (324 base pairs), f-5'-GGTCAGTAACATCCTCCTC-3', r-5'-GACCCATTTGTTCATCTCTC-3', ms-5'- AGGTATCACTGTACTTTAAGC-3' rs394643 (365 base pairs), f-5'-CACGAGGGAACTGGGAACG-3', r-5'-CGCCTTACCCAGCTCCAG-3', ms-5'-GACGTCCAACCATAAGTGGG-3'. PCR conditions were as follow: In final volume of 6 µl, 20 ng of genomic DNA were added to a mixture containing a final concentration of dNTP (Amersham Pharmacia Biotech Inc.), 30µM each; *Taq* DNA polymerase (QUIAGEN), 0.3 U; buffer 1X [10 X: TRIS-HCl, KCL, $(NH_4)_2SO_4$ and 15 mM $MgCl_2$; pH 8.7 (20°C)]; $MgCl_2$, 2.25 mM; flanking primers, 50 nM each. Following a 5-min denaturation step at 95°C, 30 PCR amplification cycles were performed as follow: denaturation at 95°C, 20 sec; annealing 60°C, 1 min; for 10 cycles and denaturation at 95°C, 20 sec; annealing at 57°C, 1 min; for the remaining 20 cycles. In the same well, the PCR mixture dNTP's was digested using Shrimp Alkaline Phosphatase (USB), 0.2 U (final volume: 11 µl) for 15 min at 37°C follow by 20 min at 80°C. Mini-sequencing assay was performed in a final volume of 16 µl (in the same well); dTTP/ddNTP mix (dTTP, ddATP, ddCTP and ddGTP) for rs553359 and dGTP/ddNTP mix (dGTP, ddATP, ddTTP and ddCTP) for rs 394643 (dNTP and ddNTP are from Amersham Pharmacia Biotech Inc.), 1.56 µM each; IRDye tag primer, 3.125 nM (LICOR); Thermosequenase (USB), 0,3 U; 0.6 X buffer (10X: Tris-HCl, 260 mM, MgCl2, 65 mM, pH 9.5) were added to microplates. Following 2 min denaturation step at 95°C, 30 PCR amplification cycles were performed as follow: denaturation at 95°C, 10 sec; annealing at 60°C, 30 sec; extension at 72°C, 5 sec. Detection was done on a LICOR automated sequencer model 4200.

Sequencing

The promotor and the coding regions of the PLTP gene were sequenced in 19 subjects with different genotypes for the intronic variants and with different degrees of obesity. All exons and exon-intron splicing boundaries were amplified from genomic DNA by use of specific primers derived from the 5' and 3' ends of intronic sequence. We also sequenced up to 230 base pairs located downstream relative to the first transcriptional initiation site which is responsible for the full promotor activity[34]. Because of the particular genomic structure of the PLTP gene,

characterized by small intron, some exons were amplified within the same fragment. Table 2 presents the specific primers of each fragment with their product size. All primers were designed using the Primer 3.0 software available on the Whitehead Insitute/MIT Center for Genome Research server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Amplification was performed by polymerase chain reaction using the thermal cycler, model PTC-200 (MJ Research, Watertown, MA). The cycler was programmed at 95^{o}C for 5 min followed by 35 cycles of the following 3 min: 1 min at 95^{o}C for denaturation, 1 min at annealing temperature, and 1 min at 72^{o}C for elongation. The program was then completed with 10 min at 72^{o}C. The annealing temperature was optimized for each pairs of primers by performing a 53^{o}C to 67^{o}C gradient assay using stock DNA (see Table 2). PCR conditions were as follow: reaction volume was 50 μL including 0.2 μL of AmpliTaq®DNA polymerase (Perkin-Elmer Cetus), 5 μL of 10X PCR buffer and 2.5 mM of $MgCl_2$ as recommended by the manufacturer, 0.2 mM of dNTPs, 8.4 μL of each primer at a final concentration of 7.5 μM and 6 μL of genomic DNA at a final concentration of 20 ng/μL. PCR products were purified by the ABI ethanol-EDTA precipitation protocol, collected using a Beckman-Coulter Allegra 6R centrifuge, and resuspended in a 50% HiDi-formamide solution. Sequence reactions were performed using the BigDyeTH Terminator v3.1 kit and samples were run on ABI Prism® 3730/XL DNA Analyzer automated sequencers (Applied Biosystems, Foster City, CA). The sequences were then assembled and analyzed using the Staden preGAP4 and GAP4 programs[35].

Linkage disequilibrium

Prior to calculate linkage disequilibrium, haplotype frequencies were estimated using the EH+ program available at this address: http://www.iop.kcl.ac.uk/IoP/Departments/PsychMed/GEpiBSt/software.shtml. Thereafter, these parameters were used to calculate pairwise measures of linkage disequilibrium (D') using the 2LD program available at the same address.

Statistical analysis

Hardy-Weinberg equilibrium for genotype frequencies was evaluated using a χ^2 test. We used the FBAT program to test association with either single SNPs or haplotypes and obesity-related phenotypes[36] (http://www.biostat.harvard.edu/~fbat/default.html). The FBAT program performed family-based test of association that is efficient and robust to population admixture, phenotype distribution and ascertainment based on phenotype. It can also handle missing parental genotypes and/or missing phase in both offspring and parents for haplotype analysis. The approach holds as well for multi-locus and multi-allelic markers. The haplotype test is ideal for candidate gene studies with tightly linked markers (no recombination between the markers). Because of the high proportion of obese individuals in our cohort, the affection status was set as follows: 2 for affected (BMI $\geq$ 30 kg/m^2), 1 for unaffected (BMI < 30 kg/m^2) and 0 for unknown. These values allowed unaffected subjects to contribute to the analyses when association is tested with the dichotomous phenotype[37]. The minimum number of informative families necessary to compute the test statistics was set to 10. We first tested association with the global test (mode = multi-allelic) for intron 1 and intron 12 polymorphisms with the dichotomous and the quantitative phenotypes. For the global test, the FBAT program gives a χ^2 statistic and its one-sided corresponding p-value. To know the effect of the transmited allele on the traits values, an univariate FBAT test was performed for each allele. This test provided a Z-statistic with the corresponding p-value. A positive Z-statistic is indicative of a high risk allele and a negative Z-statistic is indicative of a protective allele. This univariate FBAT statistic (Z-statistic) was also used to make inference regarding the effect of haplotypes of the PLTP gene on obesity-related phenotypes.

Results

A total of 811 subjects above 18 years old have been genotyped for the c.-87G>A and the c.1175+68T>G polymorphisms. For the c.-87G>A polymorphism, 215 subjects were homozygotes GG, 413 were heterozygotes GA and 183 were homozygotes AA. For the c.1175+68T>G polymorphism, 332 subjects were homozygotes TT, 382 were heterozygotes TG and 97 were homozygotes GG. The genotype distribution of both polymorphisms was in Hardy-Weinberg equilibrium (c.-87G>A, $\chi^2 = 0.33$, p=0.85; c.1175+68T>G, $\chi^2 = 0.65$, p=0.72). The two variants were in linkage disequilibrium with a D' coefficient of 0.69 ($p < 0.001$).

Associations between single SNPs and the obesity-related phenotypes were tested using a family-based association test. The results of these analyses are presented in Table 3. The χ^2 statistics with its one df corresponding p-value are presented for each obesity-related phenotypes. In addition, the effect of the low frequency allele (allele A for the c.-87G>A polymorphism and allele G for the c.1175+68T>G polymorphism) on the trait values are indicate, based on the Z-statistic provided by the univariate test performed for each allele. Of interest to note, since bi-allelic markers are under study, the p-value associated with the Z-statistic is the same to the one calculated by the χ^2 statistic (the two tests are equivalent). The SNP located in intron 1 (c.-87G>A) showed significant association with obesity, weight, BMI, waist circumference and fat-free mass. The Z-statistics for allele A were positive for every obesity-related phenotypes suggesting that the transmission of this allele increases the risk of being obese. On the other hand, no associations were observed between the c.1175+68T>G polymorphism and phenotypes under study.

Haplotype association tests for family-based studies were also performed (Figure 2). For each haplotype, the Z-statistic is presented for the dichotomous (obesity) and the nine quantitative phenotypes. The haplotype frequencies were as follow: GT = 0.468, AG = 0.286, AT = 0.180 and GG = 0.066. The AT haplotype is significantly and positively associated with the dichotomous obesity phenotype, waist circumference, percent body fat and fat-free mass ($p < 0.05$), suggesting a high risk haplotype. The same trend ($p < 0.1$) is also observed for BMI, weight, fat mass and subcutaneous adipose tissue areas. On the other hand, the transmission of the GT haplotype

seems to be protective against obesity but only the waist circumference phenotype reached statistical significance ($p < 0.05$).

In an attempt to identify a functional mutation in the PLTP gene that could explain the observed results, we sequenced the coding and the promotor regions of the gene. In addition to the two genotyped polymorphisms (rs394643 and rs553359), we identified three SNPs (Figure 1): 1- two heterozygous subjects for a C>G substitution in intron 1 (c.-601C>G) of the gene which was already in dbSNP (rs2294213), 2- one heterozygous subject carries a C>T substitution in intron 2 (C.100+42C>T), and 3- one heterozygous subject carries a G>A substitution in exon 6 (c.537G>A) which is a synonymous change. These three SNPs are located at position 84449, 83707 and 80249 in the DNA genomic sequence AL008726 (GenBank accession number), respectively. This attempt decreases the likelihood that a functional mutation in the PLTP gene, in linkage disequilibrium with the tested markers, is responsible for the significant association observed.

Discussion

In this study, we performed family-based tests of association between PLTP genetic variants and obesity-related phenotypes. Two SNP polymorphisms were genotyped, one located in intron 1 (c.-87G>A) and the second in intron 12 (c.1175+68T>G). Single marker association tests revealed significant associations between SNP in intron 1 and several indices of adiposity, including BMI (both as a quantitative trait and as a dichotomous trait with a threshold of BMI ≥ 30 kg/m^2), weight and waist circumference. In these analyses the transmission of the low frequency allele (A allele) was associated with increased trait values. Despite of being in linkage disequilibrium with the SNP in intron 1, the SNP in intron 12 provided no evidence of association with any obesity-related phenotypes. Since multimarker haplotypes are likely to yield more genetic information than the study of a single marker, we also performed haplotype association tests for family-based study. Again significant associations were observed for some haplotypes. Indeed, the transmission of the AT haplotype increases the likelihood of being obese, whereas the transmission of the GT haplotype seems to be protective.

It is recognized that a significant association test could be the result of a functional variant in the gene in linkage disequilibrium with the tested marker. We thus verified this hypothesis by sequencing the promotor and the coding regions of the PLTP gene in subjects with different genotypes for the intronic variants and with different degrees of obesity. This attempt was undertaken to identify a mutation that could provide functional meaning to the results observed with intronic variants. Three additional SNPs were identified, the first one located in the 5' untranslated region (c.-601C>G), the second one in intron 2 (c.100+42C>T) and the last one in exon 6 (c.537G>A) (Figure 1). None of these SNPs changed the amino acid sequence of the protein and their low frequencies make them unlikely to be the genetic variants responsible for the association. We thus ended up with a limited explanation. We might first suspect the intronic markers tested to be functional. Recently, it has been demonstrated that a silent substitution in intron 11 of the lamin A gene causes a rare disorder, called Hutchinson-Gilford Progeria Syndrome, that result in premature ageing and shortened lifespan[38]. Intronic SNPs have also been associated with rheumatoid arthritis[39] and myocardial infarction[40]. Accordingly, if a silent mutation could produce such severe phenotypes, more subtle effect from this type of mutation

might also be expected. However, the burden of the proof remains without functional studies evaluating the effect of the intronic variants on the protein products.

Different mechanisms could explain the association between PLTP and obesity-related phenotypes. First, the messenger and the activity of PLTP have been consistently related to obesity[4,12-15]. However, these studies used correlations to investigate the relationship and cannot determine a cause and effect. Although, it has been suggested that PLTP activity is influenced directly by body weight[12,16], we might also suspect that PLTP itself is responsible for this tight relationship. Important functions governing lipid homeostasis have been ascribed to PLTP. In addition to its well established role in mediating the transfer of phospholipids between triglyceride rich lipoproteins and HDL in the intravascular compartment[8], the hepatic PLTP in mice has been shown to play a major role in regulating the secretion of apoB-containing lipoproteins[24]. Furthermore, peripheral PLTP is also known to enhance cellular lipids efflux. Indeed, Wolfbauer et al.[41] have demonstrated that PLTP increases cholesterol and phospholipid efflux from cholesterol-loaded human fibroblasts. Subsequently, the same group reported similar observations in murine macrophages and hamster kidney cells and shown that PLTP mediates its effect via the ATP-binding cassette transporter A1 (ABCA1) pathway[25]. This process might play an important role in enhancing flux of lipids from tissues. Given the expression of ABCA1[42] and PLTP[4] in the adipose tissue, such transport mechanism could prevent lipid storage into adipocytes, which is the long-term process leading to obesity.

On the other hand, it is possible that the PLTP allele significantly associated with obesity-related phenotypes in our study is not responsible for the effect. In fact, the association may arise because of a second gene allele in linkage disequilibrium with the PLPT marker locus. A first glance of genes located in proximity of the PLTP gene revealed multiple candidate genes[43]. First, two genes, the melanocortin 3 receptor (MC3R) and the adenosine deaminase (ADA), located approximately 10.3 Mb and 1.3 Mb from the PLTP gene, respectively, have been significantly associated with obesity-related phenotypes[44,45]. The agouti signaling protein (ASIP), is also located within the relevant region of human chromosome 20. The ASIP is the human orthologue of the mouse agouti gene, which is a single-gene mutation model of obesity[46]. Finally, three candidate genes located in the vicinity of the PLTP locus namely the CCAAT enhancer binding protein (CEBPB), the protein tyrosine phosphatase-1B (PTPN1), and the growth hormone

releasing hormone (GHRH) have been revealed as obesity-modifying genes in knockout and transgenic experiments[47-49]. Accordingly, considering the number of interesting candidate genes potentially implicated in body weight and fatness nearby the PLTP locus, it is highly plausible that the significant associations observed in the present study are due to an allele in a second gene in linkage disequilibrium with the marker locus.

In conclusion, we reported for the first time significant associations between PLTP genetic variants and obesity-related phenotypes. The associations were carry out using both single locus and haplotypes analyses based on family study. By sequencing all the exons and the promotor region of the gene, we were unable to identify a mutation that could explain the significant associations with the intronic variants. Although, some evidence suggested that PLTP itself may be responsible for the association, the number and the relevance of candidate genes surrounding the PLTP locus highly suggested that an allele in a second gene in linkage disequilibrium with the PLTP markers is responsible for the association. Further studies will be required to elucidate this uncertainty.

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Table 1. Characteristics of the Subjects.

			Range	
	N	**Mean ± SD**	**Minimum**	**Maximum**
Age (years)	811	42.6 ± 16.7	18.2	93.5
Weight (kg)	805	75.7 ± 21.6	35.0	181.1
BMI (kg/m^2)	805	27.7 ± 7.7	16.8	64.9
Waist circumference (cm)	779	88.8 ± 18.5	57.9	164.5
Percent body fat	651	28.2 ± 10.9	2.9	59.8
Fat mass (kg)	651	22.6 ± 14.4	1.7	108.3
Fat-free mass (kg)	651	52.8 ± 10.7	30.7	94.9
Abdominal adipose tissue areas (cm^2)	557			
Total	-	408 ± 230	46	1129
Visceral	-	118 ± 82	15	469
Subcutaneous	-	290 ± 175	16	872

Table 2. PCR primers for genomic amplification of PLTP promotor and exons.

Exons	Oligonucleotides	Product size (bp)	Annealing Temperature (^{o}C)
Promotor and exon 1	L 5'-TCAGGTCCTAAATCTCTCCCATTA-3' R 5'-GCAGGAAGACATGGATAATTGTAA-3'	517	55
Exon 2 and 3	L 5'-CCATCCGGTTTCTTAAGTCTTG-3' R 5'-CATATGGAGGCAAGATTATTGGTT-3'	605	60
Exon 4	L 5'-AAATGCATATGCCTGTTTCTGTTA-3' R 5'-AGCAGACAAATTTCCTGTCGTT-3'	339	60
Exon 5	L 5'-CCAGGAAGTGACAGAGCTGAG-3' R 5'-GATAGGAAGGTTTGGTAGGATGTG-3'	409	60
Exon 6 and 7	L 5'-AGAAATGAGCACTTCACCCAAAAT-3' R 5'-GCCACTATTTCCCTAGTCACTGAT-3'	454	55
Exon 8	L 5'-TCATCAGAGCTGTGCTTTAAGAAG-3' R 5'-AAGGATGTATCCTCATTTTATGGG-3'	293	60
Exon 9 and 10	L 5'-AGACAGGAACAGCCATGACAAG-3' R 5'-AGACACCTGCTGTCAGTCCAG-3'	541	60
Exon 11 and 12	L 5'-AATGCAGAGGAGTCAGACTTTACC-3' R 5'-TATTTGCAATATGAGAGTCCCCAG-3'	624	57
Exon 13, 14 and 15	L 5'-AGTCTAAGATTAAAATGGGAGGGG-3' R 5'-GAAAGTTGTAGCACTTGGAAAGGT-3'	681	64
Exon 16	L 5'-CTTAAAGAATGCCCTTTATGATGC-3' R 5'-TGAGTTCCTGAAAGACAAACACTC-3'	472	64

L, left; R, right

Table 3. Global tests of association between SNPs in the PLTP gene and obesity–related phenotypes.

Phenotypes	c.-87G>A			c.1175+68T>G		
	χ^{2}*	Z†	p-value	χ^{2}*	Z†	p-value
Obesity§	5.21	2.28	0.022	0.02	0.14	0.889
Weight (kg)	4.41	2.10	0.036	0.16	0.40	0.687
BMI (kg/m^2)	4.66	2.16	0.031	0.12	0.35	0.727
Waist circumference (cm)	5.27	2.30	0.022	0.14	0.37	0.708
Fat percentage	2.35	1.53	0.125	0.65	-0.81	0.419
Fat mass (kg)	1.47	1.21	0.225	0.29	-0.54	0.591
Fat-free mass (kg)	4.05	2.01	0.044	0.00	-0.05	0.963
Abdominal adipose tissue areas (cm^2)						
Total	1.97	1.40	0.161	0.80	-0.89	0.371
Visceral	0.87	0.93	0.352	0.03	-0.17	0.866
Subcutaneous	2.26	1.50	0.133	1.20	-1.10	0.273

*Chi-square with 1 df.

†Z-statistic of the low frequency allele, positive Z-statistics are indicative of a high risk allele and negative values are indicative of a protective allele.

§Obesity is a dichotomous trait based on BMI criteria.

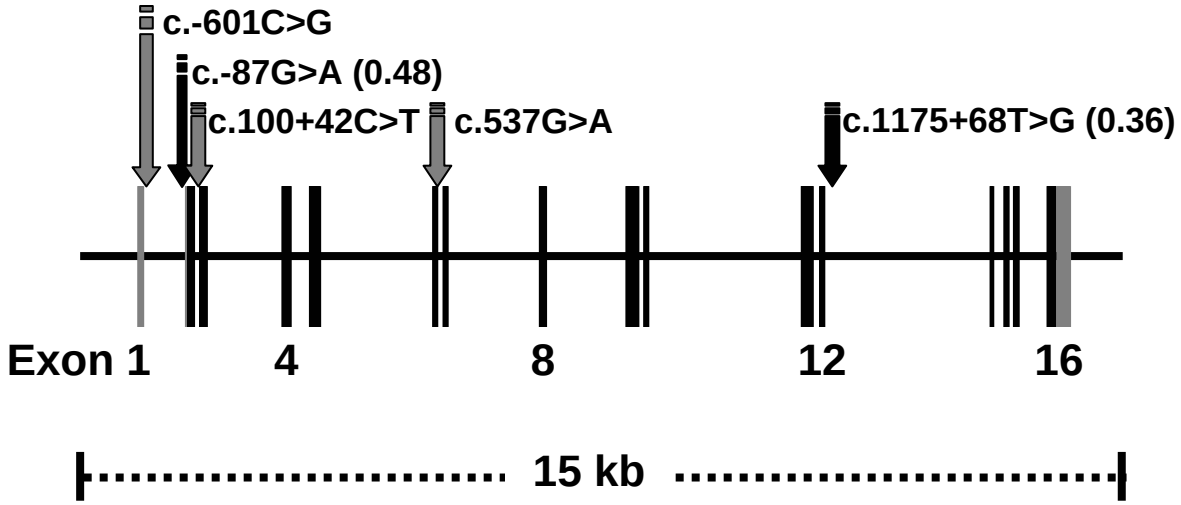

Figure 1. Genomic organization of the PLTP gene. The location of the genotyped variants are indicated with black arrows. The additional variants identified by sequencing are indicated with grey arrows. The 16 exons are shown as vertical bars whose width corresponds to their base-pairs length. Coding regions are in black and untranslated regions are in grey. Values in parentheses are the frequencies of the rare allele.

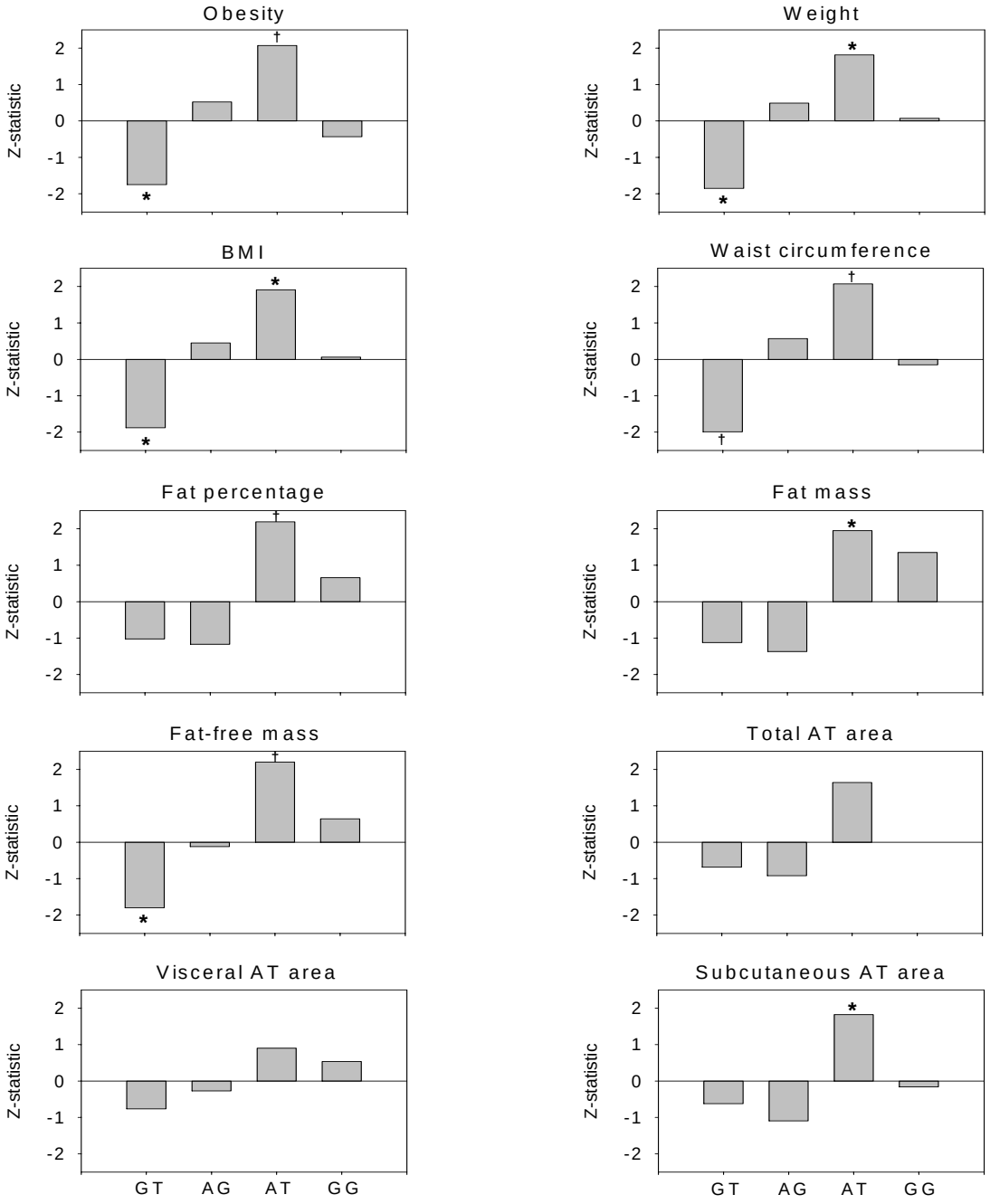

Figure 2. Haplotype-specific association tests in the PLTP gene and obesity related-phenotypes. The test was conducted on 10 phenotypes indicated above each graph. The phenotype labelled "obesity" is a dichotomous trait based on BMI above and below 30 kg/m^2. Each bar represents the Z-statistic for one of the four haplotypes constructed with the two polymorphisms (c.-87G>A and c.1175+68T>G). A positive Z-statistics are indicative of a high risk haplotype and negative values are indicative of a protective haplotype. The haplotypes frequencies were as follow: GT = 0.468, AG = 0.286, AT = 0.180, GG = 0.066. AT, adipose tissue. *$p < 0.1$, $^{\dagger}p < 0.05$.

Chapter 5.

Genome-Wide Linkage Scan Reveals Multiple Susceptibility Loci Influencing Lipid and Lipoprotein Levels in the Québec Family Study

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L'objectif de cette étude était d'identifier les régions chromosomiques contenant les gènes influençant les niveaux de lipides et de lipoprotéines plasmatiques. Un criblage génomique a donc été effectué chez 930 sujets participant à l'Étude des familles de Québec. La plus forte évidence de liaison a été retrouvée sur le chromosome 12q14.1 pour les niveaux de cholestérol-HDL avec un rapport de cotes logarithmique (LOD) de 4,06. Plusieurs loci ont été identifiés pour les niveaux de cholestérol-LDL incluant 1q43 (LOD = 2,50), 11q23.2 (LOD = 3,22), 15q26.1 (LOD = 3,11), et 19q13.32 (LOD = 3,59). Pour les niveaux de triglycérides, trois marqueurs localisés sur les chromosomes 2p14, 11p13 et 11q24.1 ont présenté des évidences suggestives de liaison (LOD > 1,75). En conclusion, ce criblage génomique a permis d'identifier plusieurs régions chromosomiques influençant les lipides et lipoprotéines plasmatiques. Les gènes en causes restent à être déterminés.

Genome-Wide Linkage Scan Reveals Multiple Susceptibility Loci Influencing Lipid and Lipoprotein Levels in the Québec Family Study

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Running title: Genome scan on blood lipids

Abbreviations: Apo, apolipoprotein; FCHL, familial combined hyperlipidemia; BMI, body mass index; QTL, quantitative trait locus; LOD, logarithm of the odds; IBD, identical by descent; ABC, ATP-binding cassette; ACAD8, Acyl-CoA dehydrogenase family, member 8; CPT1A, Carnitine palmitoyltransferase 1A; CYP, Cytochrome P450; FABP1, Fatty acid binding protein 1; LIPE, Hormone-sensitive lipase; LRP, Low-density lipoprotein receptor-related protein; GGPS1, Geranylgeranyl diphosphate synthase 1; SOAT, Sterol O-acyltransferase; UCP, Uncoupling protein; CETP, cholesterol ester transfer protein.

Abstract

A genome-wide linkage study was performed to identify chromosomal regions harboring genes influencing lipid and lipoprotein levels. Linkage analyses were conducted for four quantitative lipoprotein/lipid traits, i.e. total cholesterol, triglyceride, HDL-C and LDL-C concentrations, in 930 subjects enrolled in the Québec Family Study. A maximum of 534 pairs of siblings from 292 nuclear families was available. Linkage was tested using both an allele sharing and a variance component linkage methods. The strongest evidence of linkage was found on chromosome 12q14.1 at marker D12S334 for HDL-C with a logarithm of the odds (LOD) score of 4.06. Chromosomal regions harboring quantitative trait loci (QTLs) for LDL-C included 1q43 (LOD = 2.50), 11q23.2 (LOD = 3.22), 15q26.1 (LOD = 3.11) and 19q13.32 (LOD = 3.59). In the case of triglycerides, three markers located in 2p14, 11p13 and 11q24.1 provided suggestive evidence of linkage (LOD > 1.75). Tests for total cholesterol levels yielded significant evidence of linkage at 15q26.1 and 18q22.3 with the allele sharing linkage method, but the results were non-significant with the variance component method. In conclusion, this genome scan provides evidence for several QTLs influencing lipid and lipoprotein levels. Promising candidate genes were located in the vicinity of the genomic regions showing evidence of linkage.

Supplementary key words: genome scan, linkage, genetics, blood lipids, quantitative trait locus, triglyceride, cholesterol.

Introduction

Studies investigating the genetics of blood lipids and lipoproteins have clearly established that genetic factors contribute to these phenotypes (1,2). Until recently, the molecular bases of blood lipids have been mainly investigated using a candidate gene approach. Although genes accountable for several monogenic dyslipidemias have been identified (3), those underlying the variation in the population at large remain to be found. These results have motivated several investigators to use the genome scan approach to identify chromosomal regions harboring genes controlling lipoprotein/lipid levels. Such an approach has the ability to find quantitative trait-loci (QTLs) without being dependent on an understanding of the physiology governing the traits. Genome scans can generate useful leads and hypotheses whose usefulness is greatly enhanced when the findings are replicated in independent samples (4).

To date, the results of full genome scans for lipoprotein/lipid traits have produced a number of significant findings. For total cholesterol, the results from the Pima Indian community have provided evidence of linkage on chromosome 19p (5). The 1q region was also suggested to contain a locus influencing cholesterol level in obese families (6). A cholesterol-lowering gene was mapped as well on 13q from an extended Israel family and replicated by the same investigators with a healthy white twin cohort (7). Loci controlling LDL-C were reported on 19q in the Hutterites community (8) and on 11p in the NHLBI Family Heart Study (9). For HDL-C, several major loci were mapped, including 5q in the NHLBI Family Heart Study (10), 8q in Finnish families (11), 9p in Mexican Americans (12) and 6q in the Framingham Study (13). However, the most promising location for an HDL-C locus is on 16q22-q23 from linkages in both the Mexican Americans (14) and combined Dutch and Finnish families (15). The Finnish families have also suggested a low HDL-C locus within this region (11). Finally, a putative locus for familial low HDL-C has also been identified near the apo AI-/C-III/A-IV gene cluster on 11q23 (16). The search for loci influencing triglyceride levels has been similarly fruitful. Genome-wide evidence of linkage has been reported on 2q in Hutterites (17), 10p in Finnish families (18), 15q in a second set of Mexican Americans ascertained for type 2 diabetes (19) and 19q in Utah Caucasian families (20).

Based on these observations, it is clear that lipid and lipoprotein traits are influenced by several loci. However, additional genome scans are required to strengthen previous observations and

identify the most promising regions underlying the genetic components of these phenotypes. Thus, the purpose of this study was to identify the genomic regions influencing total cholesterol, LDL-C, HDL-C and triglyceride levels in a cohort of French-Canadian families.

Methods

Subjects

The Québec Family Study (QFS) is an ongoing investigation of French-Canadian families studying the genetics of obesity and its comorbidities (21). There are four phases in the QFS and the forth phase is currently in progress. The first phase includes the data collection that took place from 1979 to 1981 on families randomly ascertained. In phase 2, a sample of families from phase 1 were remeasured and additional families, ascertained through obese proband, were recruited and incorporated in the cohort. In the third phase, members of the phase 2 cohort were remeasured and the children of the adult offspring were recruited when they reach 10 years of age. DNA analysis are available for subjects in phase 2 and over. In the current study, the subjects were participants from phase 2 and phase 3 in order to maximize the number of subjects available for transversal analysis. The serum lipid and lipoprotein concentrations were available for 930 members of 292 nuclear families. This sample represents an half and half mixture of random sampling and ascertainment through obese probands. The characteristics of the subjects in the four sex-by-generation groups (fathers, mothers, sons, and daughters) are reported in Table 1. All subjects were free of familial lipid disorders requiring lipid lowering drugs. The Institutional Review Board of the Laval University approved all procedures and all subjects gave informed-written consent.

Phenotypes

Blood samples were collected in the morning from an antecubital vein after a 12-hour overnight fast. The plasma was separated immediately after blood collection by centrifugation at 3000 rpm for 10 minutes for the measurement of plasma lipoprotein/lipid levels. Cholesterol (22) and triglyceride (23) concentrations were determined enzymatically using a Technicon RA-500 automated analyzer (Bayer, Tarrytown, NY, USA). HDL fraction was obtained after precipitation of LDL in the infranatant (>1.006 g/mL) with heparin and $MnCl_2$ (24). The cholesterol content of the infranatant fraction was measured before and after the precipitation step for the measurement of HDL-C and for the calculation of LDL-C. Body mass index (BMI) was determined by weight (kg)/ height (m^2).

Genotyping

A total of 443 markers spanning the 22 autosomal chromosomes with an average intermarker distance of 7.2 centimorgans were genotyped as described previously (25). These markers included 337 microsatellite markers (dinucleotide, trinucleotide, and tetranucleotide repeats) and 106 polymorphisms in 65 candidate genes. The results were stored in a local dBase IV database, GENEMARK, which inspects results for Mendelian inheritance incompatibilities within nuclear families and extended pedigrees. The OMIM gene map (http://www.ncbi.nlm.nih.gov/htbin-post/Omim/getmap) and the bioinformatic site from the University of California, Santa Cruz (http://genome.ucsc.edu/) were used to identify candidate genes.

Linkage Analyses

The triglyceride and cholesterol variables were $\log_{10}$ transformed to normalize their distribution prior to adjustment for covariates. Lipid and lipoprotein traits were adjusted for the effects of age including squared and cubic terms to allow for non-linearity, as well as for gender and BMI. The adjustments were performed using a stepwise multiple regression procedure retaining only significant terms ($p < 0.05$). Separate regression models were used for each of six age-by-sex (<30, 30-50, and ≥50 years, in male and female) groups. Regression parameters were estimated after exclusion of outliers (± 3 SD), and residuals were computed for all subjects. Residual scores were then standardized to a mean of 0 and an SD of 1 before genetic analyses. Subjects whose values were greater than 4 SD from the mean and were separated by more than 1 SD from the nearest internal score were excluded from the analysis because they were considered to be sparse outliers (4 subjects for total cholesterol, 1 for triglyceride, 2 for LDL-C and 3 for HDL-C). Adjustments of the phenotypes were performed using SAS (version 8.02).

We conducted quantitative trait linkage analyses using two different methods. We used the new Haseman-Elston regression-based method (26) which models the trait covariance between sibpairs, instead of the squared sibpair trait difference used in the original method. It regresses the mean-corrected sib pair product on the number of alleles shared identical by descent (IBD). Singlepoint and multipoint estimates of alleles shared IBD were generated using the GENIBD

software and linkage was tested using the SIBPAL2 software from the S.A.G.E. 4.0 statistical package (S.A.G.E., 2001) (27). The maximum number of sibpairs was 534. In the alternative method, the phenotypic covariance among members of a family is assumed to result from the additive effects of linkage due to a QTL (q), a residual familial component due to polygenes (g) and an individual-specific random environmental component (e). Hypothesis testing was performed by the likelihood ratio test, which test the null hypothesis that the additive genetic variance due to the QTL (σ_q) equals zero ($\sigma_q = 0$) by comparing the likelihood of this restricted model with that of a model in which σ_q is estimated ($\sigma_q \neq 0$). The difference in minus twice the log-likelihoods is approximately distributed as a 50:50 mixture of a χ^2 and a point-mass distribution at zero. The LOD score was computed as $\chi^2/(2 \log_e 10)$. These analyses were performed using the quantitative transmission disequilibrium test (QTDT) computer program (28). We used a LOD score of ≥ 3.00 ($p \leq 0.0001$) to indicate adequate evidence of linkage and a LOD threshold of ≥ 1.75 ($p \leq 0.0023$) as suggestive (29).

Results

Prior to the genome scan analysis, total cholesterol, triglyceride, LDL-C and HDL-C levels were adjusted in a stepwise manner for the effects of age, age^2, age^3, gender and BMI. These covariates accounted for 0 to 15.3%, 5.7 to 30.2%, 0 to 10.6% and 6.6 to 32.2% of the total phenotypic variation in total cholesterol, triglyceride, LDL-C and HDL-C, respectively, depending on the age-by-sex groups (see Methods).

An overview of the variance component-based linkage results for the LDL-C, HDL-C and triglyceride phenotypes is given in Figure. Numerous peaks with LOD score above 1.75 are observed for LDL-C, including chromosome 1q43, 3q23, 11q13-q24, 13q32, 15q26, 18q21 and 19q13. The highest peak among them is located on chromosome 19q13 with a LOD score of 3.59 for a marker within the gene coding apo E. The peak on chromosome 11q was quite broad, and encompassed a 1-LOD support interval (1 LOD unit reduction from the peak) of about 40 cM. In contrast, only one chromosomal region reaches the significance level of linkage for HDL-C. However, this peak located on chromosome 12q14 provided the highest LOD score observed in the study (LOD = 4.06). In the case of triglycerides, four genomic regions exceeded the 1.75 LOD score threshold, including 2p14, 5q14, 11p13 and 11q24. Although interesting, these peaks did not reach the magnitude of those observed for LDL-C and HDL-C. Remarkably, the two peaks for triglycerides on chromosome 11 did not overlap with the large one observed for LDL-C.

Linkage was also tested using singlepoint and multipoint allele sharing method. All chromosomal regions with a variance component-based LOD score ≥ 1.75 or an allele sharing-based p value ≤ 0.0023 are reported in Table 2 for the four lipid traits. Six, 17, 9 and 13 markers showed suggestive evidence of linkage with at least one of the methods used for total cholesterol, LDL-C, HDL-C and triglycerides, respectively. Among all these markers, only seven of them provided suggestive evidence of linkage (LOD ≥ 1.75 or $p \leq 0.0023$) with both the allele sharing and the variance components-based linkage methods. These markers are underlined in Table 2 and correspond to chromosome regions 1q43, 15q26.1 and 19q13.32 for LDL-C, 12q14.1 for HDL-C and 2p14, 11p13 and 11q24.1 for triglycerides. All singlepoint and multipoint results around these chromosomal regions are provided in the supplementary Table. Although some markers

provided fairly good evidence of linkage with total cholesterol, results were inconsistent across linkage methods (Table 2). Positional candidate genes in the seven regions identified as the most promising one in addition to the large 11q region for LDL-C are summarized in Table 3.

Discussion

The present study confirms the existence of multiple loci influencing blood lipids and lipoproteins. Based on this genome-wide scan, evidence of linkage was found on chromosome regions 1q43, 11q13-q24, 15q26.1 and 19q13.32 for LDL-C, 12q14.1 for HDL-C and 2p14, 11p13 and 11q24.1 for triglycerides. Some of these regions have been previously linked to lipid-related phenotypes while others represent new findings. In genome-wide linkage studies, independent replication of positive findings is important to distinguish between true and false positives (4). For complex traits, determining whether a given study has replicated an initial study's findings is difficult. It has been demonstrated that the location estimate may be many centimorgans away from the true locus (30). Given this variation in position, it is hard to distinguish between random variation around a single locus and the presence of multiple genetic signals. Despite these limitations, we present in Table 4 the positive findings reported by previous genome scans on lipid-related phenotypes that are located around (and potentially replicated) the chromosomal regions identified in the current study.

The peak observed on 1q43 for LDL-C in this study represents a newly identified locus. The 1q region is a well recognized region for FCHL (31-33) and has also been linked to cholesterol (6), and Apo AII levels (34) as well as with Lp(a) concentrations (35). However, our peak on 1q is more distal from the centromere compared to the other studies. Multiple peaks were observed on chromosome 11, including two for triglycerides and another large one for LDL-C. Genome-wide evidence of linkage has also been demonstrated on this chromosome for LDL-C in the NHLBI family heart study (9). In addition, suggestive linkages have been reported for total cholesterol levels in the Rochester family heart study (36), for FCHL in Dutch families (37), and for elevated apo B levels in Finnish families (18) (Table 4). In addition, dense markers linkage analysis restrict to a specific region (11q23) of chromosome 11 has provided evidence of linkage with hypoalphalipoproteinemia (16). For chromosome 15, the peak observed on the q-terminal side for LDL-C overlapped with the newly identified locus for autosomal recessive hypercholesterolemia observed in Sardinian families (38). A QTLs for unesterified HDL_{2b}-C reported in the San Antonio family heart study is also located close to the LDL-C signal observed in the present study (39). One of the strongest signals in this genome scan was found on 19q13 with LDL-C. This region has produced genome-wide evidence of linkage with different lipid-

related phenotypes before including LDL-C among the Hutterites population (8), triglyceride in Utah families (20), and apo E levels in the Rochester family heart study (36). A suggestive QTL that influence variation in cholesterol concentrations of large LDL particles (LDL-2) has also been mapped at this location in the San Antonio family heart study (40). The highest LOD score for the present genome scan was observed on 12q with HDL-C. No QTLs for lipid-related phenotypes have been reported around this area suggesting that this locus represents a newly identified region influencing HDL-C levels. Finally, despite being of lesser magnitude, the 2p region provided consistence evidence of linkage for triglyceride levels. This region is near to the locus suggested for low-HDL cholesterol (11), unesterified HDL_{2a}-C level (39) and triglyceride/HDL ratio (41).

In contrast, some of the most promising regions linked to lipid-related phenotypes reported so far were not replicated. This is the case for total cholesterol on 19p (5), for HDL-C-related phenotypes on 5q (10), 9p (12), and 16q (14,15), and for triglyceride levels on 10p (18), and 15q (19). The lack of replication in these regions is not surprising and can be due to a variety of reasons. First, the previous genome scan studies were conducted in populations with a variety of ethnic backgrounds and that were ascertained for different reasons. Thus, there may be etiological heterogeneity. Second, it is conceivable that similar phenotypes may not have common etiologies and different lipoprotein/lipid genes may operate in different subsets of families. In addition, multiple interacting loci or environmental factors are likely to participate in the regulation of these phenotypes. As a consequence, complex genetic and environmental contexts may be required for lipoprotein/lipid genes to be expressed. Accordingly, replication of a previously significant linkage can be difficult to achieve when dealing with complex quantitative traits such as blood lipids.

Other than the single gene defects known to cause dyslipidemia (3), little is known about the specific major genetic determinants of blood lipids. From this genome-wide scan analysis, a number of promising candidate genes can be located in the vicinity of the genomic regions showing evidence of linkage. These candidates as well as their distances from the peak are provided in Table 3. First, the strong evidence of linkage on chromosome 19q comes from a marker located within the apo E gene. It is well known that genetic variations in this particular gene modulate plasma lipid levels (42). Nevertheless, there are other genes within this region that

worth mentioning. Indeed, the apo E gene lies in a cluster of apolipoprotein genes containing apo C-I, C-II and C-IV. These apolipoproteins are constituents of lipoproteins and serve as activators for enzymes. The hormone sensitive lipase (LIPE) gene is also located under the 19q peak. Finally, the large genetic distance (35 Mb) separating the LDL receptor gene from the peak rules out its possible involvement in this signal.

Interesting candidate genes are also located under the broad peak observed on 11q. The width of this peak, covering more than one third of the total chromosome (LOD ≥ 1.00), may be due to the major effect of a gene or may indicate overlapping peaks that are due to more then one gene. The ACAT1 gene, known to be involved in the esterification of intracellular cholesterol, is located near the highest point of the peak (115.7 cM). Even closer to the signal is the apo A-I/C-III/A-IV gene cluster. Additional candidate genes are located in the 80 cM region of the peak (Table 3). Thus, the number of candidate genes in that region suggests the existence of more than one gene being causative. Promising genes were also located within the HDL-C locus on 12q. Particularly interesting is the apo F gene which encodes a protein product known to inhibit the CETP-mediated transfer of triglyceride and cholesterol between plasma lipoproteins (43). However, the LDL receptor-related protein 1, known to bind apo E-containing lipoproteins, is also close to the signal.

In summary, despite the candidate genes/regions identified so far, the specific loci acting on the variability of serum lipids in individuals who have not been selected for lipid disorders are still unknown. This genome scan presented evidence of linkage for lipid-related traits on 8 chromosomal regions, including 1q43, 11q13-q24, 15q26.1 and 19q13.32 for LDL-C, 12q14.1 for HDL-C and 2p14, 11p13 and 11q24.1 for triglyceride levels. Most of these regions have been linked to lipoprotein/lipid traits before. However, the highest signal (LOD = 4.1 at 12q14.1) observed in this genome scan for HDL-C level represents a newly identified region. Interesting candidate genes are located within this chromosomal region and the other regions identified. These positional candidate genes represent hypotheses to be tested in future studies.

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Table 1. Characteristics of Genomic Scan Participants by Gender and Generation Groups.

Variables	**Fathers** **n = 194**	**Mothers** **n = 261**	**Sons** **n = 213**	**Daughters** **n = 262**
Age (years)	55.5 ± 10.1	54.8 ± 12.5	26.7 ± 9.7	27.9 ± 10.6
BMI (kg/m^2)	28.4 ± 5.8	28.7 ± 8.0	26.4 ± 7.2	26.8 ± 8.8
Cholesterol (mmol/L)	5.42 ± 0.85	5.44 ± 1.08	4.47 ± 0.91	4.48 ± 0.80
LDL-C (mmol/L)	3.49 ± 0.78	3.35 ± 0.95	2.77 ± 0.78	2.64 ± 0.70
HDL-C (mmol/L)	1.07 ± 0.27	1.37 ± 0.35	1.11 ± 0.25	1.28 ± 0.30
Triglyceride (mmol/L)	1.96 ± 1.12	1.62 ± 0.81	1.31 ± 0.69	1.23 ± 0.56

Values are means ± SD.

BMI, body mass index.

Table 2. Summary of P Values < 0.0023 from the Allele Sharing Method (Singlepoint and Multipoint) or LOD Scores > 1.75 from the Variance Component Method.

Phenotypes	Chr.	Distance (cM)	Marker*	p values		LOD score
				Singlepoint	Multipoint	
Cholesterol	1	239.8	D1S547	0.000208	0.000437	1.21
	2	241.2	D2S2968	0.042940	0.030438	2.04
	15	90.8	D15S652	**0.000077**	0.036295	0.73
	16	20.9	D16S403	0.001993	0.018806	0.96
	18	71.9	ATA82B02	**0.000059**	0.002177	1.15
	20	4.5	D20S482	0.001804	0.039883	0.46
LDL-C	1	230.4	D1S3462	0.026331	0.005501	1.79
	1	239.8	D1S547	**0.000008**	**0.000078**	2.50
	3	145.1	D3S1764	0.028576	0.012546	2.79
	11	79.8	D11S911	0.327252	0.004652	2.57
	11	82.3	D11S2002	0.012992	0.001407	2.81
	11	107.8	D11S2000	0.040486	0.001069	2.55
	11	115.7	DRD2	0.009048	0.001213	**3.22**
	11	118.5	UCP3	0.325612	0.022330	2.06
	13	97.0	D13S793	0.007766	0.015280	1.85
	15	90.8	D15S652	0.000315	0.000288	**3.11**
	18	62.0	D18S38	0.051564	0.005764	2.04
	18	62.5	MC4R	0.150490	0.005014	2.24
	18	71.9	ATA82B02	0.001517	0.017365	1.01
	19	57.5	D19S178	0.012600	0.000617	**3.24**
	19	58.6	APOE	**0.000005**	0.000241	**3.59**
	19	62.8	GYS1	0.194859	0.001568	2.93
	20	4.5	D20S482	**0.000013**	0.000690	1.64
HDL-C	2	171.7	D2S1776	0.001052	0.005608	0.35
	12	53.4	D12S398	0.042114	0.004348	1.93
	12	61.0	D12S334	0.002204	0.000746	**4.06**
	12	68.5	D12S375	0.002196	0.001407	0.88
	16	7.4	D16S287	0.000251	0.313383	0.21
	18	12.3	D18S542	0.061955	0.000717	1.19
	18	13.3	MC5R	0.121424	0.002061	0.94
	20	45.9	D20S197	0.015407	0.001505	1.01
	20	47.0	D20S176	0.627925	0.000466	0.81
Triglyceride	1	111.2	D1S2860	0.000586	0.001153	1.39
	1	117.7	ATP1A1	0.247956	0.002042	1.05
	1	229.3	AGT	0.000893	0.008674	0.82
	2	67.8	D2S441	**0.000009**	0.001346	2.32
	4	149.6	UCP	**0.000043**	0.189994	0.33
	5	73.5	D5S1501	0.036299	0.004120	2.23

Phenotypes	Chr.	Distance (cM)	Marker*	p values		LOD score
				Singlepoint	Multipoint	
	5	73.9	CART	**0.000068**	0.015052	1.67
	7	151.6	NOS	0.000848	0.072359	0.21
	8	20.0	LPL	0.001273	0.032691	0.55
	8	88.3	D8S1119	0.001159	0.126362	0.31
	11	31.3	ATA34E08	0.022495	0.000330	1.69
	11	34.9	D11S1392	0.000105	**0.000009**	2.11
	11	125.6	**D11S4464**	0.001204	0.001890	1.93

P value ≤ 0.0001 or LOD score ≥ 3.00 are in bold.

*Markers showing suggestive evidence of linkage (P value ≤ 0.0023 or LOD score ≥ 1.75) with the three linkage methods used are in bold.

Table 3. Positional Candidate Genes Within Chromosomal Regions Showing Suggestive Evidence of Linkage with the Three Linkage Methods.

Phenotypes	Chr. Region	Marker	LOD score	Candidates genes (distance from the marker in Mb*)
LDL-C	1q43	D1S547	2.50	ABCB10 (-11.7), GGPS1 (-6.2)
	11q14.1	D11S2002	2.81	LRP5 (-11.6), CPT1A (-11.2), UCP2 (-6.3), UCP3 (-6.2)
	11q23.2	DRD2	3.22	ACAT1/SOAT1 (-5.5), APOA1 (3.4), APOC3 (3.4), APOA4 (3.4), APOA5 (3.3)
	15q26.1	D15S652	3.11	CYP11A (-18.3)
	19q13.32	APOE	3.59	LRP3 (-11.7), LIPE (-2.5), APOC4 (0), APOE (0), APOC1(0), APOC2 (0)
HDL-C	12q14.1	D12S334	4.06	SOAT2 (-7.3), APOF (-4.4), LRP1 (-3.5), CYP27B1 (-3.2)
Triglyceride	2p14	D2S441	2.32	FABP1 (19.6)
	11p13	D11S1392	2.11	ABCC8 (-17.6), LRP4 (12.6)
	11q24.1	D11S4464	1.93	ACAT1 (-15.4), APOA1 (-6.6), APOC3 (-6.6), APOA4 (-6.6), APOA5 (-6.6), ACAD8 (11)

*Distance separating the marker and the candidate genes are taken from the bioinformatic site of the University of California, Santa Cruz (http://genome.ucsc.edu/). Negative and positive values indicate that the gene is located downstream or upstream from the marker, respectively.

ABC, ATP-binding cassette; ACAD8, Acyl-CoA dehydrogenase family, member 8; APO, Apolipoprotein; CPT1A, Carnitine palmitoyltransferase 1A; CYP, Cytochrome P450; FABP1, Fatty acid binding protein 1; LIPE, Hormone-sensitive lipase; LRP, Low density lipoprotein receptor-related protein; GGPS1, Geranylgeranyl diphosphate synthase 1; SOAT, Sterol O-acyltransferase; UCP, Uncoupling protein.100

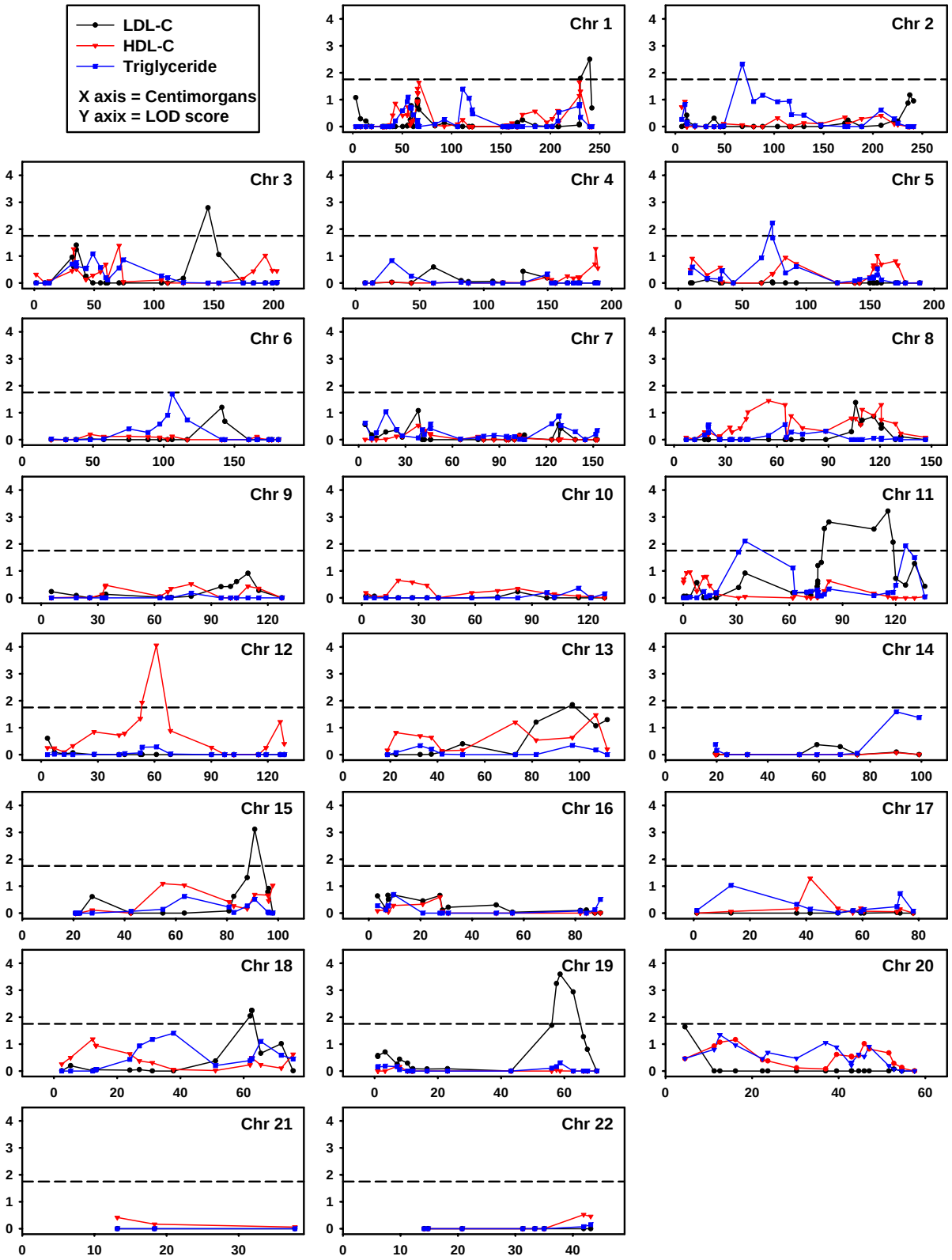
Table 4. Possible Replication of the Current Chromosomal Regions Identified with those from Previous Genome Scans on Lipid-Related Phenotypes.

Chr	Location (Mb)*	Study	Phenotypes	Lod Score
1q	239.8	This report	LDL-C	2.5
2p	68.4	This report	Triglyceride	2.3
	73.1	Finnish Families (11)	HDL-C	2.1
	75.5-88	North Eastern Indian (41)	TG/HDL ratio	1.9
	85.1	San Antonio FHS (39)	Unesterified HDL_{2a}-C	2.3
11	30.2	Rochester FHS (36)	Cholesterol	1.8
	36.2	This report	Triglyceride	2.1
	46.3	NHLBI FHS (9)	LDL-C	3.7
	60.9	Dutch Families (37)	FCHL	2.6
	79.8-130.6	This report	LDL-C	3.2
	125.6	Finnish Families (18)	Apo B	1.8
	125.6	This report	Triglyceride	1.9
12q	61	This report	HDL-C	4.1
15q	81.5	San Antonio FHS (39)	Unesterified HDL_{2b}-C	2.5
	88.3-92.7	Sardinian Families (38)	FH	3.3
	89	This report	LDL-C	3.1
19q	31	Hutterites (8)	LDL-C	p=0.0001
	34.7-58.6	Rochester FHS (36)	Apo E	4.2
	35.8-45.1	San Antonio FHS (40)	LDL-2	1.9
	45.1-46.1	Utah Families (20)	Triglyceride	3.2
	46.1	This report	LDL-C	3.6

*The physical distance is the location of the marker(s) that define the peak or the closest to the signal and is obtain from the genome browser of the University of California, Santa Cruz (http://genome.ucsc.edu).

FHS, family heart study; FH, familial hypercholesterolemia.

Figure 1. Variance component-based linkage results for all autosomal chromosomes with LDL-C, HDL-C and triglyceride phenotypes. LOD scores are presented on the y-axis and genetic distance is presented on the x-axis in centimorgans. The three traits are adjusted for the effects of age, age^2, age^3, gender and BMI. The horizontal dashed line represent a LOD score of 1.75.


LDL-C
HDL-C
Triglyceride
X axis = Centimorgans
Y axix = LOD score
Chr 1
Chr 2
Chr 3
Chr 4
Chr 5
Chr 6
Chr 7
Chr 8
Chr 9
Chr 10
Chr 11
Chr 12
Chr 13
Chr 14
Chr 15
Chr 16
Chr 17
Chr 18
Chr 19
Chr 20
Chr 21
Chr 22

Chapter 6.

What Have we Learned from Genomewide Scans on Lipid-Related Phenotypes so Far? Fixing Perspective with a New Genomewide Search on Apo B and Apo AI Levels in the Québec Family Study.

Yohan Bossé, Yvon C Chagnon, Jean-Pierre Després, Treva Rice, D.C. Rao, Claude Bouchard, Louis Pérusse, Marie-Claude Vohl.

Un grand nombre de criblages génomiques sur les variables lipidiques ont été rapportés dans la littérature. À cet effet, nous avons créé une banque de données contenant les résultats des criblages génomiques effectués jusqu'à ce jour. Cette synthèse va permettre aux investigateurs de positionner leurs prochains résultats sans être obligés de digérer la grande quantité d'articles scientifiques. L'utilité de cette banque de données a ensuite été démontrée avec un nouveau criblage génomique sur les niveaux d'apolipoprotéine (apo) B, d'apoB-LDL et d'apoAI, chez 679 sujets. Deux nouveaux loci ont été identifiés, soit le 18q21.32 et le 3p25.2 pour les niveaux d'apoB-LDL et d'apoAI, respectivement. La banque de données nous a permis de dévoiler que ce dernier est un nouveau locus relié aux lipides sanguins. Cet exercice nous a aussi permis de constater qu'une grande portion du génome est maintenant couverte avec des évidences de liaison.

REVIEW

What Have we Learned from Genomewide Scans on Lipid-Related Phenotypes so Far? Fixing Perspective with a New Genomewide Search on Apo B and Apo AI Levels in the Québec Family Study.

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Short title: Genome Scans on Lipid-Related Phenotypes

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Abstract

The genetic dissection of complex inherited diseases is a major challenge. Although the success rate is highly limited in finding potentially relevant genes, a large body of data based on genomewide scan strategies is now available for a variety of diseases and related phenotypes. This can perhaps be best appreciated in the field of lipid and lipoprotein levels. The amount of information generated from genomewide scans on lipid-related phenotypes may soon overwhelm even the most dedicated investigators. We have created a database containing the results from whole-genome scans undertaken to date. This synthesis may be helpful to investigators in positioning new findings without having to digest a large body of scientific papers. The usefulness of this database is then demonstrated by performing a new autosomal genomic scan on apolipoprotein (apo) B, LDL-apo B and apo AI levels, measured in 679 subjects of 243 nuclear families. Linkage was tested using both allele sharing and variance component methods. Only two loci provided support for linkage with both methods, including a LDL-apo B locus on 18q21.32 and an apo AI locus on 3p25.2. Adding those findings to the database highlighted the fact that the former is a first time reported lipid-related locus, whereas the later has been observed before. However, displaying all data on the same map revealed that a large portion of the genome is now covered with loci supported by at least suggestive evidence of linkage.

Keywords: Genome scans, lipid-related phenotypes, compendium, apolipoproteins, lipoproteins, quantitative trait locus, cardiovascular risk factors, linkage, dyslipidemia.

Introduction

Mapping genes involved in complex human diseases is one of the major challenges in human genetics. With the increasing incidence of chronic diseases in industrialized societies, finding these genes is clinically and economically relevant. During the past few years, considerable research resources have been deployed to study the genetic etiology of complex human diseases in order to better understand their pathogenesis and, ultimately, improve prevention strategies, diagnostic tools, and therapies[1]. Enthused by the early success in the identification of genes responsible for monogenic diseases, many investigators have embraced genome scan strategies. This trend has resulted in an enormous amount of information, which is now typically difficult to synthesize and interpret for a given complex disease.

The importance ascribed to lipid and lipoprotein levels in risk estimation and in the treatment of CHD[2] has stimulated molecular studies to investigate the genetic etiology underlying human variation in these traits. A large number of genomewide screens on serum lipid-related phenotypes have been performed to date and a review of such studies seems timely. Since linkage results must be replicated to be credible[3], a compendium of published QTLs may facilitate the identification of replicated findings. To provide an example on how such information can be useful, we are adding herein the results of a new genome scan on apolipoprotein (apo) B and apo AI levels to this compendium.

Apo B and apo AI levels are good markers of CHD risk[4,5]. A number of studies have clearly established that genetic factors contribute to interindividual differences in apo levels. An elegant study comparing identical and fraternal twins reared together with twins reared apart has shown that a large portion of the variance in apo B and apo AI levels is attributable to genetic factors, with heritability estimates above 50%[6]. In addition, based on complex segregation analyses, major gene effects have been reported for these two phenotypes[7,8]. Mutations in genes that encode apo B, LDL receptor and ABCA1 have been implicated in monogenic disorders altering plasma apolipoprotein levels including familial hypobetalipoproteinemia (OMIM 605019), familial hypercholesterolemia (OMIM 143890) and hypoalphalipoproteinemia (OMIM 604091). However, these mutations do not account for the variation in plasma apo B and apo A levels in the general population. In an attempt to identify the responsible genes, a large number of association and linkage studies have been performed with candidate genes. However, these

studies have been difficult to interpret due to conflicting results, lack of replication, and the occurrence of positive findings only in specific subgroups. Perhaps the highest linkage signal for apo B levels was reported in Dutch pedigrees on chromosome 1p31 (LOD = 4.7)[9]. Other suggestive linkages (LOD > 1.7) have been found on chromosome 12q24 for apo AI[10] and on 1p, 11q24, 21q21 and Xq23 for apo B[11,12]. However, other genomewide scans failed to identify QTL for apo B levels[10,13]. To search for additional loci influencing apo B and apo AI levels or to replicate previous findings, we performed an autosomal genome scan among 243 nuclear families participating in the Québec Family Study.

Materials and Methods

Population

Subjects were participants of the Québec Family Study (QFS) — an ongoing project with French-Canadian families investigating the genetics of obesity and its comorbidities[14]. In this study, a total of 679 subjects of 243 nuclear families had apolipoprotein measurements available. This cohort represents a mixture of random sampling and ascertainment through obese (BMI > 32 kg/m^2) probands. Table 1 presents the characteristics of subjects in each of the sex and generation groups. The study was approved by the Laval University Medical Ethics Commitee, and all subjects provided written informed consent. All procedures followed were in accordance with institutional guidelines.

Apolipoprotein measurements

Blood samples were obtained from an antecubital vein in the morning after a 12-hour overnight fast. The apo measurements were performed with the rocket immunoelectrophoretic method[15]. Apo B concentrations were measured in plasma whereas LDL apo B and apo AI concentrations were measured in the infranatant (d > 1.006 g/ml) obtained after separation of very-low density lipoprotein from the plasma by ultracentrifugation. The measurements were calibrated with reference standards obtained from the Center for Disease Control (Atlanta, GA, USA).

Linkage analysis

A total of 443 markers spanning the 22 autosomal chromosomes with an average intermarker distance of 7.2 centimorgans were genotyped as described in Chagnon et al.[16]. The apo traits were adjusted for the effects of age (up to cubic polynomial to allow for non-linearity), gender and body mass index (BMI) using a stepwise multiple regression procedure retaining only significant covariates ($p < 0.05$) as described previously[17]. Adjustments of the phenotypes were performed using SAS (version 8.2).

We conducted quantitative trait linkage analyses using both an allele sharing and a variance component methods. For the allele sharing method, we used the new Haseman-Elston regression-

based method[18] which models the mean-corrected cross product of the sibs' trait values, instead of the squared sib pair trait difference used in the original method[19]. Two-point and multipoint (at 1 cM interval) estimates of alleles shared IBD were generated using the GENIBD software and linkage was tested using the SIBPAL2 software from the S.A.G.E. 4.0 statistical package[20]. The maximum number of sib pairs was 347. Empirical p values of the test statistic were also computed using a Monte Carlo permutation procedure with 10000 replicate permutations for genomic regions containing two-point linkage markers with suggestive evidence of linkage ($p<0.0023$). Linkage was also performed with a variance component model using the quantitative transmission disequilibrium test (QTDT) computer program[21]. Under this model, a phenotype is influenced by the additive effects of a QTL (q), a residual familial component due to polygenes (g) and a residual nonfamilial component (e). Hypothesis testing was performed by the likelihood ratio test. The likelihood of the null hypothesis is obtained by restricting the additive genetic variance due to the QTL (σ_q) equal to zero ($\sigma_q = 0$). The test is conducted by contrasting this restricted model with the alternative where σ_q is estimated ($\sigma_q \neq 0$). The difference in minus twice the log-likelihoods between the null and alternate hypotheses is approximately distributed as a χ^2 which allowed LOD score computation as $\chi^2/(2 \log_e 10)$. We have taken a LOD score of ≥ 3.00 ($p \leq 0.0001$) as evidence of linkage and a LOD of ≥ 1.75 ($p \leq 0.0023$) as evidence of suggestive linkage[22]. We have also retained LOD scores ≥ 1.18 ($p \leq 0.01$) to identify potential independent confirmation of a previously reported significant linkage[23].

Database

The initial search for genomewide scan publications on lipid-related phenotypes was accomplished with keywords (genome scan + lipoprotein and linkage + lipoprotein + genome) at the bioinformatic site of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The publication list was completed and verified by examination of both the discussion section and the reference list of the publication found in the initial search. The search focused on results published before the end of April 2003 and excluded abstract presented at meetings.

A whole-genome scan excel database for lipid-related phenotypes was established. The database contained bibliographic details (first author, source and years), study population (ethnicity), ascertainment scheme, phenotypic traits, sample-size details (number of individuals, sib pairs and families), linkage analysis methods, and results. Any evidence of linkage, from suggestive and better, (LOD score ≥ 1.7 or P value ≤ 0.0023) was treated as an observation (a hit). Results were entered in the database with the name of the linked marker/gene, its location (megabase and chromosomal band), and its maximum LOD score or Z score or P value. For most studies, markers were provided in the papers and were those defining the peak or were the closest to the signal. When the marker's name or the specific location of the QTL (hits) was not available in the original manuscript, the authors were contacted and asked to provide the missing information. To identify possible replication and compared loci across studies, the location of each linked marker/gene was positioned on a single map provided by the human genome browser of the University of California, Santa Cruz (assembly, June 2002) (http://genome.ucsc.edu). When a two-stage strategy was reported in the publication, the lowest P value attained at any phase of the analytical strategy was considered. Similarly, when multiple linkage methods were used in the same publication, the most significant result was kept for the database.

To evaluate whether QTLs were randomly distributed across the genome, we regressed the observed hit ratio against the expected hit ratio as reported previously[24]. The observed hit ratio of each chromosome was obtained as: (number of hits on a specific chromosome / number of hits across all chromosomes) x 100, and the expected hit ratio of each chromosome was obtained as: (number of genes on a specific chromosome / total number of genes in the genome) x 100. The gene content of each chromosome and for the whole genome are from Venter et al.[25]. A significant association (positive slope) between the observed and expected hit ratio would suggest that the positive linkage reported in the literature are distributed randomly across the genome. In contrast, if the association is missing, it would suggest that the observed hits are concentrated within specific chromosomes containing the genes controlling lipid and lipoprotein levels.

Results

Genome scan on apo B, LDL-apo B and apo AI

Detailed results for all chromosomes and phenotypes are available in supplementary information. Table 2 summarizes the markers showing weak to moderate evidence of linkage ($p \leq 0.01$ or LOD score ≥ 1.18) with the allele sharing (two-point and multipoint) and the variance component linkage methods. The highest variance component LOD score was obtained for LDL-apo B on chromosome 18q21.32 (LOD = 2.05) (Figure 1). Hits were also observed by the variance component method for total apo B on 6p22.3-p21.1 and 6q23.1, for LDL-apo B on 2q35 and 11q22.3, and for apo AI on 3p25.2.

In this study, the new Haseman-Elston linkage method yielded more genetic loci which are summarized in Table 2. However, most of the strong linkage evidence observed with the allele sharing linkage method (both in two-point and multipoint) were not supported by the variance component method. Only two loci, one at 18q21.32 (marker D18S38, Figure 1) for LDL-apo B and the other at 3p25.2 (D3S1259, Figure 2) for apo AI were supported by both the allele sharing and the variance component methods. These findings were added to the accumulating database derived from the published genomewide scans for lipid-related phenotypes.

Descriptive statistics of the database

The database included 32 citations published during the 1998 through 2003 period. Phenotypes incorporated in the database and the number of genome scans for each phenotype are presented in Table 3. The most frequently studied phenotypes were total cholesterol (n = 10), LDL-C (n = 11), HDL-C (n = 18) and triglyceride (n = 16). Studies on familial hypercholesterolemia, familial combined hyperlipidemia and familial hypobetalipoproteinemia typically used a disease affliction status (affected or unaffected) based on lipid and non-lipid criteria. The other phenotypes were treated as either quantitative or qualitative variables. The study design, the sample size as well as linkage methods varied greatly between studies. Only 15.6% of the investigations were conducted among families ascertained randomly. The remaining were ascertained based on specific clinical criteria such as familial combined hyperlipidemia, familial hypercholesterolemia, familial hypobetalipoproteinemia, CHD, myocardial infarction, low HDL-C concentrations,

hypertension, obesity and type 2 diabetes. Few studies were from genetically isolated populations, such as the Hutterites, North-Eastern Indian and the Pima Indians.

Table 4 presents a summary of the loci providing evidence of linkage from the compendium of whole-genome scans. A total of 152 hits were identified which suggests that an average of 4.8 positive loci per study reached the suggestive threshold of significance ($p \leq 0.0023$ or LOD $\geq$ 1.7). This number is very similar to what has been observed for other complex traits when positive loci are summarized from a number of studies[24]. In order to evaluate whether positive loci were randomly distributed across the genome, we plotted the observed number of hits against the expected number of hits for chromosome 1 to 22 (Figure 3) (see Materials and Methods). A close relationship between positive loci and theoretical genes-content was apparent. This suggests that the null hypothesis of random linkage across the genome cannot be rejected. On the other hand, some chromosomes showed an increased number of observed hits, relative to expected. Indeed chromosomes 21, 13, 15 and 2 had an observed to expected hit ratios of 2.7, 2.4, 1.8 and 1.5, respectively.

Discussion

The avalanche of information anticipated from whole-genome linkage scans[23] has certainly been confirmed for the field of blood lipids and lipoproteins. The accumulating information may soon be overwhelming even for the scientists. Here we have produced a summary of the loci providing evidence of linkage from published genomewide scans carried out on blood lipid-related phenotypes (Table 4). We believe that such compendium will be useful to others in the field. For instance, it may help investigators to access quickly the data on linkage for a specific genomic region or a particular phenotype. We have integrated all linkage signals on the same map to facilitate comparisons across studies.

To provide an example of the usefulness of this compendium, we performed a new genome-wide search on apo B, LDL-apo B and apo AI levels. The results suggested the existence of a susceptibility locus for LDL-apo B on 18q21.32 and a second one for apo AI on 3p25.2. Additional linkages were observed with the allele sharing linkage method but the lack of consistency across linkage methods made the significance of the findings quit doubtful. From Table 4, we can easily identify the other QTLs that have been reported in the same regions from previous genomewide scan studies. Interestingly, the apo AI locus on 3p overlaps with the locus for low HDL-C levels reported in Finnish families[26] and with the locus for LDL-3 (phenotype defined as the cholesterol concentration in small LDL particles) observed in Mexican Americans[27]. The region is also close to the locus for familial hypobetalipoproteinemia[28]. In contrast, the LDL-apo B locus (18q21.32) observed in this study represents a newly identified locus. Although some genomewide scans have been performed on apo B levels before[9-11], this study was the first to investigate the LDL-apo B subfraction. Genomewide scans with subphenotypes have been successful in the past[27,29] and may explain the identification of this new locus on 18q21.32.

Our biggest challenge in the compilation of Table 4 was the choice of a significance level for inclusion of a linkage result. This question is related to the ongoing debate concerning significance levels appropriate for reporting evidence of linkage from genome-wide scans on complex traits[23,30-34]. With the emergence of genomewide scans to identify loci underlying complex traits, geneticists have proposed a refinement of the originally proposed 3 LOD score threshold[35]. While some advocated a continuation of the more stringent guideline in order to

control false positives[23], others suggested more flexible guidelines to hunt down genes with small effects believed to be involved in complex traits[31]. Rao et al.[32] proposed a middle ground, for the purpose of carrying out follow up studies, to deal with both false positive and false negative claims. The recommendation was to increase our tolerance from one false positive in 20 genomic scans assuming a continuous map, as suggested by Lander and Kruglyak[23], to one per scan assuming a more realistic map density of 400 markers, and to additionally rely on replication. These modifications set the nominal p value to 0.0023 which corresponds to a LOD score of 1.75[22,36]. However, it is interesting to note that this new threshold corresponded to what was called *putative* linkage by Thomson[31] and *suggestive* linkage by Lander and Kruglyak[23]. Accordingly, all point-wise significance levels below this threshold were included in Table 4.

For complex traits, independent replication of an earlier finding gives substantial credibility to the results. However, determining whether a given study has replicated an earlier finding is not simple particularly when different markers have been used. When do we accept that two location estimates in a genomic region representing the same QTL? This issue has been addressed before and it has been proposed that the location estimate may sometime be several centimorgans away from the true locus[37]. In fact, the 95% CI of the location estimate can span tens of centimorgans depending on family size and number, penetrance of locus, and heterogeneity. Based on the above, the cumulative evidence from genomewide screens for lipid-related phenotypes is now covering a very large portion of the genome (Table 4). It is likely that the entire genome will eventually be covered with at least suggestive evidence of linkage in a few years and replication of findings will be guaranteed in future genomewide scans. This phenomenon is not unique to lipid-related phenotypes. The evolution of the human obesity gene map is a good example of this trend, with more than 300 genes, markers, and chromosomal regions that have now been associated or linked with human obesity phenotypes[38].

Despite the large number of QTLs reported to date, a coherent and comprehensive pictures of the loci contributing to variation in lipid and lipoprotein has not been achieved. This is demonstrated by the inability to reject the hypothesis of random positive linkage (Figure 3). We have learned that the genetic mechanisms underlying the predisposition to favorable or unfavorable plasma lipoprotein-lipid levels are more complicated than previously thought. The emergence of such a large number of potential susceptibility loci for lipid-related phenotypes may make it necessary to

revisit the criteria for claiming linkage or linkage replication. It is commonly accepted, that a p value less than 0.01 from an independent study sample is sufficient to declare replication of an earlier significant linkage[23]. However, given the large number of genome scan reports and the inability to precisely localize the locus[37], many regions are likely to be replicated solely by chance. For example, more than 30 loci reached the $p < 0.01$ threshold in the present genome scan study on apo levels and many of them could therefore be considered replicated linkage. New strategies to deal with these issues are urgently needed.

In summary, the identification of gene for complex human diseases and their associated biological traits has had limited success thus far. This limited of success may be explained by genetic heterogeneity, incomplete penetrance, epistasis, phenocopy and pleiotropy[39], and undoubtedly other factors. In this paper, we provide a compendium of previous results from genome scan studies on lipid related-phenotypes. We have recorded a large number of loci covering a large portion of the genome. The number of false positives is difficult to assess but is likely to be high since positive findings are more frequently published. Accordingly, even though a single tool summarizing the extensive literature on the subject may prove to be useful, it should be used with caution since the probability of claiming replication just by chance is getting high. In the same paper, we also report a new genome scan on apo levels. Linkage was tested using both an allele sharing and a variance component methods. Many loci provided weak to moderate evidence of linkage but only two QTLs were supported by both analytical methods.

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Table 1. Characteristics of Genome Scan Participants by Gender and Generation Groups.

Variables	**Fathers** **n = 132**	**Mothers** **n = 175**	**Sons** **n = 164**	**Daughters** **n = 208**
Age (years)	54.1 ± 9.7	50.9 ± 9.2	27.2 ± 10.8	28.8 ± 11.6
BMI (kg/m^2)	29.5 ± 6.3	30.5 ± 8.5	27.4 ± 7.8	28.3 ± 9.4
Total apo B (g/L)	1.13 ± 0.22	1.02 ± 0.24	0.89 ± 0.23	0.87 ± 0.20
LDL-apo B (gl/L)	1.00 ± 0.20	0.90 ± 0.21	0.80 ± 0.21	0.77 ± 0.19
Apo AI (g/L)	1.20 ± 0.17	1.33 ± 0.20	1.19 ± 0.16	1.24 ± 0.17

Values are means ± SD.

BMI, body mass index.

Table 2. Summary of LOD Scores ≥ 1.18 or P Values ≤ 0.01.

				p value*		LOD score
Trait	**Chr band†**	**Distance (cM)**	**Marker**	**Two-point (empirical p value)**	**Multipoint**	
Total apo B	2q33.3	208.3	D2S1384	0.004439	NS	NS
	6p22.3	30.8	D6S2439	NS	NS	1.32
	6p21.33	38.0	TNFα	NS	NS	1.28
	6p21.1	48.1	D6S1017	NS	NS	1.34
	6q23.1	143.2	D6S1040	NS	NS	1.35
	15q26.1	90.8	D15S652	0.007851	NS	NS
	18q21.32	62.0	D18S38	0.005121	NS	NS
	19q13.2	56.0	LIPE	0.009221	0.009682	NS
	19q13.32	58.6	APOE	**0.002271** (0.022600)	NS	NS
	20p13	4.5	D20S482	0.002712	NS	NS
LDL-apo B	1q42.2	230.4	D1S3462	NS	0.009261	NS
	1q43	239.8	D1S547	0.005565	0.006285	NS
	2q35	221.7	D2S434	NS	NS	1.31
	4q21.23	88.0	D4S1534	0.007840	NS	NS
	7q32.1	127.9	D7S1875	0.008742	NS	NS
	11q22.3	107.8	D11S2000	NS	NS	1.28
	12p13.32	3.3	D12S372	0.006271	0.009195	NS
	13q32.1	97.0	D13S793	0.006976	NS	NS
	18q21.32	62.0	D18S38	0.004792	0.007469	**2.05**
	18q21.32	62.5	MC4R	NS	NS	1.33
	19q13.2	56.0	LIPE	**0.001798** (0.097400)	0.003889	NS
	19q13.32	58.6	APOE	**0.001581** (0.051000)	NS	NS
	20p13	4.5	D20S482	**0.000051** (0.003200)	0.005086	NS
Apo AI	3p25.2	12.6	D3S1259	**0.000022** (0.040200)	**0.000161**	1.21
	4q31.1	149.6	UCP1	NS	**0.000800**	NS
	4q31.21	153.0	D4S1586	NS	**0.000001**	NS
	5q21.3	138.0	D5S1453	**0.000084** (0.038600)	NS	NS
	5q31.3	158.7	D5S1480	**0.001577** (0.009200)	NS	NS
	5q33.2	169.8	D5S497	0.003630	NS	NS
	7p22.2	4.2	D7S3056	0.002641	NS	NS
	9q31.3	100.2	D9S1835	**0.000086**	NS	NS

			(0.052600)		
9q33.3	115.2	D9S282	**0.000450**	NS	NS
			(0.011689)		
10q21.1	58.2	D10S1221	**0.001651**	NS	NS
			(0.005800)		
11p15.1	18.6	SUR	**0.000900**	NS	NS
			(0.001300)		
11q13.2	72.1	D11S4136	NS	**0.001624**	NS
12q24.21	115.0	D12S2070	**<u>0.000026</u>**	**0.000176**	NS
			(0.021000)		
12q24.23	119.1	D12S395	NS	0.008953	NS
13q33.3	106.9	D13S796	NS	**<u>0.000008</u>**	NS
15q11.2	21.9	D15S63	**<u>0.000001</u>**	NS	NS
			(0.001100)		
16p13.13	3.4	D16S748	NS	**0.002019**	NS
16p13.11	6.5	D16S405	NS	**<u>0.000091</u>**	NS
16p12.3	7.4	D16S287	NS	0.005713	NS
16p13.11	7.6	D16S764	**0.000428**	NS	NS
			(0.072000)		
16p11.2	30.8	D16S753	NS	0.007298	NS
16q12.1	49.3	D16S261	NS	**0.000518**	NS
16q12.2	55.6	D16S3253	NS	**<u>0.000006</u>**	NS
16q22.2	81.9	D16S2624	**0.001318**	NS	NS
			(0.336600)		
19q12	43.1	D19S433	NS	0.005224	NS
20q13.2	51.6	D20S480	NS	0.002670	NS
20q13.2	52.7	D20S120	NS	0.004233	NS
22q13.31	41.9	D22S274	0.006556	NS	NS

*Markers showing suggestive evidence of linkage (P value ≤ 0.0023 or LOD score ≥ 1.75) are in bold and markers showing evidence of linkage (P value ≤ 0.0001 or LOD score ≥ 3.00) are in bold and underline;

NS, p > 0.01 or LOD score < 1.18;

†Chromosome bands are from the human genome browser of the University of California, Santa Cruz (http://genome.ucsc.edu/).

Table 3. Whole-Genome Scans on Lipid-Related Phenotypes.

Phenotypes	No. of Studies	References
Cholesterol	10	10-12,40-46
LDL-C	11	13,27,40-42,44-49
HDL-C	18	10,12,13,26,40-43,45,46,48-55
Triglyceride	16	10-13,26,40,41,43,45,46,48,49,51,54,56,57
Non-HDL-C	1	13
CH/HDL-C ratio	1	10
LDL-C/HDL-C	2	45,55
TG/HDL-C ratio	4	10,45,55,57
TG/Apo CIII	1	13
Total apo B	5	10-13, this study
LDL-apo B	1	This study
Apo AI	2	10, this study
Apo AII	1	10
Apo CII	1	10
Apo CIII	2	10,13
Apo E	1	10
Lp (a)	2	48,49
FCHL	3	11-13
FH	5	44,58-61
FHBL	1	28
HDL subfractions	1	29
LDL subfractions	1	27
LDL-PPD	2	17,54

FCHL, familial combined hyperlipidemia; FH, familial hypercholeterolemia; FHBL, familial hypobetalipoproteinemia; LDL-PPD, LDL peak particle diameter; CH, cholesterol; TG, triglyceride.

Table 4. Evidence for the Presence of Linkage with Lipid-Related Phenotypes from Genomewide Scan Studies

Markers or Genes	Location (Mb)**	Chromosome Band**	Samples	Phenotypes	p, Z or Lod values	References
D1S1608, 3735	4.3-65.1	1p36.32-p31.3	31 subjects; 1 kindred	FH	Lod = 6.8	58
D1S214, 228	6.5-13.4	1p36.31-p36.21	576 subjects; 42 families	LDL-C	Lod = 2.4	45
D1S2826, 513	18.1-31.1	1p36.13-p35.2	74 subjects; 1 kindred	FH	Lod = 3.1	44
D1S552, 2843	18.8-20.1	1p36.13-p36.12	Twin and parents	Cholesterol	Lod = 1.8	44
				LDL-C	Lod = 1.9	44
D1S2725, 2787	21.7-27.3	1p36.12-p35.3	17 subjects; 2 families	FH	Lod = 5.3	60
D1S233, 193	31.3-43	1p35.2-q34.2	576 subjects; 42 families	Ratio LDL/HDL	Lod = 2.1	45
D1S2892, 2722	40.2-41.6	1p34.2	1 pedigree; 12 families	FH	Lod = 3.1	61
D1S405	58.7	1p32.1	383 sib pairs; 75 families	Triglyceride	Z = 3.1	40
LEPR	65.9	1p31.2	681 subjects; 236 nuclear families	LDL-PPD	Lod = 2.6	17
D1S1665	74.4	1p31.1	269 subjects; 48 families	Apo B (qualitative)	Lod = 2.0	12
D1S484	158.6	1q23.3	383 sib pairs; 75 families	Cholesterol	Z = 3.4	40
D1S1679	160	1q23.3	1406 subjects; 513 families	Lp(a)	Lod = 3.8	49
D1S104	161.3	1q23.3	269 subjects; 48 families	Triglyceride (qualitative)	Lod = 2.8	12
				FCHL	Lod = 2.5	12
D1S2623*	180.4	1q25.3	649 sib pairs	HDL-C	Lod = 2.1	41
D1S547	239.8	1q43	930 subjects; 292 nuclear families	LDL-C	Lod = 2.5	46
D2S2211*	7.3	2p25.1	649 sib pairs	Cholesterol	Lod = 2.2	41
D2S2952	7.9	2p25.1	269 subjects; 48 families	Apo B (qualitative)	Lod = 1.8	12
				Triglyceride (qualitative)	Lod = 1.8	12
			240 subjects; 18 families	FCHL	Lod = 2.6	13
D2S423	9.7	2p25.1	269 subjects; 48 families	FCHL	Lod = 2.2	12
			29 families	HDL-C (qualitative)	Lod = 3.4	26
D2S1788	36.2	2p22.3	547 sibs; 188 nuclear families	Triglyceride	Lod = 1.7	43
D2S441	68.4	2p14	930 subjects; 292 nuclear families	Triglyceride	Lod = 2.3	46
D2S1394	73.1	2p13.2	25 families	HDL-C (qualitative)	Lod = 2.1	26
D2S286, 2216	75.5-88	2p12-p11.2	535 subjects; 99 families	Ratio TG/HDL	Lod = 1.9	55
D2S1790	85.1	2p11.2	477 subjects; 10 pedigrees	Unesterified HDL_{2a}-C	Lod = 2.3	29
D2S410	113.4	2q14.1	485 subjects; 1 pedigree	Triglyceride	p = 0.000006	56
			451 subjects; 1 pedigree	Triglyceride	p = 0.000006	48
D2S1391	183.2	2q32.1	201 subjects; 42 families	Triglyceride (qualitative)	Lod = 2.3	11.
D2S1384	203.4	2q33.3	681 subjects; 236 nuclear families	LDL-PPD	Lod = 2.3	17
D2S338	235.3	2q37.2	31 subjects; 1 kindred	FH	Lod = 2.2	58
D2S338, 125	235.3-240.3	2q37.2-q37.3	576 subjects; 42 families	HDL-C	Lod = 2.3	45
D2S2968	236.1	2q37.3	930 subjects; 292 nuclear families	Cholesterol	Lod = 2.0	46
D3S2387, 2403	1-13.1	3p26.3-p25.2	470 subjects; 10 pedigrees	LDL-3	Lod = 2.6	27
D3S1304	6.8	3p26.1	25 families	HDL-C (qualitative)	Lod = 2.1	26
D3S1259	12	3p25.2	679 subjects; 243 nuclear families	Apo AI	p = 0.000022	This study
D3S2407, 1578	40.7-52.9	3p22.1-p21.2	38 subjects; 1 family	FHBL	Lod = 3.3	28
D3S2406	71.7	3p13	547 sibs; 188 nuclear families	Triglyceride	Lod = 1.8	43
D3S2406, 2459	71.7-98.9	3p13-q12.3	1702 subjects; 332 families	Ratio TG/HDL-C	Lod = 1.8	57
D3S1271	97.4	3q12.2	535 subjects; 99 families	Ratio TG/HDL	Lod = 2.1	55
D3S2459, 1310	98.9-111.6	3q12.3-q13.31	31 subjects; 1 kindred	FH	Lod = 1.9	58
D3S1764	136.1	3q23	930 subjects; 292 nuclear families	LDL-C	Lod = 2.8	46
D3S3053	168.8	3q26.31	590 sibs; 201 nuclear families	HDL-C	Lod = 2.6	43

Markers or Genes	Location (Mb)**	Chromosome Band**	Samples	Phenotypes	p, Z or Lod values	References
D3S1754, 1311	174.4-193	3q26.32-q29	470 subjects; 10 pedigrees	LDL-3	Lod = 4.1	27
D4S3007*	6.7	4p16.1	622 sib pairs	HDL-C	Lod = 2.0	41
D4S2397	27.4	4p15.2	681 subjects; 236 nuclear families	LDL-PPD	Lod = 2.2	17
D4S3248	59.8	4q13.1	477 subjects; 10 pedigrees	Unesterified HDL_{3a}-C	Lod = 2.6	29
D4S1647, 1644	99.7-142.6	4q23-q31.21	470 subjects; 10 pedigrees	LDL-3	Lod = 4.1	27
D4S2623	111.3	4p25	269 subjects; 48 families	Triglyceride (qualitative)	Lod = 1.8	12
D4S2368	169.5	4q32.3	1482 subjects; 232 pedigrees	Apo AII	Lod = 2.4	10
D5S2849	3.5	5p15.33	2799 subjects; 500 families	LDL-C	Lod = 1.9	47
D5S593*	4.4	5p15.33	649 sib pairs	HDL-C	Lod = 2.7	41
D5S1470	32.3	5p13.3	1027 subjects; 101 families	HDL-C	Lod = 3.6	50
D5S2500	58.9	5q11.2	1482 subjects; 232 pedigrees	Apo AII	Lod = 2.1	10
D5S427	63.1	5q12.1	576 subjects; 42 families	Cholesterol	Lod = 2.1	45
D5S1501	77.3	5q14.1	681 subjects; 236 nuclear families	LDL-PPD	Lod = 2.4	17
			930 subjects; 292 nuclear families	Triglyceride	Lod = 2.2	46
D5S1505	118.8	5q23.1	240 subjects; 18 families	HDL-C	Lod = 2.4	54
D5S1456	169.3	5q35.1	477 subjects; 10 pedigrees	Unesterified HDL_{2b}-C	Lod = 2.8	29
D5S211, 408	173.5-180.1	5q35.2-q35.3	576 subjects; 42 families	LDL-C	Lod = 2.0	45
D6S282	43.2	6p21.1	535 subjects; 99 families	HDL-C	Lod = 2.0	55
D6S257	55.9	6p12.1	96 subjects; 1 pedigree	Cholesterol (qualitative)	Lod = 2.0	42
D6S1717*	99.5	6q16.2	622 sib pairs	HDL-C	Lod = 1.8	41
D6S1003, 1277	144.2-163.7	6q24.2-q27	470 subjects; 10 pedigrees	LDL-3	Lod = 2.9	27
D6S305	161.6	6q26	451 subjects; 1 pedigree	Lp(a)	$p < 0.000001$	48
			1406 subjects; 513 families	Lp(a)	Lod = 27.0	49
D7S691, 479	41.7-94.9	7p14.1-q21.3	418 subjects; 27 families	Triglyceride	Lod = 2.1	51
D7S520*, 820	63.4-82.3	7q11.21-q21.11	470 subjects; 10 pedigrees	LDL-3	Lod = 2.1	27
D7S653, 471	70.5-110.5	7q11.22-q31.1	418 subjects; 27 families	HDL-C	Lod = 1.7	51
D7S1824, 688	138.3-146.8	7q34-q36.1	418 subjects; 27 families	Triglyceride	Lod = 1.9	51
D7S2195, 3058	142.1-152.8	7q35-q36.2	1702 subjects; 332 families	Triglyceride	Lod = 1.8	57
D7S2195, 3058	142.1-152.8	7q35-q36.2	1702 subjects; 332 families	Ratio TG/HDL-C	Lod = 2.5	57
D8S1477	32	8p12	477 subjects; 10 pedigrees	Unesterified HDL_{2b}-C	Lod = 2.1	29
D8S259, 1121	33-35.6	8p12	472 subjects; 10 families	HDL-C	Lod = 2.0	53
D8S1132	106.4	8q23.1	25 + 29 families	HDL-C (qualitative)	Lod = 4.7	26
D8S1128	127.6	8q24.21	477 subjects; 10 pedigrees	Unesterified HDL_{2a}-C	Lod = 4.9	29
D9S921	10.7	9p23	269 subjects; 48 families	HDL-C (qualitative)	Lod = 2.1	12
D9S925, 741	18.5-23.4	9p22.2-p21.3	415 subjects; 27 families	HDL-C	Lod = 3.4	52
IFNA	21.8	9p21.3	485 subjects; 1 pedigree	Triglyceride	$p = 0.000043$	56
D9S1122	70.7	9q21.2	1406 subjects; 513 families	Triglyceride	Lod = 1.9	49
D10S1220	51.5	10q11.23	269 subjects; 48 families	Triglyceride (qualitative)	Lod = 3.3	12
D10S568	52.6	10q21.1	269 subjects; 48 families	HDL-C (qualitative)	Lod = 2.0	12
D10S1221	56.3	10q21.1	201 subjects; 42 families	Triglyceride (qualitative)	Lod = 3.2	11
D10S520, 521	95.3-108.3	10q23.33-q25.1	2799 subjects; 500 families	LDL-C	Lod = 2.5	47
D10S169	131.3	10q26.3	201 subjects; 42 families	FCHL	Lod = 2.3	11
				Cholesterol (qualitative)	Lod = 2.6	11

Markers or Genes	Location (Mb)**	Chromosome Band**	Samples	Phenotypes	p, Z or Lod values	References
D11S1324*	30.2	11p14.1	1482 subjects; 232 pedigrees	Cholesterol	Lod = 1.8	10
D11S1392	36.2	11p13	930 subjects; 292 nuclear families	Triglyceride	Lod = 2.1	46
D11S1993	46.3	11p12	2799 subjects; 500 families	LDL-C	Lod = 3.7	47
D11S1985	60.9	11q12.1	240 subjects; 18 families	FCHL	Lod = 2.6	13
D11S911, 912	79.8-130.6	11q13.5-q24.3	930 subjects; 292 nuclear families	LDL-C	Lod = 3.2	46
D11S4464	125.6	11q24.1	201 subjects; 42 families	Apo B (qualitative)	Lod = 1.8	11
			930 subjects; 292 nuclear families	Triglyceride	Lod = 1.9	46
D12S334	61	12q14.1	930 subjects; 292 nuclear families	HDL-C	Lod = 4.1	46
PAH	102.4	12q23.2	477 subjects; 10 pedigrees	Unesterified HDL_{2a}-C	Lod = 2.1	29
D12S1091, 378	104.2-123.4	12q23.3-q24.31	383 sib pairs; 75 families	Triglyceride	Z = 3.0	40
D12S2070	115	12q24.21	1482 subjects; 232 pedigrees	Apo AI	Lod = 2.0	10
D13S171, 263	31.2-40.1	13q13.1-q14.11	576 subjects; 42 families	HDL-C	Lod = 2.0	45
D13S1493	32	13q13.1	25 + 29 families	HDL-C	Lod = 1.9	26
			1027 subjects; 101 families	HDL-C	Lod = 2.4	50
D13S800	71.8	13q22.1	201 subjects; 42 families	Triglyceride (qualitative)	Lod = 1.9	11
D13S156, 158	72.6-102.3	13q22.1-q33.1	96 subjects; 1 pedigree	Cholesterol (qualitative)	Lod = 5.7	42
			222 pairs of twins	Cholesterol	p = 0.0002	42
				LDL-C	p = 0.0002	42
				HDL-C	p = 0.004	42
D13S1300, 1266	91-101.5	13q31.3-q33.1	74 subjects; 1 kindred	FH	Lod = 3.1	44
D13S793	96.3	13q32.1	930 subjects; 292 nuclear families	LDL-C	Lod = 1.9	46
D14S53	74.4	14q24.3	681 subjects; 236 nuclear families	LDL-PPD	Lod = 2.8	17
D15S11, 659	20.5-41.9	15q11.2-q21.1	418 subjects; 27 families	Triglyceride	Lod = 3.9	51
D15S1007	29	15q14	535 subjects; 99 families	Ratio LDL/HDL	Lod = 1.7	55
D15S1040*	29.4	15q14	649 sib pairs	Triglyceride	Lod = 1.9	41
ACTC, D15S659	30.4-41.9	15q14-q21.1	470 subjects; 10 pedigrees	LDL-1	Lod = 1.8	27
D15S659	41.9	15q21.1	240 subjects; 18 families	LDL-PPD	Lod = 2.2	54
D15S643	55.3	15q22.2	477 subjects; 10 pedigrees	Unesterified HDL_{2a}-C	Lod = 3.3	29
D15S653	81.5	5q25.3	477 subjects; 10 pedigrees	Unesterified HDL_{2b}-C	Lod = 2.5	29
D15S963, 207	88.3-92.7	15q26.1-q26.2	5 families	FH	Lod = 3.3	59
D15S652	89	15q26.1	930 subjects; 292 nuclear families	LDL-C	Lod = 3.1	46
D16S769	25.6	16p12.1	269 subjects; 48 families	Triglyceride (qualitative)	Lod = 1.9	12
D16S3136	41.1	16q12.1	535 subjects; 99 families	Ratio TG/HDL	Lod = 1.7	55
D16S2624, 518	62.6-69.1	16q22.2-q23.1	472 subjects; 10 families	HDL-C	Lod = 4.3	53
D16S518, 3091	69.1-73.8	16q23.1-q23.3	560 subjects; 73 families	HDL-C (qualitative)	Lod = 3.6	12
D16S3091	73.8	16q23.3	25 + 29 families	HDL-C (qualitative)	Lod = 2.2	26
D17S938*	6.6	17p13.2	622 sib pairs	Triglyceride	Lod = 1.8	41
D17S1290	56.1	17q23.2	1406 subjects; 513 families	LDL-C	Lod = 2.3	49
D17S1291	63.8	17q24.1	383 sib pairs; 75 families	Triglyceride	Z = 2.6	40
D17S1535, 928	72.5-79	17q25.1-q25.3	31 subjects; 1 kindred	FH	Lod = 2.7	58
D17S1301	72.7	17q25.1	681 subjects; 236 nuclear families	LDL-PPD	Lod = 6.8	17
044XG3	77.7	17q25.3	2799 subjects; 500 families	LDL-C	Lod = 2.3	47
D17S928	79	17q25.3	1482 subjects; 232 pedigrees	Ratio total-C/HDL-C	Lod = 2.5	10

Markers or Genes	Location (Mb)**	Chromosome Band**	Samples	Phenotypes	p, Z or Lod values	References
D18S843	8.7	18p11.22	451 subjects; 1 pedigree	Lp(a)	p = 0.000069	48
D18S38	58.4	18q21.32	679 subjects; 243 nuclear families	LDL-apo B	Lod = 2.1	This study
D19S247, 209	3.2-3.4	19p13.3	576 subjects; 42 families	Ratio TG/HDL	Lod = 2.1	45
D19S1034, 219	6.2-46.6	19p13.3-q13.32	998 sibs; 292 nuclear families	Cholesterol	Lod = 3.9	43
D19S916	9.2	19p13.2	38 subjects; 1 family	FHBL	Lod = 1.7	28
D19S714, 433	16.1-31	19p13.12-q12	470 subjects; 10 pedigrees	LDL-1	Lod = 2.3	27
D19S433	31	19q12	451 subjects; 1 pedigree	LDL-C	p = 0.00011	48
D19S245, 254	34.7-58.6	19q13.11-q13.43	1482 subjects; 232 pedigrees	Apo E	Lod = 4.2	10
D19S587, 178	35.8-45.1	19q13.12-q13.31	470 subjects; 10 pedigrees	LDL-2	Lod = 1.9	27
D19S178, APOCII	45.1-46.1	19q13.31-q13.32	576 subjects; 42 families	Triglyceride	Lod = 3.2	45
APOE	46.1	19q13.32	930 subjects; 292 nuclear families	LDL-C	Lod = 3.6	46
D20S103	0.5	20p13	38 subjects; 1 family	FHBL	Lod = 1.8	28
D20S900*	7.3	20p12.3	622 sib pairs	Triglyceride	Lod = 2.8	41
D20S171	57.5	20q13.32	25 + 29 families	HDL-C (qualitative)	Lod = 1.9	26
D21S1437	18.3	21q21.1	201 subjects; 42 families	Apo B (qualitative)	Lod = 2.2	11
D21S263	28.8	21q22.11	535 subjects; 99 families	Ratio LDL/HDL	Lod = 2.0	55
D21S1246	37.4	21q22.2	2799 subjects; 500 families	LDL-C	Lod = 2.7	47
D21S1260*	39.4	21q22.3	622 sib pairs	Cholesterol	Lod = 2.3	41
D21S1411*	40.7	21q22.3	622 sib pairs	LDL-C	Lod = 1.7	41
D22S1161	45.6	22q13	31 subjects; 1 kindred	FH	Lod = 2.0	58
DXS6804	107.3	Xq23	201 subjects; 42 families	Apo B (qualitative)	Lod = 1.9	11

Status as April 2003.
When two markers per line are shown, these give the interval within which the peak is located.
*When the authors provided only the location of linkage (in genetic distance) without mentioning the name of the marker, we identified a possible marker within the region showing evidence of linkage from the genetic map used by the authors.
**The physical and genetic location of markers and genes are from the genome browser of the University of California, Santa Cruz (http://genome.ucsc.edu).
NA, non available; Apo, apolipoprotein; FH, familial hypercholesterolemia; FCHL, familial combined hyperlipidemia; FHBL, familial hypobetalipoproteinemia; LDL-PPD, Low-density lipoprotein peak particle diameter; LDL-1,2,3,4, cholesterol concentration in 4 LDL size fractions (LDL-1, 26.4 to 29.0 nm; LDL-2, 25.5 to 26.4 nm; LDL-3, 24.2 to 25.5 nm; and LDL-4, 21.0 to 24.2 nm).

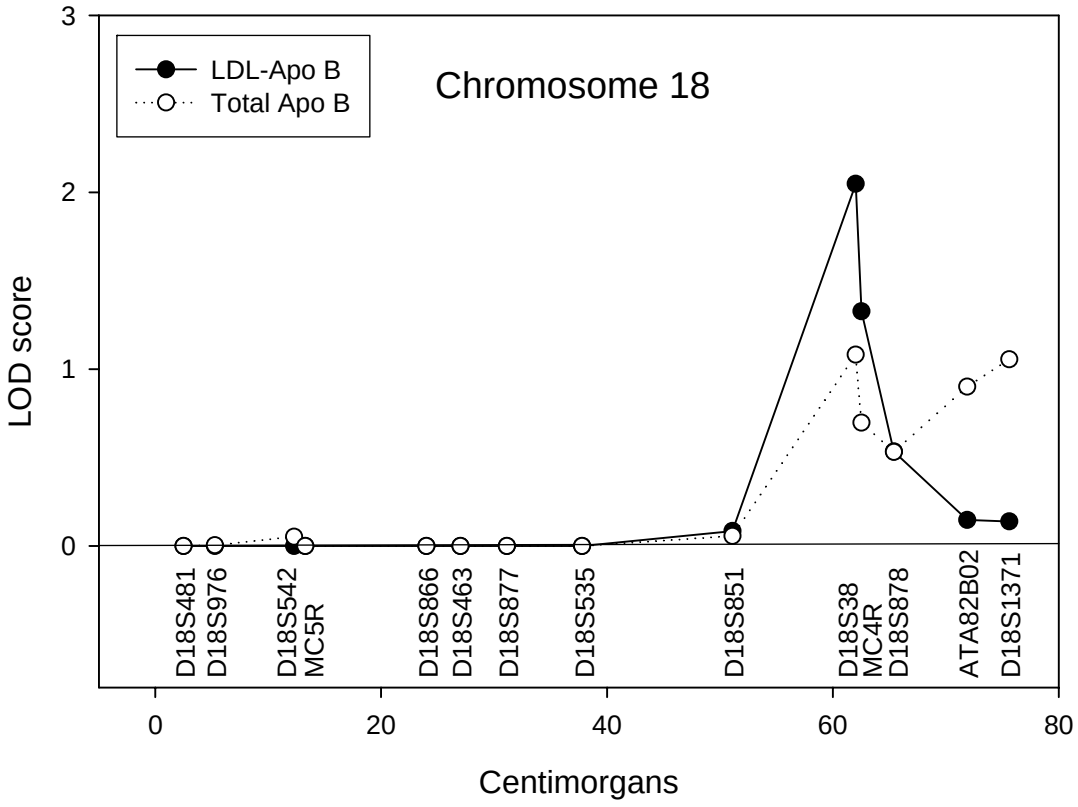

Figure 1. Variance component-based linkage results for chromosome 18 with the total apo B and the LDL-apo B phenotypes. The two traits are adjusted for the effects of age, age^2, age^3, gender and BMI.

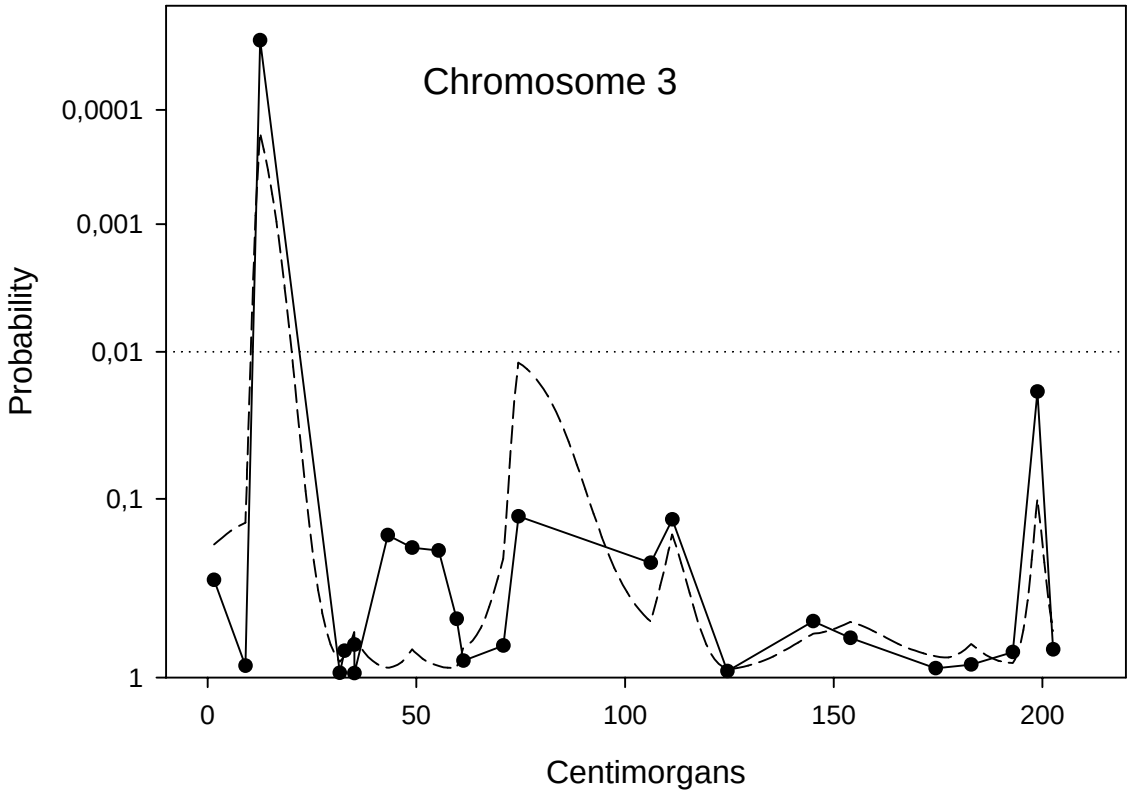

Figure 2. Two-point (solid line) and multipoint (dashed line) sib pairs linkage analysis for chromosome 3 with the apo AI phenotype. Apo AI is adjusted for the effects of age, age^2, age^3, gender and BMI. The horizontal dot line is a reference corresponding to a p value = 0.01.

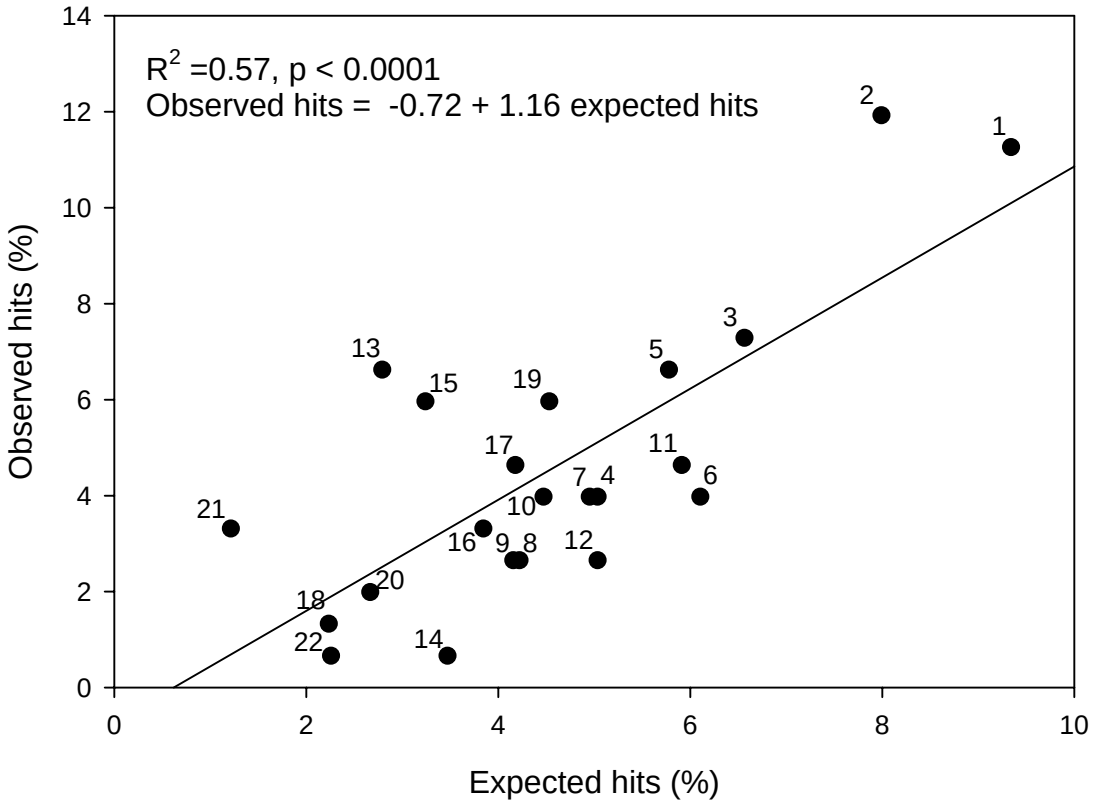

Figure 3. Regression analysis of observed and expected hits on the autosomal chromosomes. The observed hit ratio of each chromosome was obtained as: (number of hits on a specific chromosome / all 152 hits) x 100, and the expected hit ratio of each chromosome was obtained as: (number of genes on a specific chromosome / total number of genes in the genome) x 100. The gene content of each chromosome and the genome are from Venter et al.[25]

Chapter 7.

Heritability of LDL Peak Particle Diameter in the Québec Family Study

Yohan Bossé, Marie-Claude Vohl, Jean-Pierre Després, Benoît Lamarche, Treva Rice, D.C. Rao, Claude Bouchard, Louis Pérusse

L'objectif de cette étude était de vérifier l'existence de facteurs familiaux influençant le diamètre principal des particules LDL (DP-LDL). Le DP-LDL a été mesuré par électrophorèse sur gradient de gel de polyacrylamide chez 681 sujets. Le DP-LDL a été ajusté pour l'âge (DP-LDL1), l'âge et l'indice de masse corporelle (IMC) (DP-LDL2), ou l'âge, l'IMC et les triglycérides (DP-LDL3). Les résultats suggèrent que la cellule familiale explique 47.4, 46.7 et 48.9% de la variance totale de ces phénotypes, respectivement. Le patron de corrélations familiales indique aucune corrélation entre époux alors que des corrélations significatives sont observées entre les parents et les enfants et les frères et sœurs avec une héritabilité maximale de 59%, 58% et 52% pour DP-LDL1, DP-LDL2 et DP-LDL3, respectivement. Ces résultats suggèrent que la taille des particules LDL est fortement similaire à l'intérieur des familles et que la ressemblance familiale semble être principalement attribuable à des facteurs génétiques.

Heritability of LDL Peak Particle Diameter in the Québec Family Study

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Short title: Familial resemblance for LDL size

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Abstract

LDL size has been associated with the risk of coronary heart disease. The objective of the present study was to verify whether familial factors influence LDL peak particle diameter (LDL-PPD), a quantitative trait reflecting the size of the major LDL subclass. LDL-PPD was measured by 2-16% polyacrylamide gradient gel electrophoresis in 681 members of 236 nuclear families participating in the Québec Family Study. LDL-PPD was adjusted for either age (LDL-PPD1), age and body mass index (LDL-PPD2) or age, body mass index and plasma triglyceride levels (LDL-PPD3), separately in men and women. The residual scores were used to test for familial aggregation using an ANOVA as well as computing maximum likelihood estimates of familial correlations. The ANOVA revealed that family lines accounted for 47.4, 46.7 and 48.9% of the variance in the LDL-PPD1, LDL-PPD2 and LDL-PPD3 phenotypes, respectively. The pattern of familial correlations revealed no significant spouse correlations but significant parent-offspring and sibling correlations for the three LDL-PPD phenotypes with maximal heritability estimates of 59%, 58% and 52% for LDL-PPD1, LDL-PPD2 and LDL-PPD3, respectively. These results suggest that LDL-PPD strongly aggregates in families and that the familial resemblance appears to be primarily attributable to genetic factors. Genes responsible for this genetic contribution remain to be identified.

Key words : genetics, lipoproteins, LDL size

Introduction

Data from case-control [Austin et al., 1994] and prospective [Gardner et al., 1996; Lamarche et al., 2001; Stampfer et al., 1996] studies suggest that small dense LDL particles are associated with increased risk of coronary heart disease (CHD). Defining the genetic and environmental factors modulating LDL particle size may be helpful in understanding its relationship with CHD. Fisher and collaborators [Fisher et al., 1975] were the first to report a genetic effect on LDL subclass phenotypes. Their finding of significant parent-offspring correlations but no spouse resemblance was interpreted as strong support for a genetic determination. Two large twin studies also provided evidence of heritability for LDL particle size. The first study was based on 109 monozygotic (MZ) and 113 dizygotic (DZ) male twin pairs, aged between 59 and 70 years, participating in the third examination of the National Heart, Lung, and Blood Institute Twin Study [Lamon-Fava et al., 1991]. The heritability estimate was performed on the weighted LDL type measured by gradient gel electrophoresis which takes into account both the major and satellite bands. The weighted LDL type intraclass correlation coefficient was higher in MZ twins (0.58) than in DZ twins (0.32), with a heritability of 52% prior to controlling for covariate effects and 39% after adjusting for BMI, alcohol consumption, cigarette smoking and physical activity. Similar results were obtained when only the major LDL band was used as a variable. The second study was based on 203 MZ and 145 DZ pairs of adult female twins, participating in the second examination of the Kaiser Permanente Women Twins Study [Austin et al., 1993b]. In this study, the heritability estimate was performed using the LDL Peak Particle Diameter (LDL-PPD) measured by gradient gel electrophoresis. This phenotype is a continuous variable reflecting the size of the major LDL subclass. Again the intraclass correlation coefficient was higher in MZ twins (0.71) than in DZ twins (0.44) and the heritability coefficient was estimated to reach 54%.

Twin studies represent a powerful design to detect the presence of a genetic effect. However, heritability estimates derived from twin studies should always be interpreted with caution as they assume that the difference in the correlations between MZ and DZ twins is entirely ascribed to genetic factors. This is analogous to saying that both types of twins have been and are exposed to similar environmental conditions. However, failure to meet this assumption typically leads to an overestimation of the heritability. Consequently, estimates derived from twin studies may represent the upper bound estimates of heritability, and other study designs should be used to

verify these estimates. Thus far, only one study provide heritability estimates of LDL-PPD based on a family study design [Edwards et al., 1999]. The cohort was based on two family studies, the first ascertained through hyperlipidemic proband surviving a myocardial infarction and the second ascertained through hypertriglyceridemic proband. The heritability coefficient was 34% for the LDL-PPD adjusted for age and gender effects. However, heritability estimates for a multifactorial phenotype derived from such high-risk families may not properly quantify the strength of the familial resemblance for the majority of the population. Thus, the purpose of the present study was to assess the heritability of LDL-PPD based on subjects participating in the Québec Family Study (QFS).

Methods

Population

The QFS is a prospective study monitoring several phenotypes among French-Canadian families with the aim of investigating the genetics of obesity and its comorbidities [Bouchard, 1996]. For the present study, a total of 681 individuals (aged 41.1 ± 17.7 years) including 285 men and 396 women from 236 nuclear families were available. These families included a subsample of 100 families randomly ascertained with regards to obesity, while the remaining families were ascertained through one or more obese probands. Table 1 presents the characteristics of subjects in each of the sex and generation groups. The majority of the families consisted of families with both parents and at least one child (43%), families composed exclusively of siblings (25%) and families with data on mothers and offspring (19%). The study was approved by the Laval University Medical Ethics Commitee, and all subjects provided written informed consent. All the procedures followed were in accordance with institutional guidelines.

Phenotypes measurements

LDL-PPD were measured by electrophoresis with 8X8-cm nondenaturing 2-16% polyacrylamide gradient gels as described in details eslewhere [St-Pierre et al., 2001]. Triglyceride levels were assayed from blood samples collected in the morning after a 12-hour overnight fast. Total triglyceride concentrations were determined enzymatically with commercial kits as previously described [Perusse et al., 1989]. Body mass index (BMI) was calculated as weight (kg)/height (m^2).

Data Adjustments

Before computing familial correlations, the LDL-PPD was adjusted for the effects of age and other covariates in both the mean and variance, as explained elsewhere [Perusse et al., 1997]. Briefly, LDL-PPD was regressed on up to a cubic polynomial in age (age, age^2 and age^3) and other covariates using a stepwise multiple regression procedure (mean regression), performed separately in each age- (<30, 30-50, and ≥50 years) by-sex (male vs. female) group and retaining

only terms that were significant at the 5% level. To generate regression equations that were not affected by extreme scores, individuals with phenotypic values beyond ± 3 SD from the mean were identified and temporarily set aside. After estimation of the regression parameters for every group, these outliers were added back for computation of residual scores. One female subject with a residual score above 4 SD and with more then 1 SD from the previous highest score was excluded from the analysis because she was a distinct outlier. The phenotype used to estimate familial correlations was the residual from the mean regression standardized to a zero mean and unit variance. Three LDL-PPD phenotypes based on three different adjustment procedures were computed: LDL-PPD1 adjusted for age effects, LDL-PPD2 adjusted for age and BMI effects and LDL-PPD3 adjusted for age, BMI and plasma triglyceride effects. Table 3 presents the significant covariates for each phenotype within each age-by-sex group. Descriptive statistics and phenotype adjustments were performed using SAS (version 8.02).

Familial Correlation Model

The presence of familial resemblance was first tested using an ANOVA comparing between- versus within-family variance. This test was performed with the general linear model including the LDL-PPD phenotype as the dependent variable and the family line (family number) as the independent variable. The familial correlation model was based on four groups of individuals (fathers=F, mothers=M, sons=S, and daughters=D) leading to 8 correlations: 1 spouse (FM), 4 parent-offspring (FS, FD, MS, and MD), and 3 sibling (SS, DD, and SD) coefficients. Correlations were estimated using maximum likelihood methods of the computer program SEGPATH [Province & Rao, 1995]. A general (Model 1) and eight reduced models (Models 2 through 9) testing specific null hypotheses were fitted to the data. Null hypotheses were tested using the likelihood ratio test, which is the difference in minus twice the log-likelihoods (-2 ln L) between the general and a reduced model. The likelihood ratio is approximately distributed as a χ^2, with the degrees of freedom being the difference in the number of parameters estimated in the two models being contrasted. Nonrejected models ($P > 0.05$) were combined into a single test with the aim of finding the most parsimonious model. This model is the one that best fit the data with the fewest parameters. The most parsimonious model was chosen from among all non-rejected alternatives using the Akaike's Information Criterion [Akaike, 1974] (AIC), which is -2

ln L plus twice the number of estimated parameters. The most parsimonious model is the one with the smallest AIC value.

The specific hypotheses tested in each model were the following. Sex and generation differences in correlations were first considered by testing the hypotheses of no sex differences in offspring in Model 2 (FS = FD, MS = MD, SS = DD = SD, df = 4), no sex differences in offspring or parents in Model 3 (FS = FD = MS = MD, SD = SS = DD, df = 5), and no sex nor generation differences in Model 4 (FS = FD = MS = MD = SD = SS = DD, df = 6). In Model 5, all eight correlations were equated to yield to the so-called environmental model (FM = FS = FD = MS = MD = SD = SS = DD, df = 7). The remaining models tested the strength of the familial resemblance, including the hypotheses of no sibling correlation in Model 6 (SS = DD = SD = 0, df = 3), no parent-offspring correlations in Model 7 (FS = FD = MS = MD = 0, df = 4), no spouse correlation in Model 8 (FM = 0, df = 1) and finally no familial resemblance at all in Model 9 (FM = FS = FD = MS = MD = SD = SS = DD = 0, df = 8). The maximal heritability (h^2) was computed using the correlations from the most parsimonious model according to the following equation :

$$h^2 = [(r_{sibling} + r_{parent\text{-}offspring})(1 + r_{spouse})] / [(1 + r_{spouse}) + 2\,(r_{spouse})(r_{parent\text{-}offspring})]$$

This maximal heritability is defined as the percent of variance due to all additive familial effects (including both genetic and nongenetic) and is adjusted for the degree of spouse resemblance. The 95% confidence intervals associated with the heritability coefficient was also calculated using the same equation as above by substituting the standard errors obtained from the estimates of the familial correlation.

Results

Prior to familial correlation estimation, LDL-PPD phenotypes were adjusted in the six age-by-sex groups (see Methods) for the effects of age, BMI and triglyceride. There were no significant age effects for LDL-PPD phenotypes, while BMI accounted for less than 10% of the variance. Triglyceride levels had significant effects in each group accounting for between 8 to 48% of the variance in LDL-PPD. The ANOVA (results not shown) revealed that there were about two times more variance between families than within families and that family lines accounted for 47 to 49% of the variance in the LDL-PPD phenotypes. A summary of the correlation model results is presented in Table 2. For each model, the P values and the AIC values are shown. For all three phenotypes the hypotheses of no sibling (Model 6), no parent-offspring (Model 7) and no familial (Model 9) correlations are strongly rejected. On the other hand, the no spouse (Model 8) correlation is accepted for each LDL-PPD phenotype. This pattern of correlations is consistent with the hypothesis that the familial resemblance in LDL size is primarily attributable to genetic factors. For LDL-PPD1 and LDL-PPD2 all tests, except the no spouse correlation, are rejected leaving model 8 as the most parsimonious one. Concerning the LDL-PPD3 phenotype, four models in addition to the no spouse correlation model are accepted: Model 2 for no sex differences in offspring; Model 3 for no sex differences in offspring or parents; Model 4: for no sex nor generation differences; and Model 5: for the environmental model. To determine the most parsimonious model in such case, we combined all nonrejected null hypotheses. This was done by combining the best sex/generation models (Models 2 through 4) with the best model for the level of familial correlations (Models 6 through 9). The best sex/generation model was model 4 (FS = FM = MS = MD = SD = SS = DD) and the best model for the strength of familial correlations was model 8 (FM = 0). As shown in Table 2, the combined test of no sex nor generation differences (Model 4) and no spouse correlation (Model 8) did fit by likelihood ratio test (P = 0.366) and also provided the smallest AIC value (9.63). This combination of models 4 and 8 (i.e., no sex or generation differences and no spouse resemblance) was then chosen as the most parsimonious hypothesis for the LDL-PPD3.

Maximum likelihood estimates of the familial correlations under the general and most parsimonious models are presented in Table 3 for the three LDL-PPD phenotypes. Table 3 also presents the maximal heritability coefficients calculated from the most parsimonious models. For

the LDL-PPD1, LDL-PPD2 and LDL-PPD3 the heritabilities are 59%, 58% and 52%, respectively. For all phenotypes, heritability estimates are based on models in which there is no spouse correlation, suggesting that only genetic factors account for these estimates. These estimates of heritability are expressed as a percentage of the residual variance (ie, after removing effects associated with covariates). Heritability can also be expressed as a percent of total variance. This is done by multiplying the heritability estimates by the residual variance after adjustment for the covariates. Table 4 presents the percentage of the total variance explained by the heritability component and the total variance explained by both heritability and covariates. The residual heritability (h^2) is fairly similar among the three phenotypes. However, the total variance explained by heritability (h^2*) is about 20% lower for LDL-PPD3 as compared to the other phenotypes. This decrease is attributed to removing the variability in LDL-PPD that is shared with triglyceride (i.e., covariance). On the other hand, the total variance explained by both heritability factor and covariates is 5% greater for LDL-PPD3 as compared to LDL-PPD1 and LDL-PPD2.

Discussion

The present study is the first to provide heritability estimates of LDL peak particle diameter (LDL-PPD) using families randomly ascertained with regards to their lipid and lipoprotein profile. Three LDL-PPD phenotypes derived by adjusting variously for the effects of age (LDL-PPD1), age and BMI (LDL-PPD2), and age, BMI and triglycerides (LDL-PPD3) were considered. The results suggest that these phenotypes strongly aggregate in families and are characterized by significant maximal heritability estimates of 59%, 58% and 52%, respectively. In addition, the lack of significant spouse correlation, combined with significant parent-offspring and sibling correlations, suggests that genetic factors are likely the major determinants of the familial aggregation.

Twin studies have demonstrated that approximately 50% of the variability in LDL size is attributed to genetic factors [Austin *et al*., 1993b; Lamon-Fava *et al*., 1991]. The coefficient was lower when it was derived from 85 families ascertained through hyperlipidemic proband participating in the Genetic Epidemiology of Hypertriglyceridemia (GET) study. This high-risk CHD family study design suggested that approximately one third of the residual variance in LDL-PPD (h^2=34%) was attributable to additive genetic effects [Edwards *et al*., 1999]. The results of the present study are similar to those reported from twin studies.

Others studies have attempted to uncover the genetics architecture underlying the small dense LDL phenotype. A number of studies have investigated the inheritance of the trait using complex segregation analysis [Austin et al., 1990; Austin et al., 1993a; Austin et al., 1988; Bredie et al., 1996; de Graaf et al., 1992; Friedlander et al., 1999; Vakkilainen et al., 2002]. Despite using different types of family structures, different criteria for proband ascertainment and the use of different techniques to characterize LDL heterogeneity, these studies were consistent in finding a major gene effect influencing the phenotype. Additionally, numerous candidate gene studies have been tested for their potential association or linkage with the small dense LDL phenotype. Unfortunately, due to the inability to replicate positive findings, results derived from these candidate gene studies are inconclusive so far.

LDL-PPD and triglyceride levels are traits with large genetic and environmental correlations [Edwards *et al*., 1999]. In the present study, the total variance explained by heritability was lower when LDL-PPD was adjusted for triglycerides. In fact, the heritability factors explained 39% of the total variance in LDL-PPD3 compared to 59% and 57% in LDL-PPD1 and LDL-PPD2, respectively. We assume this reduction is caused by the removal of some of the shared additive genetic and environmental contributions of triglycerides to the variance in LDL-PPD. Such adjustments have the potential to eliminate pleiotropic effects of genes and to narrow the contribution of heritable factors to that specific to the phenotype of interest. This type of adjustment can also be useful in finding genetic loci contributing to LDL size. Indeed, it has been proposed that removing the effects accounted for by covariates and using the residual trait in linkage analysis may increase the likelihood of detecting genes unique to that trait [Comuzzie et al., 1997].

Overall, the results presented in this study indicate a strong familial aggregation with maximal heritability estimates above 50% for LDL-PPD. The pattern of familial correlations suggests that this effect is primarily attributable to genetic factors. Molecular studies are warranted to identify genes responsible for this large genetic contribution.

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Table I. Descriptive Statistics of LDL Peak Particle Diameter and Covariates in Each of the Sex and Generation Groups.

Variables	**Fathers**	**Mothers**	**Sons**	**Daughters**
	n = 137	**n = 194**	**n = 148**	**n = 202**
LDL-PPD(angstroms)	261.2 ± 5.4	264.3 ± 5.2	262.6 ± 4.5	264.4 ± 4.5
Age (years)	55.5 ± 9.2	55.5 ± 12.7	26.6 ± 10.0	28.2 ± 10.7
BMI (kg/m^2)	28.9 ± 6.3	28.7 ± 8.0	26.5 ± 6.9	27.6 ± 8.7
Triglyceride (mmol/L)	1.98 ± 1.23	1.79 ± 2.40	1.35 ± 0.71	1.27 ± 0.59

Values are mean ± SD.

LDL-PPD, LDL peak particle diameter; BMI, body mass index.

Table II. Summary of Goodness of Fit Tests for LDL Peak Particle Diameter Phenotypes.

Models	LDL-PPD1		LDL-PPD2		LDL-PPD3	
	P*	**AIC†**	**P**	**AIC**	**P**	**AIC**
1. General		16.00		16.00		16.00
2. No sex differences, offspring	0.007	22.07	0.009	21.48	0.221	13.72
3. No sex differences, offspring or parents	0.008	21.60	0.012	20.72	0.312	11.95
4. No sex nor generation differences	0.015	19.73	0.021	18.89	0.420	10.03
5. Environmental model	0.007	21.42	0.009	20.62	0.199	11.82
6. No sibling correlation	<0.001	41.76	<0.001	41.58	<0.001	38.60
7. No parent-offspring	<0.001	47.67	<0.001	45.07	<0.001	46.15
8. No spouse correlation	0.382	14.76	0.482	14.49	0.191	15.71
9. No familial correlations	<0.001	67.01	<0.001	63.53	<0.001	64.95
Parsimonious models						
Models 8	**0.382**	**14.76**	**0.482**	**14.49**		
Models 4 and 8					**0.366**	**9.63**

* P = P values from the likelihood ratio χ^2 test; a significant value ($P < 0.05$) indicates rejection of the null hypothesis a compared to the general model.

†AIC = Akaike's Information Criterion; the most parsimonious model is the one with the smallest AIC and is shown in bold.

Table III. Familial Correlations (±SE) and Maximal Heritability Under the General and the Most Parsimonious Models.

Parameter	**LDL-PPD1**	**LDL-PPD2**	**LDL-PPD3**
General model			
FM	0.08 ± 0.09	0.07 ± 0.09	0.12 ± 0.09
FS	0.34 ± 0.08	0.34 ± 0.08	0.32 ± 0.08
FD	0.08 ± 0.08	0.08 ± 0.08	0.31 ± 0.08
MS	0.28 ± 0.09	0.27 ± 0.09	0.27 ± 0.09
MD	0.37 ± 0.06	0.35 ± 0.06	0.28 ± 0.07
SD	0.27 ± 0.08	0.26 ± 0.08	0.24 ± 0.08
SS	0.55 ± 0.09	0.55 ± 0.09	0.47 ± 0.09
DD	0.20 ± 0.08	0.20 ± 0.08	0.21 ± 0.08
Parsimonious model			
FM	0	0	0
FS	0.33 ± 0.08	0.33 ± 0.08	0.26 ± 0.03
FD	0.05 ± 0.07	0.06 ± 0.08	0.26 ± 0.03
MS	0.26 ± 0.09	0.25 ± 0.09	0.26 ± 0.03
MD	0.37 ± 0.06	0.35 ± 0.07	0.26 ± 0.03
SD	0.26 ± 0.08	0.26 ± 0.08	0.26 ± 0.03
SS	0.55 ± 0.09	0.55 ± 0.09	0.26 ± 0.03
DD	0.20 ± 0.08	0.20 ± 0.08	0.26 ± 0.03
Maximal heritability (CI)*	59% (43-75%)	58% (42-75%)	52% (46-58%)

*CI = 95% confidence intervals are calculated as described in the Methods section.

Table IV. Percentage of Variance Explain by Covariates and Heritability.

	Covariates		**Heritability**		
	Range*	**Average (R^2)**	**Residual (h^2)**	**Total (h^2*)†**	**Total Variance Explained‡**
LDL-PPD1 (%)	0	0	59	59	59
LDL-PPD2 (%)	0-3.9	1.7	58	57	59
LDL-PPD3 (%)	3.0-47.9	25.0	52	39	64

*The percentage of variance accounted for by covariates varies in age-by-sex groups (see Table 3).

†The percentage of the total variance explained by the heritability is calculated by the following formula: $h^2* = h^2 \text{ X } (1-R^2)$, in which h^2 is the heritability calculated in Table 5 and R^2 is the variance due to covariates.

‡The total variance explained is computed as $R^2 + h^2*$.

Chapter 8.

Evidence for a Major Quantitative Trait Locus on Chromosome 17q21 Affecting LDL Peak Particle Diameter

Yohan Bossé, Louis Pérusse, Jean-Pierre Després, Benoît Lamarche, Yvon C Chagnon, Treva Rice, D.C. Rao, Claude Bouchard, and Marie-Claude Vohl

Des études d'héritabilité et de ségrégation ont démontré que la taille des particules LDL est caractérisée par une grande contribution génétique et la présence d'un gène à effet majeur. L'objectif de cette étude était d'identifier les régions chromosomiques influençant le diamètre principal des particules LDL (DP-LDL). Un criblage génomique a donc été effectué chez 681 sujets participant à l'Étude des familles de Québec. La plus forte évidence de liaison a été retrouvée sur le chromosome 17q21.33 avec un rapport de cote logarithmique (LOD) à 6.76 pour le DP-LDL ajusté pour l'âge, l'indice de masse corporelle et les niveaux de triglycérides. Des évidences de liaison suggestive (LOD > 1.75) ont aussi été retrouvées sur les régions 1q31, 2q33.2, 4p15.2, 5q12.3 et 14q31. Ces résultats suggèrent fortement la présence d'un locus majeur sur le chromosome 17q ainsi que de plusieurs autres loci prometteurs influençant le DP-LDL.

Evidence for a Major Quantitative Trait Locus on Chromosome 17q21 Affecting LDL Peak Particle Diameter

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Abstract

Background—Several lines of evidence suggest that small dense low-density lipoprotein (LDL) particles are associated with the risk of coronary heart disease. Heritability and segregation studies suggest that LDL particle size is characterized by a large genetic contribution and the presence of a putative major genetic locus. However, association and linkage analyses have been thus far inconclusive in identifying the underlying gene(s).

Methods and Results—An autosomal genome-wide scan for LDL peak particle diameter (LDL-PPD) was performed in the Québec Family Study. A total of 442 markers were genotyped with an average intermarker distance of 7.2 centimorgans. LDL-PPD was measured by gradient gel electrophoresis in 681 subjects from 236 nuclear families. Linkage was tested using both sibpair- and variance components-based linkage methods. The strongest evidence of linkage was found on chromosome 17q21.33 at marker D17S1301 with a LOD score of 6.76 using variance components method for the phenotype adjusted for age, BMI and triglyceride levels. Similar results were obtained with the sibpair method ($p < 0.0001$). Other chromosomal regions harboring markers with highly suggestive evidence of linkage ($p \leq 0.0023$; LOD ≥ 1.75) includes 1p31, 2q33.2, 4p15.2, 5q12.3 and 14q31. Several candidate genes are localized under the peak linkages, including apolipoprotein H on chromosome 17q, the apolipoprotein E receptor 2 and members of the phospholipase A_2 family on chromosome 1p as well as the HMG-CoA reductase on chromosome 5q.

Conclusions—This genome-wide scan for LDL-PPD indicates the presence of a major QTL located on chromosome 17q and others interesting loci influencing the phenotype.

Condensed abstract

In order to identify genetic loci involved in LDL peak particle diameter (LDL-PPD), an autosomal genome-wide scan was performed in 681 subjects enrolled in the Québec Family Study. A variance-component linkage analysis revealed a strong evidence of linkage on chromosome 17q21.33 at marker D17S1301 for LDL-PPD adjusted for age, BMI and triglyceride levels (LOD = 6.76). Other chromosomal regions harboring markers with highly suggestive evidence of linkage for LDL-PPD includes 1p31, 2q33.2, 4p15.2, 5q12.3 and 14q31 (LOD > 1.75). Several candidate genes were located in the vicinity of the genomic regions showing evidence of linkage.

Key words: genome scan, LDL size, genetics, lipoproteins, candidate genes.

A number of case-control as well as prospective studies reveal an increased risk of coronary heart disease (CHD) in patients with small, dense low density lipoprotein (LDL) compared with those having larger, more buoyant LDL particles[1]. Heritability studies, based on twins, suggested that approximately one third to one half of the variation in the LDL peak particle size can be attributed to genetic influences[2,3]. Complex segregation analyses of small dense LDL phenotypes have been performed with data from different types of family structures, different criteria for proband ascertainment and the use of different techniques to characterize LDL heterogeneity[4-9]. Indeed, the model providing the best fit to the data included either a dominant, a recessive or an undetermined mode of inheritance for the trait. Furthermore, the allele frequency determining the small dense LDL phenotype ranges from 19 to 42%, with reduced penetrances in young males and premenopausal women. However, these studies unanimously provided evidence in favor of a gene with a major effect on LDL particle phenotypes.

Association studies with candidate genes have been inconsistent in finding genes associated with small dense LDL. The –250G→A polymorphism within the hepatic lipase promoter was associated with buoyant LDL particles[10]. However, the –514C→T polymorphism, which is in complete linkage disequilibrium with the –250G→A polymorphism[11], showed no effect on LDL particle size[12,13]. The apolipoprotein (apo) E genotype was also associated with the small dense LDL phenotype. However, some have reported smaller particles for subjects carrying the E4 allele[14-16], while others did for subjects carrying the E2 allele[17,18]. In contrast, others have show that LDL particle size did not differ among the apo E genotypes[19]. Additional candidate genes, including cholesteryl ester transfer protein (CETP)[20], microsomal triglyceride transfer protein[21], cholesterol 7alpha hydroxylase[22], apo B-100[23], apo C-III[24] and angiotensin-converting enzyme[16] were investigated for potential effects on small dense LDL phenotypes. These studies revealed either absence of an association or presence of an association only in particular subgroups.

Results from linkage studies are equivocal. After excluding linkage of small dense LDL with the apo B (the protein moiety of LDL) gene locus on chromosome 2[25,26], suggestive linkage to the LDL receptor locus on chromosome 19 has been reported[27,28]. However, subsequent sequencing of the entire coding regions of the LDL-receptor gene did not reveal any sequence variants, thus weakening the hypothesis that a mutant LDL-receptor allele is responsible for the dense LDL phenotype[29]. Other candidate loci, including hepatic lipase[12], lipoprotein lipase[30], CETP[28,31,32],

apo A1-CIII-AIV complex[28,32], and the manganese superoxide dismutase[28,32], have been shown to be linked with the small dense LDL phenotype. Unfortunately, most of these linkages have not been replicated[33,34]. Based on these results, Austin et al.[34] emphasized the necessity of finding new genetic loci, other than those harboring known candidate genes, in order to identify the genes potentially involved in determining the small dense LDL phenotype. Genome-wide scans are particularly suited for this purpose. Prior genome wide scan have focus on variation in cholesterol concentrations of LDL size fractions. Rainwater et al.[35] found two QTLs on chromosome 3 and 4 with LOD scores > 3 for LDL size fraction 3 (LDL-3), a fraction that contains small LDL particles. This study demonstrates the existence of QTLs affecting the concentration of cholesterol within a particular sub-population of LDL, but do not provide evidence of QTLs responsible for the size of the LDL particle by itself. To the best of our knowledge, the only whole genome scan on LDL particle size have been performed on 240 individuals ascertain through 18 unrelated familial combined hyperlipidemic probands[12]. Results suggest a locus, over the hepatic lipase gene on chromosome 15, with a LOD score of 2.2. Here we report the results of an autosomal genomic scan for LDL peak particle diameter (LDL-PPD) measured by gradient gel electrophoresis.

Methods

Population

The Québec Family Study (QFS) is an ongoing project composed of French-Canadian families that has been described previously[36]. In the present study, a total of 681 subjects from 236 nuclear families had available data on LDL-PPD. Table 1 presents the characteristics of subjects in each of the sex and generation groups. The study was approved by the Laval University Medical Ethics Commitee, and all subjects provided written informed consent. All the procedures followed were in accordance with institutional guidelines.

Phenotypes

LDL peak particle diameter (LDL-PPD) was measured by gradient gel electrophoresis from plasma obtained after a 12-hour fast. Details on the technique have been provided previously[37].

Genotypes

Genomic DNA was prepared by the proteinase K and phenol/chloroform technique. DNA preparation, polymerase chain reaction conditions, and genotyping are described in details elsewhere[38]. Genotypes for each marker were typed using automatic DNA sequencers and the computer software SAGA from LICOR (Lincoln, NE). The results were stored in a local dBase IV database, GENEMARK, which inspects results for Mendelian inheritance incompatibilities within nuclear families and extended pedigrees. A total of 335 microsatellite markers (dinucleotide, trinucleotide, and tetranucleotide repeats) selected from different sources, but mainly from the Marshfield panel version 8a, were available for this genome scan. The location of markers on the chromosomes in centimorgan (cM) were taken from version 9.0 of the Marshfield Institute map (http://research.marshfieldclinic.org/genetics/) and the Location Database map (http://cedar.genetics.soton.ac.uk/public_html). In addition, 107 polymorphisms in 63 candidate genes were included. The average intermarker distance for the whole set of 442 markers was 7.2 cM. The Genome Database (http://gdbwww.gdb.org/) and the OMIM gene map (http://www.ncbi.nlm.nih.gov/htbin-post/Omim/getmap) were used to identify candidate genes.

Statistical analyses

LDL-PPD was adjusted for covariates using a stepwise multiple regression procedure retaining only terms that were significant at the 5% level. Regression parameters were estimated within six age- (<30, 30-50, and ≥50 years) by-sex (male vs. female) groups after exclusion of outliers (± 4SD) and residuals were computed for all subjects. Residual scores were then standardized to a mean of 0 and a standard deviation of 1. LDL-PPD were adjusted for three different sets of covariates: 1) age up to the cubic polynomial, 2) age and BMI, 3) age, BMI and triglyceride levels. These adjustments gave three phenotypes arbitrary called LDL-PPD1, LDL-PPD2 and LDL-PPD3, respectively. Adjustment of the phenotypes were performed using SAS (version 8.02).

The search for linkage between the phenotypes and the genetic markers was performed using two different approaches. First, linkage was tested using the new Haseman-Elston regression-based method which models the trait covariance between sibpairs, instead of the squared sibpair trait difference used in the original method. It regresses the mean-corrected trait cross-product on the number of alleles shared identical by descent (IBD). Singlepoint and multi-point estimates of alleles shared IBD were generated using the GENIBD software and linkage was tested using the SIBPAL2 software from the S.A.G.E. 4.0 statistical package (S.A.G.E., 2001)[39]. The maximum number of sibpairs was 352. Linkage was also investigated using the variance components-based approach implemented in the quantitative transmission disequilibrium test (QTDT) computer software[40]. Under this approach, the phenotypic covariance among members of a family is assumed to result from the additive effects of linkage due to a major locus (a), a residual familial component due to polygenes (g) and a residual non-shared environmental component (e) that represents environmental effects unique to each family member. Linkage is tested by contrasting the null hypothesis of no linkage ($\sigma_a = 0$) to the alternative hypothesis ($\sigma_a \neq 0$) using a likelihood ratio test as described previously[41]. The LOD score was computed as $\chi^2/(2 \log_e 10)$. The interpretation of linkage evidence was considered as suggestive ($p \leq 0.01$; LOD ≥ 1.18), highly suggestive ($p \leq 0.0023$; LOD ≥ 1.75) or evidence of linkage ($p \leq 0.0001$; LOD ≥ 3.0)[42].

Results

An overview of the variance components-based linkage results for the three LDL-PPD phenotypes is given in Figure 1. Suggestive evidence of linkages ($p \leq 0.01$ or LOD scores ≥ 1.18 for at least one of the phenotype) are summarized in Table 2. The strongest evidence of linkage, which was confirmed by both linkage methods, was found on chromosome 17q21.33. As shown in Figure 2, the peak linkages were found with marker D17S1301 for LDL-PPD1 (LOD = 4.72), LDL-PPD2 (LOD = 4.70) and LDL-PPD3 (LOD = 6.76). Marker D17S1290, located 1.6 cM from D17S1301, gave also fairly good evidence of linkage for the three phenotypes.

Other chromosomes exhibiting some evidence of linkage by the variance components-based method are displayed in Figure 1. Highly suggestive evidence of linkages were observed at 1p31 (leptin receptor locus), 2q33.2 (marker D2S1384), 4p15.2 (D4S2397), 5q12.3 (D5S1501) and 14q31.1 (D14S53). Markers at the leptin receptor locus and markers D5S1501 and D14S53 also provided evidence of linkage by the sibpair method (see Table 2).

Other markers gave highly suggestive evidence of linkage ($p < 0.0023$) with at least one of the linkage methods. For instance, marker D16S261 provided evidence of singlepoint linkage with the three phenotypes. The markers VWFP1 on chromosome 22q11.21 provided evidence of singlepoint and multipoint linkage for the three phenotypes. On the other hand, marker D4S1627 yielded highly suggestive evidence of linkage for LDL-PPD1 and LDL-PPD2 with the variance component method. D5S1457 at 5p12 shows highly suggestive evidence of linkage in multipoint analysis for the three phenotypes and in singlepoint for LDL-PPD3. Finally, several markers provided highly suggestive evidence of linkage with LDL-PPD3 only, including D1S198, D2S434, IRS1 (2q36.3), ADRB2 (5q31), TNFα (6p21.3), D8S1110, D9S1121, D16S410 and ACEDI (17q23).

Discussion

The primary objective of this study was to identify QTLs affecting LDL-PPD variation. The results provide evidence for a major locus affecting LDL-PPD located on chromosome 17q21. Interestingly, none of the candidate genes located in the area of this QTL were previously tested. The marker D17S1301 located on chromosome 17q21.33 was strongly linked with the LDL peak particle diameter, whether adjusted or not for covariates. However, the evidence for linkage was stronger when the phenotype was adjusted for plasma triglycerides, indicating that triglyceride levels may attenuate the penetrance of the locus. Marker D17S1290 located 1.6 cM from D17S1301 also provided good evidence of linkage ($1.34 \leq LOD \leq 2.63$). The apolipoprotein H (APOH) gene, also referred to as β2-glycoprotein I, is encoded under the peak linkage on 17q21. ApoH is a single chain glycoprotein that exists in plasma both in a free form and in combination with lipoprotein particles. It has been implicated in several physiologic pathways, including lipid metabolism, coagulation, and the production of antiphospholipid antibodies. This apolipoprotein activates lipoprotein lipase[43] and genetic variations in this gene has been associated with variation in HDL-cholesterol and triglyceride levels[44-46]. The angiotensin-converting enzyme (ACE) is also located in this genomic region. This enzyme cleaves the final intravascular step resulting in the vasoactive peptide, angiotensin II. Angiotensin II has been shown to bind specifically to LDL[47], which produces a modified form of LDL which is taken up by macrophages at an enhanced rate, leading to cellular cholesterol accumulation[48]. In the present study, the insertion/deletion polymorphism in intron 16 of the ACE gene provided evidence of linkage with LDL-PPD1 (LOD = 1.46), LDL-PPD2 (LOD = 1.57) and LDL-PPD3 (LOD = 2.35). Figure 2 shows the approximate location of candidate genes surrounding the major peak on chromosome 17.

Several other chromosomal regions provided highly suggestive ($p < 0.0023$) evidence of linkage. These regions include chromosomes 1p31, 5p12-p12.3 and 14q31.1, which show evidence of linkage with both linkage methods and for all LDL-PPD phenotypes. Some promising candidate genes are located within these regions. First, the strongest evidence of linkage on chromosome 1p comes from a marker located within the leptin receptor (LEPR) gene. By modulating the hypothalamic effects of leptin on food intake and energy expenditure, genetic variants in the LEPR may affect energy balance and the size of LDL particles as a consequence of body fatness

alterations. However, adjusting the LDL-PPD for BMI did not affect the strength of the linkage. On 1p, three members of the phospholipase A_2 (PLA2) gene family are present, namely PLA_2 group IID (PLA2G2D), group V (PLA2G5) and group IIA (PLA2G2A). PLA_2 is known to hydrolyze the phospholipid monolayers of LDL particles and change their physicochemical properties and size[49]. Apolipoprotein E receptor 2 (APOER2) is also located near the locus of interest. On chromosome 5, two markers (D5S1457 and D5S1501), located 20 cM apart, provided evidence of linkage with LDL-PPD. This region contains the 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG CoA reductase, HMGCR), which is the rate limiting enzyme for cholesterol synthesis. A list of others potential candidate genes within the chromosomal regions linked to the LDL-PPD is provided in Table 3.

Among the panel of markers included in the genome scan, few candidate genes for LDL-PPD were present. First, an apo B marker gave no evidence of linkage with the phenotype. A significant linkage to apo B has been reported in a sibpair linkage analysis of dizygotic women twins[33] but other linkage studies excluded the hypothesis of linkage for the apo B locus and LDL size[25,26,28]. Second, while no linkage was found with the lipoprotein lipase (LPL) locus in the current study or in two others[28,34], a highly significant LOD score of 6.24 was obtained in another study of heterozygous LPL deficient families[30]. Third, the apo E gene gave no evidence of linkage as reported previously[28,33,34]. Finally, consistent with three other studies[32-34], the LDL receptor also was not linked to LDL-PPD in the present study. In contrast, two previous evidence linked the LDL receptor locus to LDL subclass in families ascertained through probands with the atherogenic lipoprotein phenotype[27] and in families with CHD[28]. However, no amino acid sequence changes in the LDL receptor were found in the former study[27] making it unlikely that a mutant allele in the LDL receptor gene was responsible for the linkage[29]. In the present study, negative results were also obtained with other candidate genes including paraoxonase, hormone-sensitive lipase, CD36 and the intestinal fatty acid-binding protein.

In conclusion, the results of this study reveal the presence of a major locus located on chromosome 17q21.33 influencing LDL-PPD. This finding supports results from a handful of segregation analyses indicating the presence of a putative major locus for LDL particle size. Evidence of linkage was also found on chromosome 1p31, 2q33.2, 4p15.2, 5q12.3 and 14q31.1.

These QTLs harbor a good number of candidate genes that have not been previously tested in association studies with LDL-PPD.

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Table 1. Descriptive Statistics of LDL Peak Particle Diameter and Covariates in Each of the Sex and Generation Groups.

Variables	**Fathers**	**Mothers**	**Sons**	**Daughters**
	n = 137	**n = 194**	**n = 148**	**n = 202**
LDL-PPD (angstroms)	261.2 ± 5.4	264.3 ± 5.2	262.6 ± 4.5	264.4 ± 4.5
Age (years)	55.5 ± 9.2	55.5 ± 12.7	26.6 ± 10.0	28.2 ± 10.7
BMI (kg/m^2)	28.9 ± 6.3	28.7 ± 8.0	26.5 ± 6.9	27.6 ± 8.7
Triglyceride (mmol/L)	1.98 ± 1.23	1.79 ± 2.40	1.35 ± 0.71	1.27 ± 0.59

Values are mean ± SD.

LDL-PPD, LDL peak particle diameter; BMI, body mass index.

Table 2. Results from the Genome Scan: Markers showing evidence of linkage with the LDL-PPD phenotypes according to the linkage methods used.

Marker/Chromosome location[§]/Distance (cM)	LDL-PPD1			LDL-PPD2			LDL-PPD3		
	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score
D1S203/1p31.1/82.250	0.19449	0.02915	0.97	0.20201	0.03117	0.98	0.17966	0.00777*	1.74*
D1S220/1p31.1/82.496	0.21395	0.11233	0.67	0.24852	0.12599	0.66	0.07821	0.02299	1.37*
LEPR/1p31/87.771	**0.000002**[‡]	**0.00037**[†]	**2.05**[†]	**0.000004**[‡]	**0.00046**[†]	**2.04**[†]	**0.00003**[‡]	**0.00009**[‡]	**2.56**[†]
D1S198/1p22.3/88.650	0.05148	0.00551*	1.70*	0.04796	0.00533*	1.74*	0.02932	0.00406*	2.21[†]
D2S1776/2q24.2/168.109	0.01446	0.00782*	0.66	0.01235	0.00639*	0.78	0.11668	0.07954	0.43
D2S1384/2q33.2/212.118	0.06188	0.00036[†]	1.91[†]	0.05694	0.00026[†]	2.11[†]	0.08574	0.00278*	2.27[†]
D2S434/2q36.1/233.148	0.02500	0.00708*	1.45*	0.03081	0.00878*	1.54*	0.01182	0.00873*	1.92[†]
IRS1/2q36.3/235.700	0.33181	0.00626*	1.39*	0.35506	0.00736*	1.49*	0.50910	0.01011	1.85[†]
D2S427/2q37.3/247.918	0.00374*	0.01128	0.93	0.00306*	0.00797*	1.12	0.02621	0.06192	0.74
D4S403/4p15.33/19.455	0.00888*	0.00669*	0.96	0.01018	0.00764*	0.99	0.02084	0.01663	0.83
D4S2397/4p15.2/32.246	0.03234	0.02079	2.14[†]	0.02866	0.01891	2.22[†]	0.04169	0.03278	2.11[†]

Marker/Chromosome location[§]/Distance (cM)	LDL-PPD1			LDL-PPD2			LDL-PPD3		
	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score
D4S1627/4p13/47.177	0.04631	0.01098	1.87[†]	0.04270	0.00960*	2.02[†]	0.09922	0.03438	1.21*
D4S3248/4q12/61.658	0.02666	0.01647	0.93	0.02682	0.01439	1.02	0.04046	0.00860*	1.18*
D4S3243/4q13.1/67.540	0.12255	0.02906	0.82	0.12301	0.02723	0.87	0.05296	0.00593*	1.15
D5S1986/5p13.3/32.470	0.10859	0.00258*	1.00	0.13063	0.00336*	0.97	0.30218	0.01240	0.63
D5S1470/5p12/46.124	0.00981*	0.01608	0.64	0.01003	0.01812	0.61	0.07421	0.07127	0.25
D5S1457/5p12/51.910	0.00890*	0.00149[†]	1.37*	0.00834*	0.00170[†]	1.35*	0.00126[†]	0.00042[†]	1.47*
CART/5q13-q14/63.001	0.70047	0.00558*	1.23*	0.68788	0.00549*	1.21*	0.77059	0.00778*	0.71
D5S1501/5q12.3/71.671	**0.000001**[‡]	**0.000004**[‡]	**2.40**[†]	**0.000001**[‡]	**0.000003**[‡]	**2.34**[†]	**0.000001**[‡]	**0.000008**[‡]	**2.10**[†]
ADRB2/5q31/156.383	0.01360	0.20977	0.05	0.01583	0.23011	0.04	0.00003[‡]	0.01212	0.33
TNFα/6p21.3/36.428	0.00488*	0.34210	0.08	0.00561*	0.35525	0.07	0.00023[†]	0.12755	0.35
TA184A08/6q24-q25.2/146.000	0.00300*	0.00304*	0.29	0.00309*	0.00299*	0.33	0.00845*	0.01232	0.41
D6S441/6q24.3/158.347	0.01456	0.03159	0.20	0.01611	0.03469	0.21	0.00572*	0.01122	0.55
D8S1110/8q11.1/50.229	0.01498	0.11057	0.41	0.01567	0.11257	0.44	0.00210[†]	0.06285	0.56

Marker/Chromosome location[§]/Distance (cM)	LDL-PPD1			LDL-PPD2			LDL-PPD3		
	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score
D9S925/9p22.3/15.738	0.00631*	0.00427*	1.46*	0.00420*	0.00295*	1.58*	0.06369	0.03619	0.90
D9S1121/9p22.1/20.396	0.10322	0.00627*	0.84	0.09028	0.00533*	0.91	0.02736	0.00210[†]	1.63*
D9S1118/9p21.1/26.815	0.32831	0.05855	0.39	0.31187	0.05127	0.44	0.14331	0.01152	1.32*
D9S938/9q31.1/106.261	0.03766	0.01388	1.43*	0.03897	0.01155	1.49*	0.04919	0.01748	1.08
D9S934/9q33.2/123.662	0.07024	0.01885	1.33*	0.07420	0.02006	1.35*	0.10882	0.01712	1.29*
D12S1045/12q24.33/142.100	0.00964*	0.02334	0.52	0.00867*	0.02157	0.58	0.02010	0.03435	0.40
D13S141/13q11/16.075	0.00326*	0.02313	0.54	0.00419*	0.02590	0.57	0.05748	0.10325	0.40
D13S787/13q12.11/19.370	0.66731	0.00435*	0.51	0.65641	0.00413*	0.56	0.50960	0.00491*	0.69
D14S587/14q21.3/49.255	0.00544*	0.01155	1.29*	0.00683*	0.01494	1.17	0.02507	0.06996	0.41
D14S592/14q23.2/63.513	0.01322	0.02929	1.73*	0.01486	0.03486	1.59*	0.02435	0.05471	0.71
D14S588/14q24.1/71.008	0.19152	0.06990	1.44*	0.17827	0.06968	1.38*	0.39519	0.14212	0.56
D14S53/14q31.1/82.701	**0.000008**[‡]	**0.00006**[‡]	**2.79**[†]	**0.000008**[‡]	**0.00009**[‡]	**2.65**[†]	0.000008[‡]	0.00067[†]	1.72*
D14S617/14q32.11/91.013	0.52394	0.00898*	1.01	0.53584	0.01267	0.91	0.57767	0.01788	0.66

Marker/Chromosome location[§]/Distance (cM)	LDL-PPD1			LDL-PPD2			LDL-PPD3		
	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score	p-value singlepoint	p-value multipoint	LOD score
D16S287/16p13.13/15.349	0.58176	0.02271	0.28	0.61340	0.02218	0.30	0.63070	0.00927*	0.66
D16S410/16p12.3/21.763	0.21593	0.00418*	0.41	0.20760	0.00375*	0.45	0.08646	0.00044[†]	1.00
D16S403/16p12.1/30.348	0.09036	0.00429*	0.57	0.08175	0.00346*	0.64	0.12627	0.00325*	0.62
D16S261/16q11.1/53.319	0.00144[†]	0.07305	0.02	0.00142[†]	0.07113	0.03	0.00012[†]	0.08361	0.02
D17S974/17p13.1/12.330	0.01033	0.04439	0.47	0.00956*	0.04380	0.50	0.07468	0.18376	0.27
D17S1290/17q21.32/54.190	0.00114[†]	0.00142[†]	1.39*	0.00167[†]	0.00184[†]	1.34*	**0.00110[†]**	**0.00042[†]**	**2.63[†]**
D17S1301/17q21.33/55.759	**0.00003[‡]**	**0.000001[‡]**	**4.72[‡]**	**0.00003[‡]**	**0.000001[‡]**	**4.70[‡]**	**0.00013[†]**	**0.000001[‡]**	**6.76[‡]**
ACE/17q23/64.646	0.38275	0.03378	1.46*	0.36420	0.02958	1.57*	0.25981	0.01406	2.35[†]
D17S784/17q25.3/87.166	0.02866	0.01184	0.58	0.02781	0.01087	0.65	0.01924	0.00724*	1.10
ATA82B02/18q22/77.836	0.02074	0.02561	1.38*	0.01876	0.02456	1.35*	0.03310	0.02154	1.22*
VWFP1/22q11.21/15.543	0.00017[†]	0.00323*	0.87	0.00015[†]	0.00323*	0.94	0.00007[‡]	0.00088[†]	1.13

Results from singlepoint and multipoint are report in p values and results from the variance components-based method are report in LOD scores. Markers with highly suggestive evidence of linkage ($p \leq 0.0023$ or $LOD \geq 1.75$) for the three linkage methods for one of the LDL-PPD phenotypes are indicated in bold.

*P value ≤ 0.01 or LOD score ≥ 1.18;

† P value ≤ 0.0023 or LOD score ≥ 1.75;

‡P value ≤ 0.0001 or LOD score ≥ 3.00;

§Chromosome location for marker with Dnumber or marker with gene name are from the Location Database map (http://cedar.genetics.soton.ac.uk/public_html) and the others markers are from Marshfield Institute map (http://research.marshfieldclinic.org/genetics/).

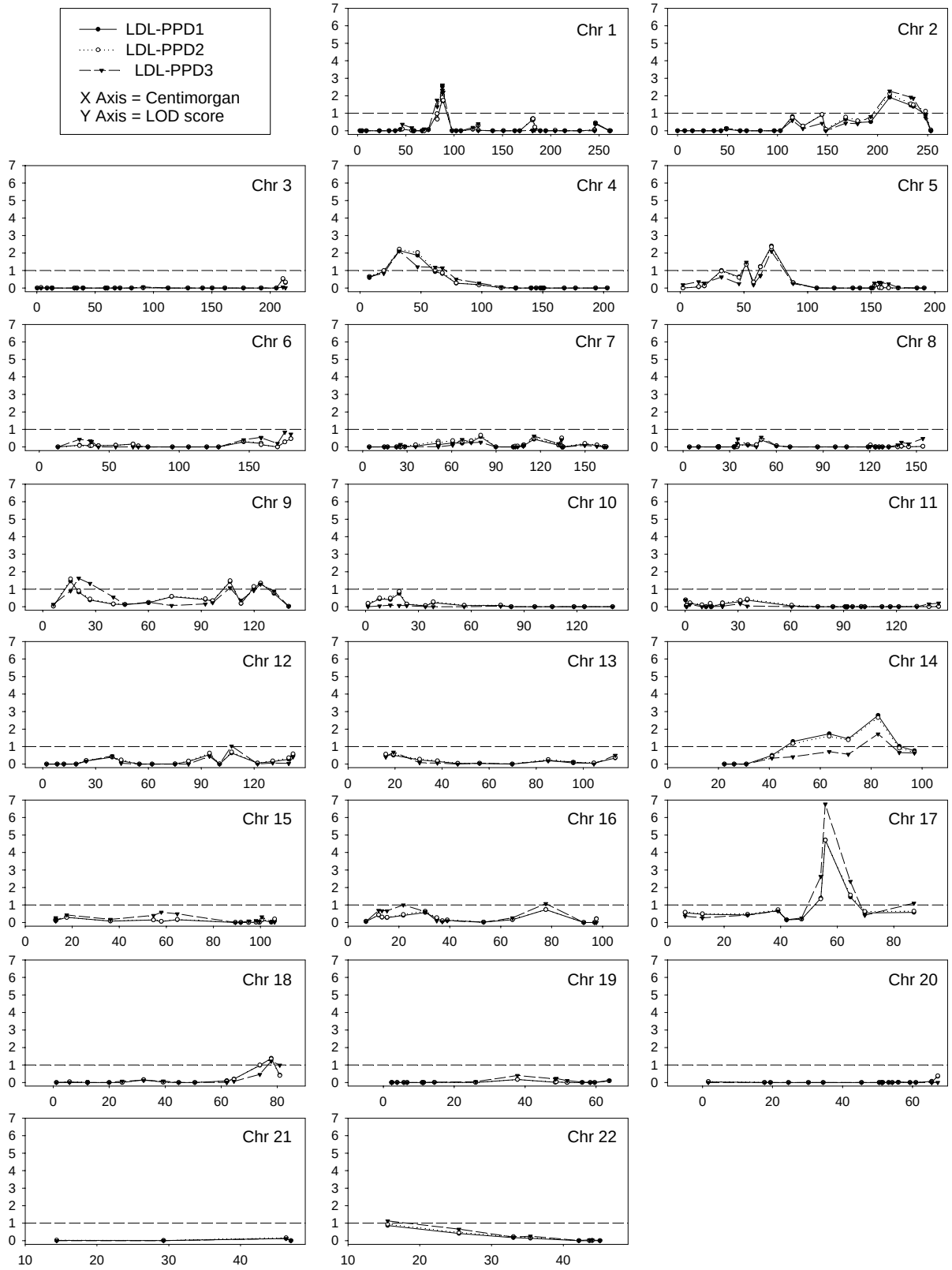
LDL-PPD1, LDL-PPD2 and LDL-PPD3 indicate LDL-PPD adjusted for: 1) age, 2) age and BMI, 3) age, BMI and triglyceride, respectively.

Table 3. Candidate genes within chromosomic regions linked to LDL-PPD.

Chr. region	Marker	LOD score			Candidates genes
		LDL-PPD1	LDL-PPD2	LDL-PPD3	
1p31	LEPR	2.05	2.04	2.56	PLA2G2D, PLA2G2A, PLA2G5, APOER2, FABP3, CPT2, ABCD3,HMGCS2.
2q33.2	D2S1384	1.91	2.11	2.27	PLA2R1, ABCB11, LRP2, ACADL, FACL3, ABCB6, IRS1, HDLBP.
4p15.2	D4S2397	2.14	2.22	2.11	LRPAP1
5q12.3	D5S1501	2.40	2.34	2.10	HMGCR, HMGCS1
14q31.1	D14S53	2.79	2.65	1.72	ABCD4, CYP46
17q21.32-q21.33	D17S1290 D17S1301	1.39 4.72	1.34 4.70	2.63 6.76	ACACA, ABCC3, ACE, APOH, ACOX1, FASN

LDL-PPD1, LDL-PPD2 and LDL-PPD3 indicate LDL-PPD adjusted for: 1) age, 2) age and BMI, 3) age, BMI and triglyceride, respectively.

Figure 1. Quantitative transmission disequilibrium test linkage results for all autosomal chromosomes with LDL-PPD phenotypes. LOD scores are presented on the y-axis and genetic distance is presented on the x-axis in centimorgans. LDL-PPD1, LDL-PPD2 and LDL-PPD3 indicate LDL-PPD adjusted for: 1) age, 2) age and BMI, 3) age, BMI and triglyceride, respectively.


LDL-PPD1
LDL-PPD2
LDL-PPD3
X Axis = Centimorgan
Y Axis = LOD score
Chr 1
Chr 2
Chr 3
Chr 4
Chr 5
Chr 6
Chr 7
Chr 8
Chr 9
Chr 10
Chr 11
Chr 12
Chr 13
Chr 14
Chr 15
Chr 16
Chr 17
Chr 18
Chr 19
Chr 20
Chr 21
Chr 22

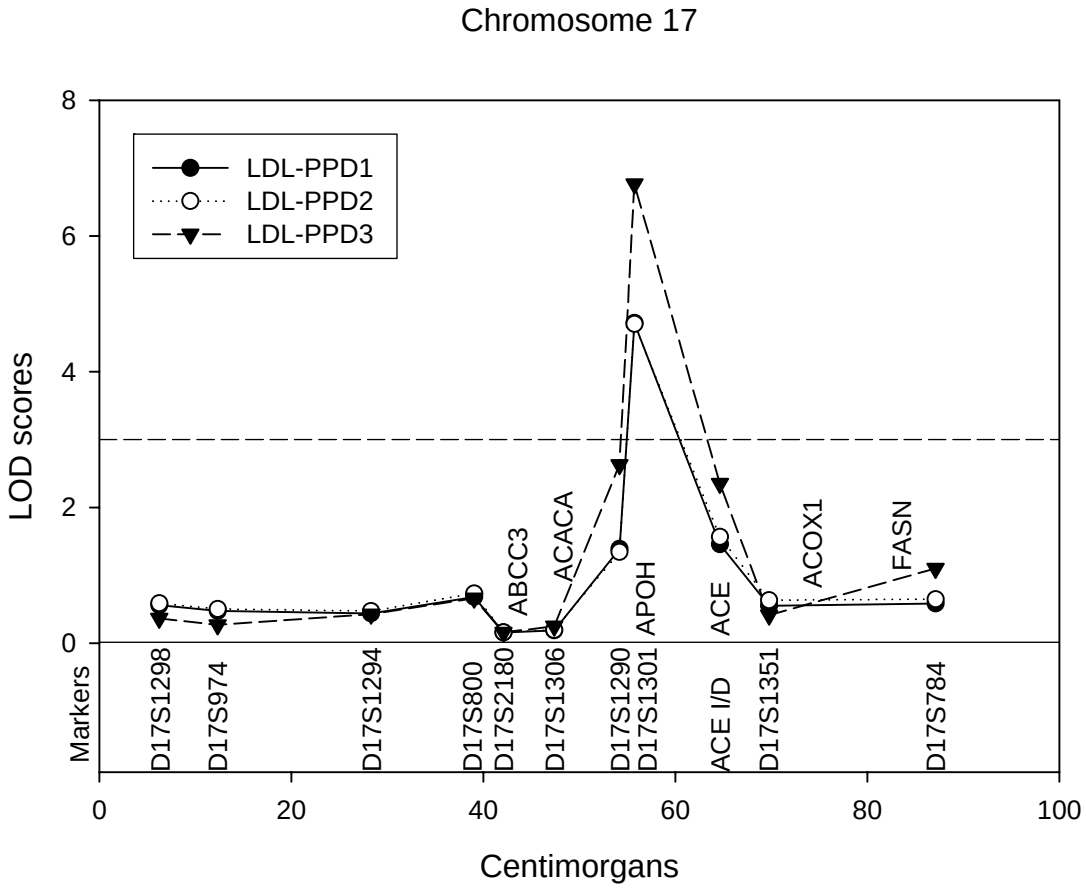


Figure 2. Quantitative transmission disequilibrium test linkage results for chromosome 17 with LDL-PPD phenotypes. Genetics markers used for linkage are indicated under the x-axis. The approximate location of candidate genes in the vicinity of the major peak are displayed on the graph. The dashed horizontal line represents a LOD score of 3.00. LDL-PPD1, LDL-PPD2 and LDL-PPD3 indicate LDL-PPD adjusted for: 1) age, 2) age and BMI, 3) age, BMI and triglyceride, respectively.

Chapter 9.

Is the major gene effect for LDL peak particle diameter on 17q caused by the apolipoprotein H gene?

Yohan Bossé, Mary F. Feitosa, Jean-Pierre Després, Benoît Lamarche, Treva Rice, D.C. Rao, Claude Bouchard, Louis Pérusse, Marie-Claude Vohl.

Des résultats antérieurs de l'Étude des familles de Québec ont révélé que le diamètre principal des particules LDL (DP-LDL) est semblable à l'intérieur des familles avec un cœfficient d'héritabilité estimé à plus de 50% et la présence d'un locus quantitatif majeur localisé sur le chromosome 17q. Dans cette étude on démontre, par analyse de ségrégation complexe, la présence d'un gène à effet majeur expliquant 52% de la variance du DP-LDL ajusté pour l'âge, l'indice de masse corporelle et les triglycérides. En séquencant le gène de l'apolipoprotéine H, localisé sur le chromosome 17q, trois mutations faux-sens ont été identifiées. Un haplotype particulier (fréquence = 20.9%) était associé avec des valeurs du DP-LDL plus élevé ($p = 0.046$). Ces résultats suggèrent que le DP-LDL est influencé par un gène à effet majeur et que le signal de liaison observé antérieurement sur le chromosome 17q pourrait être causé par le gène de l'apolipoprotéine H.

Is the major gene effect for LDL peak particle diameter on 17q caused by the apolipoprotein H gene?

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Number of figure: 1

Number of tables: 5

Abstract

Low-density lipoprotein (LDL) size, a coronary heart disease risk factor, is influenced by both genetic and environmental factors. Results from the Quebec Family Study (QFS) revealed that the LDL peak particle diameter (LDL-PPD) aggregates in families with a heritability coefficient above 50% and is affected by a major quantitative trait locus on chromosome 17q (LOD = 6.8). Complex segregation analyses have consistently demonstrated a major gene effect influencing LDL size. In the present study, we report a similar analysis in the QFS cohort, which suggests that a major gene explains 52% of the variance in age- body mass index- and triglyceride-adjusted LDL-PPD. The most intuitive positional candidate gene on chromosome 17q is the apolipoprotein H gene. Direct sequencing of the promoter, coding regions, and exon-intron splicing boundaries of this gene revealed the presence of three missense mutations and two polymorphisms in the untranslated regions. Using family-based association tests, none of these variants was individually associated with LDL-PPD. However, analysis of the haplotypes constructed from the three missense mutations, suggested that one particular haplotype (frequency = 20.9%) was associated with a significant increase in LDL-PPD trait values ($p = 0.046$). Taken together these results suggest the presence of a major gene influencing LDL-PPD and that the linkage signal previously observed on chromosome 17q may be related to the apolipoprotein H gene. Replication of the positive association between apolipoprotein H gene haplotype and LDL-PPD is warranted.

Key words: LDL peak particle diameter, apolipoprotein H, segregation analysis, family-based association test, haplotypes.

Introduction

There is considerable evidence suggesting that the presence of an increased proportion of small, dense low-density lipoprotein (LDL) particles is predictive of an increased risk of coronary heart disease (CHD)[1]. Individual variation in this new CHD risk marker has been known to be in part attributable to a number of environmental influences including, among others, dietary factors[2] and physical activity[3]. However, genetic studies have clearly demonstrated that a large part of the trait variability lies in the genes. A large number of studies have been conducted to elucidate the genetic architecture underlying the phenotype and have been the topic of a recent review[4].

Data from the Quebec Family Study (QFS) have recently confirmed the importance of genetic factors for LDL size and have produced new leads that need to be followed-up. Heritability estimates performed on QFS data suggested coefficient above 50% for LDL peak particle diameter (LDL-PPD), a quantitative trait reflecting the size of the major LDL subclass. In addition, the pattern of familial correlations revealed no significant spouse correlations but significant parent-offspring and sibling correlations, suggesting that the familial resemblance is primarily attributable to genetic factors[5]. An autosomal genomewide linkage scan was performed in order to identify the gene responsible for this genetic contribution[6]. A major quantitative trait locus (QTL) was observed on chromosome 17q for LDL-PPD adjusted for age, body mass index (BMI) and triglyceride levels (LOD = 6.8). Signals of lesser magnitude were also observed with the same phenotype on 1p, 2q, 4p, 5q and 14q, with LOD scores of 2.6, 2.3, 2.1, 2.1 and 1.7, respectively. Distinct genomic regions captured by genomewide linkage scans were also reported among families ascertained through hyperlipidemic proband, including 6q by Austin et al.[7], 15q by Allayee et al.[8] as well as 9p, 11q, 14q and 16q by Badzioch et al.[9]. Interestingly, these QTLs harbor a large number of candidate genes for LDL-PPD that have not been tested previously in linkage and association studies, and thus, provided new leads that require follow-up.

The results of the QFS genome scan generated two hypotheses that are being tested in the present study. First, the presence of a QTL affecting LDL-PPD is consistent with the major gene effect reported in segregation studies[10-16]. We speculate that the putative major gene effect observed in the later studies is responsible for the QTL on chromosome 17q. Thus we tested whether the single gene effect was also observed in QFS using complex segregation analysis. Then we have

chosen for further testing the most obvious candidate gene located on chromosome 17q, namely the apolipoprotein H gene (APOH). ApoH is a single-chain glycoprotein that exists in plasma both in a free form and in combination with lipoprotein particles. It has been implicated in several pathways, including lipid metabolism[17-20]. The second objective was thus to verify whether sequence variation in the APOH gene is responsible for the linkage signal observed on 17q.

Methods

Population

The QFS is an ongoing project of French Canadian families living in and around the Quebec City area with the aim of studying the genetics of obesity and its comorbidities[21]. This cohort represents a mixture of random sampling and ascertainment through obese (body mass index > 32 kg/m^2) probands. For the present study, LDL size characteristics were available for 680 subjects members of 236 nuclear families. The characteristics of these subjects by sex and generation groups are shown in Table 1. The QFS has been approved by the ethics committee of Laval University and all study participants provided written informed consent.

LDL peak particle diameter (LDL-PPD)

LDL-PPD was measured by gradient gel electrophoresis from plasma obtained after a 12-hours fast. Briefly, the whole plasma was loaded on nondenaturing 2-16% polyacrylamide gradient gels and exposed to electrophoresis for a prerun of 20 minutes at 70 V followed by migration at 175 V for 4 hours. Gels were then stained with sudan black, destained and size restored as described previously[22]. Gels were subsequently scanned and visualized on an electropherogram with every peak reflecting a band. The size of particles forming the bands was determined on the basis of a calibration curve constructed from the plasma standards. The estimated size of the major band was identified as the LDL-PPD. This phenotype was adjusted for age (up to the cubic polynomial), age and BMI or age, BMI and triglyceride levels. Data adjustments were performed within each of the six age-by-sex groups (<30, 30 to 50, and ≥50 years; male and female) using a stepwise multiple regression described previously[6].

Segregation analysis

Univariate segregation analysis was conducted using the Pedigree Analysis Package (PAP), version 5.0[23]. The mixed Mendelian model (model 1) assumes that a phenotype is influenced by the independent and additive contributions from a major gene, a polygenic/multifactorial background, and a nontransmitted environmental component. The major gene is biallelic (A, a), where the upper case allele, with frequency p, is associated with lower phenotypic values. The other parameters in the model are: the mean values for the three genotypes (μ_{AA}, μ_{Aa}, μ_{aa}, where the order of the means is constrained to be $\mu_{AA} \leq \mu_{Aa} \leq \mu_{aa}$); the common standard deviation within major locus genotypes (σ); the residual polygenic heritability (H), after accounting for the major gene effect; and parent-to-offspring transmission probabilities for the three genotypes (τ_{AA}, τ_{Aa}, and τ_{aa}). For a single diallelic locus, the three τ's denote the probability of transmitting allele *A* for genotypes *AA*, *Aa*, and *aa*, with Mendelian expectations of 1, 1/2, and 0, respectively. When the three τ values are equal, no transmission of the major effect is obtained. All parameters were estimated using a maximum likelihood method.

The general model (model 1) and thirteen reduced models (models 2-14) testing specific hypotheses were fitted to the data. The specific hypotheses tested in each model are the following. Three models tested the presence of familial components including a general familial component (model 2), a major gene component (model 3) and a multifactorial component (model 4). The modes of transmission were tested with recessive (model 5) and dominant (model 6) models. Two models (model 7 and 8) tested if the major effect follows Mendelian transmission probabilities (τ_{AA}, τ_{Aa}, τ_{aa}). Finally, recessive and dominant mode inheritance were also tested under the mixed (model 9 and 10), free (model 11 and 13) and equal (model 12 and 14) τ models. Nested models were tested against the mixed Mendelian model (model 1) or against each others as indicated in the last column of Table 3 using a likelihood ratio test which is the difference between the two models compared in minus twice the log-likelihoods (*-2 ln L*). The most parsimonious model of those not rejected by likelihood ratio test was determined using Akaike's Information Criterion (AIC)[24], which is computed as minus twice the log likelihood of the model plus twice the number of parameters estimated. The model with the lowest AIC indicates the most parsimonious fit to the observed data.

To claim a major gene effect with this approach, the following criteria have to be met. First, the no major gene effect (model 3) and the equal transmission probabilities (model 8) models have to be rejected. In addition, the free transmission probabilities model (model 7) has to be non-rejected. The variance accounted for by the major gene (σ^2_{mg}) was derived from this equation $(\mu_{AA} - \mu_0)^2p^2 + (\mu_{Aa} - \mu_0)^2 2p(1-p) + (\mu_{aa} - \mu_0)^2(1-p)^2$, where μ_{AA}, μ_{Aa}, μ_{aa}, and p are estimated in the parsimonious model and μ_0 are derived from the following equation $\mu_{AA}p^2 + \mu_{Aa}2p(1-p) + \mu_{aa}(1-p)^2$. The multifactorial heritability (H) estimated in the model is expressed as a function of the common residual variance (σ^2). The multifactorial heritability expressed as the percentage of the total phenotypic variance (h^2) can be computed using the equation $(H\sigma^2)/(\sigma^2 + \sigma^2_{mg})$.

Sequencing and genotyping of APOH gene

The promoter, the coding regions and the exon-intron splicing boundaries of the APOH gene were sequenced in 28 subjects having LDL-PPD in both extreme of the distribution (small < 254 Å and large > 276 Å). All exons and exon-intron splicing boundaries were amplified from genomic DNA by use of specific primers derived from the 5' and 3' ends of intronic sequence. We also sequenced up to 631 base pairs located downstream of the ATG start codon since consensus sequence elements have been localized in that region[25]. Table 2 presents the specific primers for each fragment with their product size. All primers were designed using the Primer 3.0 software available on the Whitehead Insitute/MIT Center for Genome Research server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Amplification was performed by polymerase chain reaction using the thermal cycler, model PTC-200 (MJ Research, Watertown, MA). PCR products were purified by the ABI ethanol-EDTA precipitation protocol, collected using a Beckman-Coulter Allegra 6R centrifuge, and resuspended in a 50% HiDi-formamide solution. Sequence reactions were performed using the BigDyeTH Terminator v3.1 kit and samples were run on ABI Prism® 3730/XL DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA). Sequences were then assembled and analyzed using the Staden preGAP4 and GAP4 programs[26]. Genetic variants were subsequently genotyped on the whole cohort using a mini-sequencing assay[27].

Association tests

The association between LDL-PPD and APOH variants was tested using two different statistical approaches. First, the independent effect of individual polymorphisms was tested by comparing the mean phenotype values between genotype groups using the MIXED procedure implemented in SAS (version 8.2), which takes the nonindependence of family members into account. The phenotypes were adjusted for age, gender, BMI and triglyceride levels prior to the association analyses. Secondly, we used the family-based association test (FBAT) when evaluating the association with single SNPs or haplotypes and LDL-PPD[28, 29] (http://www.biostat.harvard.edu/~fbat/default.html). The FBAT program performs family-based tests of association that are efficient and robust to population admixture, phenotype distribution and ascertainment based on phenotype. It can also handle missing parental genotypes and/or missing phase in both offspring and parents for haplotype analysis. The approach holds as well for multi-locus and multi-allelic markers. The haplotype test is ideal for candidate gene studies with tightly linked markers (no or little recombination between the markers). To test for the effect of a transmited allele on the trait values, an univariate FBAT test was performed for each allele. This test provides a Z-statistic with the corresponding p-value. A positive Z-statistic is indicative of an increasing trait value allele while a negative Z-statistic is indicative of a lowering trait value allele. This univariate FBAT statistic (Z-statistic) was also used to make inference regarding the effect of APOH haplotypes on LDL-PPD.

Family-based association tests were performed on LDL-PPD with and without adjustment for confounding factors. Adjustments were performed using a stepwise multiple regression procedure taking into account age, BMI and triglyceride levels as described previously[6]. The residuals, standardized to a mean of 0 and a SD of 1, were then used for statistical tests.

Results

Segregation analysis

Segregation analyses were performed on age-adjusted, age-BMI-adjusted, and age-BMI-triglyceride-adjusted LDL-PPD. In general, the results are quite consistent and only the results for the age-BMI-triglyceride-adjusted LDL-PPD are presented in Table 3. For the three phenotypes, the hypotheses of no familial resemblance (model 2), no major gene effect (model 3), and no multifactorial effect (model 4) are rejected, suggesting the presence of both a major gene and a multifactorial effects. In addition, the equal τ's hypotheses (model 8, 12 and 14) are rejected and the free τ's hypotheses (model 7, 11 and 13) are not rejected for the mixed, recessive mixed and dominant mixed, respectively, for the three phenotypes. Thus supporting that the trait is transmitted from parents to offspring and the transmitted effect is Mendelian in nature. In all cases, the mixed dominant Mendelian model (model 10) was not rejected and best fit the data according to the AIC values and was chosen as the most parsimonious model. With this parsimonious model, the variances accounted for by the major gene and the multifactorial component are as follow: 14% and 37% for the age-adjusted LDL-PPD; 14% and 34% for age-BMI LDL-PPD; 23% and 34% for age-BMI-triglyceride LDL-PPD.

Genetic variants in the APOH gene

A total of five genetic variants were identified in the APOH gene. Two were located within the untranslated regions of exon 1 and exon 8, namely -32C>A (rs8178822) and c.1059C>T (rs6933), respectively. The others were missense mutations found in exon 3 (rs1801692), 7 (rs4581) and 8 (rs1801690). The later have been identified before and are referred to as S88N, V247L and W316S. The frequencies of the minor alleles in the full family sample are 0.08, 0.04, 0.24, 0.06, and 0.44 for -32C>A, S88N, V247L, W316S and c.1059C>T, respectively. Genotype distributions for all variants are in Hardy-Weinberg equilibrium among genetically unrelated individuals ($p > 0.1$). Figure 1 shows the genomic organization of the APOH gene and the locations of the five genetic variants.

Genetic association analyses

Associations were first tested by comparing the mean phenotypic values between genotype groups for each genetic variant (Table 4). No significant association was observed for any of the genetic variants. For the W316S variant, only one subject was homozygous for the rare allele and was excluded from analyses.

Associations between single DNA variants and the LDL-PPD phenotype were also tested using the FBAT program. No single DNA variant showed significant association with LDL size at the 0.05 significance level (not shown). However, the number of informative families was relatively low (particularly for DNA variants with a low rare frequency allele) and may not have provided sufficient statistical power to detect an effect. We therefore used an haplotype family-based association test that took into account the genotype information of three missense mutations (S88N, V247L and W316S). Table 5 contains the haplotype patterns and frequency of the four most informative haplotypes. For each haplotype, the Z-statistic and the corresponding p value are given for LDL-PPD with and without adjustment for covariates. Haplotypes 88S/247V/316W (APOH_1), 88S/247L/316W (APOH_2), 88S/247V/316S (APOH_3) and 88N/247V/316W (APOH_4) have frequencies of 0.68, 0.22, 0.07 and 0.04, respectively. Haplotype APOH_1, APOH_3 and APOH_4 had a lowering effect on LDL-PPD phenotypes as indicated by negative Z-statistics. However, this lowering effect was not statistically significant. In contrast, haplotype APOH_2, which consists of the rare allele at V247L (exon7), and wild-type alleles at S88N (exon 3) and W316S (exon 8), was associated with a significantly greater LDL-PPD trait values. This effect was significant whether LDL-PPD was adjusted for covariates or not.

Discussion

The objectives of the present study were to test for the presence of a single gene with major effect on LDL-PPD, and to test whether genetic variants in the APOH gene was associated with this LDL phenotype. These objectives were motivated by studies consistently showing the presence of a major gene effect on LDL particle size and density[10-16], and a recent autosomal genomic scan on LDL-PPD performed in QFS[6]. Results of this genome scan revealed several QTLs with the strongest signal on 17q[6]. Based on these results, it was tempting to speculate that the putative gene detected by prior segregation analyses was located at this genomic location. We thus verified whether a major gene effect could also be detected in the QFS cohort and tested a strong candidate gene located in the 17q region, namely APOH.

Complex segregation analyses provided strong evidence suggesting the existence of a major gene effect influencing LDL-PPD. An interesting observation is that the major gene effect is amplified when LDL-PPD was adjusted for triglyceride levels (explained 52% of the variance vs 24%). This observation may simply be explained by a major gene effect that remain constant but act on a reduce variance cause by triglyceride adjustment. On the other hand, this observation may also suggest the presence of a pleiotropic gene having an effect on both LDL size and triglyceride, in addition to a gene affecting LDL size. Thus, it is possible that there are two genes, one with a pleiotropic effect on both LDL size and triglyceride, the other affecting purely LDL size.

In the present study, we used haplotypes derived from the FBAT program to test for association between LDL-PPD and genetic variants in the APOH gene. Although no single genetic variant showed a significant association with LDL-PPD, a haplotype-univariate test performed with three missense mutations revealed that one particular haplotype was associated with higher trait values. Despite its significance, this result should be interpreted with caution since no correction was applied for the number of tests performed. However, we believe that this association is likely to be true.

Testing for association using an haplotype approach is appropriate, especially when the informativeness of individual markers is low. Haplotype testing in the context of family studies is still in the developmental stage and computer programs have just become available[29, 30]. Considering the number of genetic variants identified in the present study, the most appropriate

association test was a haplotype test for family-based study. If we had elected to use only this test, the issue of multiple testing would not have arisen and the observed association would have been declared significant albeit at a low level.

Moreover, it can be argued that the association between the APOH gene and LDL-PPD has a physiological rationale. ApoH, also known as β_2-glycoprotein I, is a single chain polypeptide of 326 amino acids synthesized in the liver. The plasma concentration of apoH differs significantly among individuals, ranging from levels that are undetectable to levels as high as 35 mg/dl, with means of 20 mg/dl in Whites and 15 mg/dl in Blacks[18]. The protein exists in plasma both in a free form and in combination with lipoprotein particles, including VLDL, HDL and chylomicrons. Its role in lipoprotein metabolism is not fully understood, but it was shown to activate lipoprotein lipase[17] and clear triglycerides from plasma[31].

The missense mutations tested in the present study have been shown to be biologically functional and associated with lipid values. These variants explained a significant portion of the variation in apoH levels[18] and have also been associated with triglyceride[19, 20, 32], VLDL-C[32] and HDL-C[18] levels in some subgroups. In contrast, other studies have shown a lack of association between APOH polymorphisms and lipid traits[33-36].

Finally, an additional argument for the merit of the present finding comes from the fact that the APOH gene is located in a relatively small genomic region that has been shown to be linked to the LDL-PPD phenotype. The genomic scan signal observed on 17q makes the existence of a positional gene associated with the phenotype quite likely. However, considering the lack of association with the individual variants, we cannot exclude the possibility that the haplotype significantly associated with LDL-PPD is in linkage disequilibrium with a causative variant located elsewhere in a nearby gene.

In conclusion, complex segregation analysis supported the existence of a major gene for LDL size in QFS. This finding is consistent with similar studies performed so far[10-16]. By sequencing the promoter, splicing boundaries, and exons of a positional candidate gene, the APOH gene, we identified and genotyped five genetic variants including three missense mutations previously reported. By means of a family-based haplotype analysis, we identified a haplotype associated with larger LDL particle size. These results suggest that the APOH gene is

responsible for the QTL observed earlier on 17q and for the major gene effect detected by segregation analysis. However, replication in independent cohorts is required to secure these conclusions.

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Table 1. Descriptive statistics of the Quebec Family Study subjects by sex and generation groups.

Variables	**Fathers (n=137)**	**Mothers (n=195)**	**Sons (n=147)**	**Daughters (n=201)**
LDL-PPD (angstroms)	261.2 ± 5.4	264.3 ± 5.2	262.6 ± 4.5	264.4 ± 4.5
Age (years)	55.5 ± 9.2	55.4 ± 12.7	26.5 ± 9.9	28.1 ± 10.8
Body mass index (kg/m^2)	28.9 ± 6.3	28.6 ± 8.0	26.5 ± 7.0	27.6 ± 8.7
Triglyceride (mmol/L)	1.98 ± 1.23	1.79 ± 2.39	1.36 ± 0.71	1.27 ± 0.59
Cholesterol (mmol/L)	5.46 ± 0.92	5.46 ± 1.24	4.46 ± 0.90	4.52 ± 0.96
LDL-cholesterol (mmol/L)	3.56 ± 0.81	3.31 ± 0.90	2.76 ± 0.76	2.70 ± 0.84
HDL-cholesterol (mmol/L)	1.06 ± 0.26	1.37 ± 0.36	1.11 ± 0.24	1.25 ± 0.30

Values are means ± SD.

Table 2. PCR primers for genomic amplification of apolipoprotein H promoter and exons.

Exons	Oligonucleotides	Product size (bp)	Annealing Temperature (^{o}C)
Promoter	L 5'-TCAAATCAATTTTCAAATTCCTGA-3' R 5'-GAGATTACAGATGTGAGCAAGCAC-3'	881	60
Exon 1	L 5'-AACCATCTCCCAAAGATTTCATAA-3' R 5'-GAGATTACAGATGTGAGCAAGCAC-3'	395	60
Exon 2	L 5'-GTTGTTGAGGGGATTAGATGAGAT-3' R 5'-TAGCTTATTCCTCCAAAATACCCA-3'	409	60
Exon 3	L 5'-TATTGAACCAAAGGATGAAAATGA-3' R 5'-CAACCTAAAGGCTGAAAACAAAAT-3'	330	58
Exon 4	L 5'-GACAGCATTATACGATGGAAAAGA-3' R 5'-CATTGAGCTGTGACTGAAGAGATT-3'	303	60
Exon 5	L 5'-ATTTGATCAGTTTGCATTTTCTCA-3' R 5'-CATGGTAGATGCTCAATAAACAGC-3'	439	61
Exon 6	L 5'-GATATAAGAGGAGTGGGGACAGAA-3' R 5'-GGAAAAGTGTTGGAACAAGAAAAT-3'	440	60
Exon 7	L 5'-CCTTGACCAATTTGTGTAGGTGTA-3' R 5'-AGAGACAGACTTTCACCTTTTTGG-3'	519	60
Exon 8	L 5'-TTTGGTTTGGCTTAGCTATTTACC-3' R 5'-AACTTCACAGCCATAGAATGATGA-3'	335	64

L, left; R, right.

Table 3. Segregation analysis results for LDL-PPD adjusted for age, body mass index and triglyceride levels.

Model	μ_{AA}	μ_{Aa}	μ_{aa}	σ	p	H	*-2lnL + c*	χ^2	d.f.	P	*AIC*	Test
1. Mixed Mendelian (τ_{AA}=1, τ_{Aa}=0.5, τ_{aa}=0)	3.139 (0.245)	0.145 (0.222)	-0.783 (0.087)	0.633 (0.154)	0.186 (0.068)	0.451 (0.093)	0.07				12.07	
2. Sporadic (μ_{AA}=μ_{Aa}=μ_{aa}, p=1, *H*=0)	0.036 (0.041)	[μ_{AA}]	[μ_{Aa}]	1.067 (0.029)	[1]	[0]	99.35	99.28	4	< 0.001	103.35	2 vs 1
3. No major gene (μ_{AA}=μ_{Aa}=μ_{aa}); p=1	0.052 (0.051)	[μ_{AA}]	[μ_{Aa}]	1.064 (0.031)	[1]	0.519 (0.072)	44.29	44.22	3	< 0.001	50.29	3 vs 1
4. No multifactorial (μ_{AA}, μ_{Aa}, μ_{aa}, *H*=0)	3.136 (0.261)	0.677 (0.142)	-0.318 (0.057)	0.833 (0.035)	0.161 (0.028)	[0]	11.87	11.80	1	< 0.001	21.87	4 vs 1
5. Recessive Mendelian (μ_{AA}=μ_{Aa}, *H*=0)	2.653 (0.308)	[μ_{AA}]	-0.061 (0.039)	0.941 (0.028)	0.185 (0.007)	[0]	31.83	31.76	2	< 0.001	39.83	5 vs 1
6. Dominant Mendelian (μ_{Aa}=μ_{aa}, *H*=0)	2.921 (0.308)	-0.058 (0.038)	[μ_{Aa}]	0.932 (0.027)	0.167 (0.031)	[0]	28.68	28.61	2	< 0.001	36.68	6 vs 1
7. Mixed Free τ_{AA} (1.0*), τ_{Aa} (0.530), τ_{aa} (0.000)	2.700	0.111	-0.124	0.919	0.182	0.421	0.00	0.07	3	0.995	18.00	1 vs 7
8. Mixed Equal τ_{AA}=τ_{Aa}=τ_{aa}=p (0.220)	2.349	0.174	-0.204	0.908	0.220	0.525	21.70	21.70	3	< 0.001	33.70	8 vs 7
9. Mixed Recessive Mendelian (τ_{AA}=1, τ_{Aa}=0.5, τ_{aa}=0)	2.623 (0.264)	[μ_{AA}]	-0.052 (0.046)	0.935 (0.030)	0.021 (0.007)	0.407 (0.084)	4.94	4.87	1	0.027	14.94	9 vs 1
10. Mixed Dominant Mendelian (τ_{AA}=1, τ_{Aa}=0.5, τ_{aa}=0)	2.593 (0.267)	-0.056 (0.046)	[μ_{Aa}]	0.922 (0.030)	0.194 (0.033)	0.447 (0.091)	0.38	0.31	1	0.578	10.38	10 vs 1
11. Recessive Free τ_{AA} (1.0*), τ_{Aa} (0.401), τ_{aa} (0.000)	2.600	[μ_{AA}]	-0.055	0.931	0.023	0.410	4.22	0.72	3	0.869	20.22	9 vs 11
12. Recessive Equal τ_{AA}=τ_{Aa}=τ_{aa}=p (0.025)	2.317 (0.328)	[μ_{AA}]	-0.069 (0.047)	0.923 (0.038)	0.025 (0.009)	0.506 (0.098)	21.78	17.56	3	< 0.001	31.78	12 vs11
13. Dominant Free τ_{AA} (1.0*),	2.589	-0.055	[μ_{Aa}]	0.922	0.190	0.448	0.32	0.06	3	0.996	16.32	10 vs13

τ_{Aa} (0.528), τ_{aa} (0.0*)												
14. Dominant Equal $\tau_{AA}=\tau_{Aa}=\tau_{aa}$=p (0.223)	2.317 (0.328)	-0.069 (0.047)	[μ_{Aa}]	0.924 (0.038)	0.223 (0.040)	0.506 (0.098)	21.78	21.46	3	< 0.001	31.78	14 vs13

-2lnL + c = minus twice the log likelihood plus constant; c = 1945.63; μ_{AA}, μ_{Aa}, μ_{aa}: mean values for the three genotypes (AA, Aa and aa);

σ: standard deviation within major locus genotypes; p = allele frequency; H: the polygenic heritability; τ_{AA}, τ_{Aa}, and τ_{aa}: parent-to-offspring transmission probabilities for the three genotypes.

* The parameter reached the boundary value. [ ] The parameter was constrained to the value shown. ( ) Standard Error.

For all models, the specified tolerance on normalized gradient was met (the models reached convergence). However for models 9, 10, 11 and 13, the standard error calculation failed.

For the parsimonious models, Mixed Dominant Mendelian (model 3), the variance accounted for by the major gene was 23% with an additional 34% due to the polygenic component.

Table 4. Association of individual apolipoprotein H gene variant with LDL peak particle diameter.

	Mean values ± SE (n)			
	HMZ common allele	**HTZ**	**HMZ rare allele**	**p values***
-32C>A	262.2 ± 0.6 (552)	261.8 ± 0.7 (101)	262.7 ± 1.2 (5)	0.647
S88N	262.0 ± 0.6 (597)	262.2 ± 0.9 (45)	— (0)	0.801
V247L	262.1 ± 0.5 (379)	262.2 ± 0.6 (242)	261.6 ± 0.8 (36)	0.614
W316S	262.3 ± 0.5 (582)	262.1 ± 0.7 (78)	260.2 ± 0.7 (1)	0.764**
c.1059C>T	262.0 ± 0.6 (199)	262.0 ± 0.6 (340)	262.1 ± 0.6 (115)	0.943

*The statistical tests take into consideration the relatedness among subjects and covariates (age, body mass index and triglyceride levels).

**The only subject homozygous for the rare allele was excluded from the analysis.

HMZ, homozygotes; HTZ, heterozygotes.

Table 5. Haplotype-specific univariate family-based association test statistics (Z-statistics) for apolipoprotein H gene with LDL peak particle diameter.

Haplotype	Frequency	Number of informative families	Z-statistics (p value)	
			LDL-PPD	adj. LDL-PPD*
APOH_1 (88S/247V/316W)	0.676	112	-0.80 (0.425)	-1.29 (0.196)
APOH_2 (88S/247L/316W)	0.215	96	1.97 (0.049)	1.99 (0.046)
APOH_3 (88S/247V/316S)	0.067	38	-1.73 (0.083)	-1.20 (0.229)
APOH_4 (88N/247V/316W)	0.041	28	-0.51 (0.611)	-0.24 (0.813)

The minimum number of informative families necessary to compute the test statistics was set to ten.

*LDL-peak particle diameter adjusted for age, body mass index and triglyceride levels.

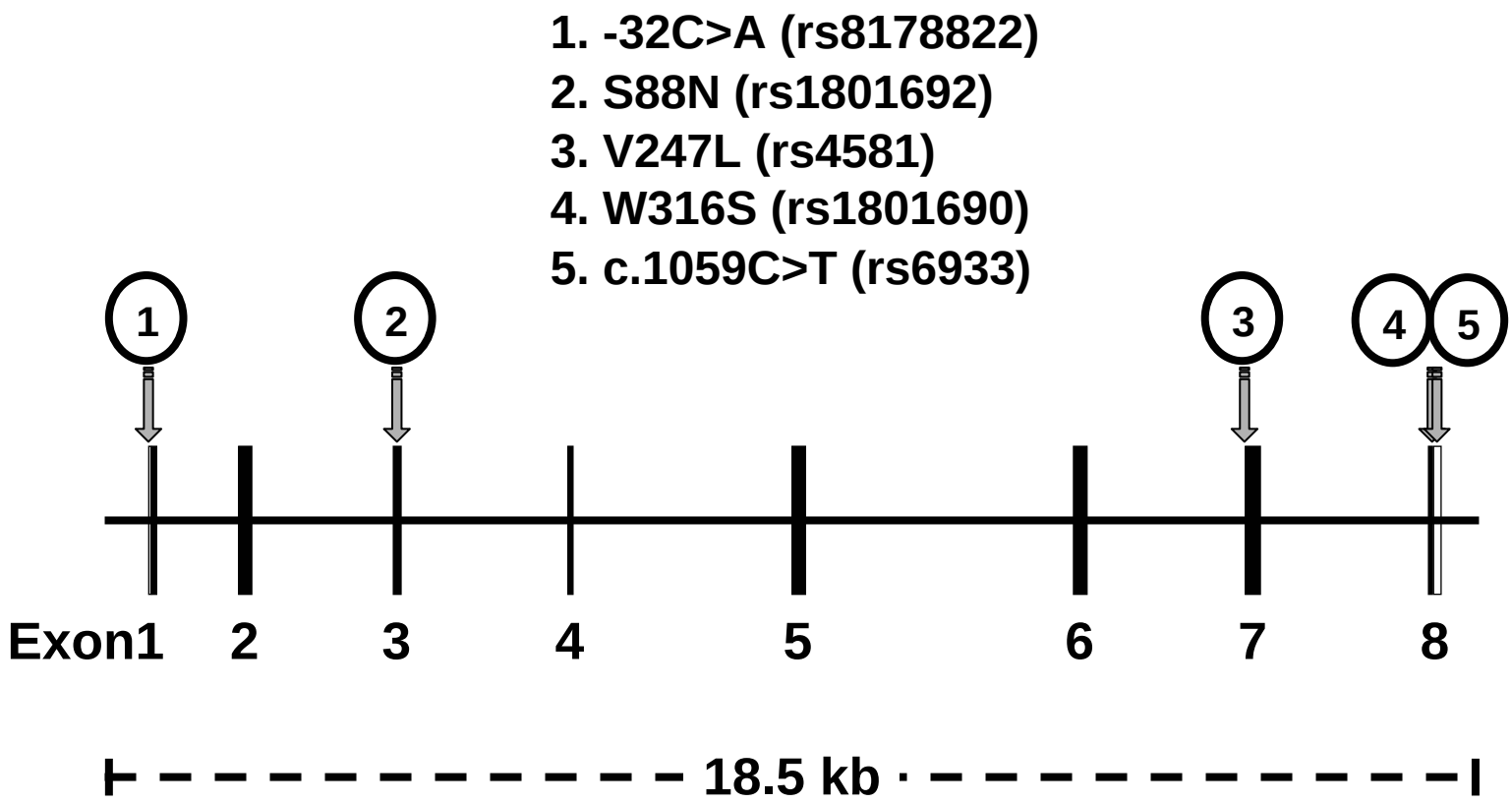

Figure 1. Genomic organization of the APOH gene, and location of the genetic variants identified in the Quebec Family Study. The eight exons are shown as vertical bars whose width corresponds to their base-pairs length. The untranslated regions located in exon one and eight are indicated as empty bars.

Chapter 10.

Genome-wide linkage scan for the metabolic syndrome reveals a major quantitative trait locus on chromosome 15q: The Quebec Family Study.

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L'objectif de cette étude était d'identifier les régions chromosomiques contenant les gènes de prédisposition au syndrome métabolique. Une analyse factorielle a été effectuée avec huit phénotypes reliés au syndrome métabolique incluant la circonférence de taille, l'indice de masse corporelle, la tension artérielle systolique et diastolique, ainsi que les mesures plasmatiques de glucose, d'insuline, de triglycérides et de cholestérol-HDL. Cette analyse a produit trois facteurs interprétés comme un facteur de syndrome métabolique, de tension artérielle et de lipides. Le facteur syndrome métabolique avait un haut degré de saturation factorielle (>0.40) avec tous les phénotypes. La cellule familiale expliquait 45.6% de la variance de ce facteur. Un criblage génomique effectué sur ce dernier indique la présence d'un locus quantitatif majeur localisé sur le chromosome 15 (86 cM, LOD = 3.15). Des évidences suggestives (LOD > 1.75) ont aussi été observées sur les régions 1p, 3p, 3q, 6q, 7p, 19q et 21q.

A quantitative trait locus on 15q for a composite metabolic syndrome variable derived from factor analysis in the Quebec Family Study.

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Abstract

The metabolic syndrome represents a cluster of cardiovascular risk factors co-occurring in the same individual. The aim of this study was to identify chromosomal regions encoding genes predisposing to the metabolic syndrome using composite factors derived from maximum likelihood-based factor analysis. Genetic data were obtained from the Quebec Family Study and included 707 subjects from 264 nuclear families. Factor analysis were performed on eight metabolic syndrome-related phenotypes including waist circumference, body mass index, systolic and diastolic blood pressure as well as plasma insulin, glucose, triglyceride and HDL-cholesterol levels. Three factors were identified and interpreted as a general metabolic syndrome, blood pressure and blood lipids, respectively. The metabolic syndrome factor had high factor loadings (>0.4) for all phenotypes and explained 42% of the total variance. An ANOVA testing for familial aggregation revealed that the family lines accounted for 45.6% of the metabolic syndrome factor variance. A genome-wide linkage scan performed with this first factor revealed the existence of a quantitative trait locus (QTL) on chromosome 15 (86 cM) with a logarithm of odds (LOD) score of 3.15. Suggestive evidences of linkage (LOD > 1.75) were also observed on chromosomes 1p, 3p, 3q, 6q, 7p, 19q, 21q. These QTLs may harbor genes contributing to the clustering of the metabolic syndrome-related phenotypes.

Introduction

The metabolic syndrome is defined as a cluster of interrelated cardiovascular risk factors observed in the same individual (1-3). There has been an increased in the number of abnormalities associated with this syndrome since its introduction more than 15 years ago (4; 5). However, all definitions include markers of glucose, lipid and blood pressure abnormalities. Obesity, and particularly abdominal obesity, is also an integral feature of the syndrome and is thought to be a major contributor to the metabolic abnormalities (1; 6).

The correlations among the multiple risk factors suggest the existence of common aetiologies. A large number of studies have used factor analysis to disentangle the metabolic and physiological basis of this clustering (7-31). They have identified two (7-13), three (14-25), four (25-31) and up to seven (31) independent factors underlying the metabolic syndrome architecture. However, several issues make the comparison among these studies difficult, including: 1-differences in study population, 2-the nature and number of variables chosen for inclusion in the modeling, 3-the number of factors extracted, and 4-the threshold for interpreting loadings (32). Nevertheless, in the aggregate, these studies suggest a three- to four-factor model including factors interpreted as representing insulin resistance, obesity, blood lipids and blood pressure with the insulin resistance and the obesity factors frequently found together. This putative metabolic syndrome factor structure was confirmed using confirmatory factor analysis (33; 34).

Heritability studies have shown that composite factors of the metabolic syndrome derived from factor analysis are under genetic influences (16; 23; 24; 30; 35). It has been postulated that a common gene, or a set of genes, may mediate the clustering of metabolic syndrome-related traits (36-38). By combining factor analysis and a candidate gene approach, Edwards et al. (39) found significant linkage between the apolipoprotein E gene and a weight/fat factor (loading on body weight, waist circumference (WC) and fasting insulin) and also between the cholesterol ester transfer protein gene and a lipid factor (loading on triglyceride, HDL-cholesterol and LDL peak particle diameter). More recently, Arya et al. (23) performed a genome-wide linkage scan on three composite factors extracted by factor analysis in nondiabetic Mexican-American families. Factor 1, loading on body mass index (BMI), fasting insulin and leptin levels (interpreted as an adiposity-insulin factor) yielded significant evidence of linkage on two loci on chromosome 6q. A third QTL was observed on chromosome 7q for factor 3 with high loadings

on HDL-cholesterol and triglyceride levels. In the present study, we have used subjects of the Quebec Family Study and performed a genome-wide linkage scan on a composite quantitative trait derived from factor analysis. The aim of this study was to identify genomic regions harboring genes influencing the variance of the metabolic syndrome-related phenotypes.

Research Design and Methods

Population

Subjects were participants of the Quebec Family Study (QFS) which is an ongoing project of French Canadian families designed to investigate the genetics of obesity and its comorbidities (40). The QFS represented a mixture of random sampling and ascertainment through obese probands (BMI $\geq$ 32 kg/m^2). In the present study, a total of 707 individuals from 264 nuclear families had complete data for the eight variables used in the identification of the metabolic syndrome factor. None of these subjects had fasting glycemia above 7.0 mmol/L or had a two hours post-glucose challenge glycemia above 11.1 mmol/L. Characteristics of the subjects are presented in Table 1. The Medical Ethics Committee of Laval University approved the protocol, and written consent was obtained from each subjects after the nature of the procedure was explained.

Phenotypes

Body weight, height, and WC were measured following standardized procedures (41). BMI was measured as weight (kg)/height (m^2). Fasting blood samples were collected, and cholesterol (42) as well as triglyceride (43) concentrations were determined enzymatically using a Technicon RA-500 automated analyzer (Bayer, Tarrytown, NY). HDL fraction was obtained after precipitation of LDL in the infranatant (>1.006 g/ml) with heparin and $MnCl_2$ (44). Plasma glucose and insulin levels were measured by standard procedures as previously described (45; 46). Subjects underwent systolic and diastolic blood pressure (SBP and DBP) measurements with a mercury sphygmomanometer and stethoscope according to the American Heart Association recommendations (47). SBP was defined as the first detectable sound, whereas DBP was measured at the disappearance of Korotkoff's sound. The blood pressure value was the mean of two consecutive measurements.

Genotyping

DNA preparation, polymerase chain reaction conditions, and genotyping are described in detail elsewhere (48). Genotypes were typed with automatic DNA sequencers and the computer software SAGA from LICOR. A total of 443 markers spanning the 22 autosomal chromosomes with an average intermaker distance of 7.2 centimorgans were available for this genome scan. These markers included 337 microsatellite markers and 106 polymorphisms in 65 candidate genes. The results were stored in a local database, GENEMARK, which inspects results for Mendelian inheritance incompatibilities.

Statistical analysis

Eight metabolic syndrome-related variables were chosen for factor analysis: WC, BMI, fasting insulin and glucose levels, SBP and DBP, as well as triglyceride and HDL-cholesterol levels. Five of them, BMI, glycemia, insulinemia, SBP and triglycerides, were log10 transformed to normalize their distribution. The factors were extracted by maximum-likelihood using PROC FACTOR procedure implemented in SAS (version 8.2, Cary, NC). This procedure generated orthogonal factors that are linear combination of the original variables. Factors were interpreted on the basis of the factor loading patterns describing the correlations between the emerging factors and the original variables.

The factor (factor 1) that accounted for the largest amount of variance had factor loadings > 0.4 for all variables and was labeled as an “overall metabolic syndrome factor”. Factor scores were then obtained for each individual and constituted the phenotype for linkage analysis. Prior to linkage analysis, the factor scores were adjusted for the effect of age (up to the cubic polynomial) in age-(<30, 30-50 and ≥50) by-sex (male and female) specific models using a stepwise multiple regression procedure retaining only significant terms ($p < 0.05$). Regression parameters were estimated after exclusion of outliers (± 3 SD), and residuals were computed for all subjects. Subjects whose residual values were greater then 4 SD from the mean and were separated by more than 1 SD from the nearest internal score were excluded from the analysis.

The presence of familial aggregation was tested using an ANOVA comparing the between-family to the within-family variances. This test was performed with the general linear model with the

overall metabolic syndrome factor as the dependent variable, and the family lines (family number) as the independent variable.

Linkage was performed with a variance component model using the quantitative transmission disequilibrium test (QTDT) computer program (49). Under this model, a phenotype is influenced by the additive effects of a QTL (q), a residual familial component due to polygenes (g) and a residual nonfamilial component (e). Hypothesis testing was performed by the likelihood ratio test. The likelihood of the null hypothesis is obtained by restricting the additive genetic variance due to the QTL (σ_q) equal to zero ($\sigma_q = 0$). The test is conducted by contrasting this restricted model with the alternative where σ_q is estimated ($\sigma_q \neq 0$). The difference in minus twice the log-likelihoods between the null and alternate models is approximately distributed as a χ^2 which allowed LOD score computation as $\chi^2/(2 \log_e 10)$. We have taken a LOD score of ≥ 3.00 ($p \leq 0.0001$) as evidence of linkage and a LOD of ≥ 1.75 ($p \leq 0.0023$) as evidence of suggestive linkage (50).

Results

Three factors accounted for 63% of the total variance (Table 2). Factor one explained 67% of the common variance and 42% of the total variance. This factor had high loadings (> 0.4) for all eight variables and can then be interpreted as the general metabolic syndrome factor. The correlation of this first factor with WC (0.97) and BMI (0.93) was especially high. Factor two had large positive loadings for SBP (0.56) and DBP (0.77) suggesting a blood pressure factor. Factor 3 had relatively high correlation with HDL-cholesterol (0.73) and triglycerides (-0.27) indicative of a lipid factor. The final communality estimates showed that all variables are relatively well accounted by the three factors, with final communality ranging from 0.19 for glycemia to 0.95 for WC. Subsequent analyses were undertaken only on the general metabolic syndrome factor (factor one).

The results of familial aggregation revealed more than two times more variance between families than within families. The family lines accounted for 45.6% of the variance in the general metabolic syndrome factor (F value = 2.18, $p < 0.0001$). Thus, the metabolic syndrome factor extracted from factor analysis significantly aggregates within families.

An overview of the linkage results for the general metabolic syndrome factor is given in Figure 1. The strongest evidence of linkage was found on chromosome 15q25 (D15S171) with a LOD score of 3.15 at 86 cM. The 1-LOD support interval extends from 69 to 95 cM. The second highest LOD score (2.60) was detected on chromosome 3p (49 cM) with marker D3S1581. Approximately, 6 cM downstream from that marker, a polymorphism located within the PPARγ gene gave highly suggestive evidence of linkage with a LOD score of 2.56. Suggestive evidences of linkage (LOD > 1.75) were also observed on chromosome 1p, 3q, 6q, 7p, 19q and 21q (Figure 1).

The linkage profile observed on chromosome 15 was then compared to that of each of the eight original variables (Figure 2). WC and BMI have a similar linkage pattern to the general metabolic syndrome factor, with LOD scores of 3.06 and 2.28, respectively, at marker D15S171. LOD scores above 1.0 were also observed on chromosome 15q for triglyceride and insulin levels as well as for DBP. LOD scores of 0.98 and 0.89 were observed for SBP and fasting glucose levels,

respectively. However, no evidence of linkage was observed in the 15q region for HDL-cholesterol levels.

Discussion

The metabolic syndrome is recognized as a constellation of metabolic disturbances present in the same individual, which tends to include obesity, hypertension, dyslipidemia and insulin/glucose disturbances (1-3). The metabolic and physiological bases for this clustering are not well elucidated but it has been hypothesized that it may have a common etiology. In the present study, we performed a genome-wide linkage scan on a general metabolic syndrome factor derived from factor analysis in order to identify genetic loci influencing the syndrome in a nondiabetic cohort. Factor analysis identified one underlying factor (labeled the general metabolic syndrome factor) with high loadings on each of the eight metabolic syndrome-related phenotypes. This suggests that a single weighted combination of these variables accounts for a large fraction of the clustering. Assessment of familial aggregation reveals that the general metabolic syndrome factor exhibits significant familial clustering. A major QTL was found on chromosome 15q suggesting the presence of a gene (or genes) contributing to the shared variance among the original variables. Evidences of linkage for WC and BMI considered individually were also observed at the same location. The stronger genetic signals with obesity-related phenotypes compared to the other traits may indicate that there is a gene acting through obesity.

The three factor model found in the present study is consistent with most of the literature (7-31). However, what is less consistent is the fact that all eight metabolic syndrome-related phenotypes had high factor loadings (>0.40) on the first factor. This discrepancy might be explained by the fact that most studies have used orthogonal rotation (varimax) following factor extraction in order to produce interpretable factors. In fact, the few studies that have not used such a rotation procedure have reported high factor loadings with the first factor for all, or almost all, metabolic syndrome-related phenotypes (13; 30). These results are in accordance with the concept that one unifying biological/physiological process underlies the clustering of cardiovascular risk variables.

Few studies have attempted to localize the “metabolic syndrome genes” using a similar approach. Loos et al. (13) performed a genome-wide search on two principal components obtained from 456 whites and 217 blacks participants of the HERITAGE Family Study. Principal component analysis was carried out on seven metabolic syndrome-related phenotypes including percent body fat, visceral adipose tissue area assessed by computed tomography, mean arterial blood pressure, and plasma HDL-cholesterol, triglyceride, glucose, as well as insulin concentrations. Suggestive

evidences of linkage ($p < 0.0023$) were found on 10p for principal component 1 and 19q for principal component 2 in Whites and on 1p for principal component 2 in Blacks. Similarly, Tang et al. (30) performed a genome scan on a metabolic syndrome factor using traditional (BMI, waist-to-hip ratio, triglyceride, HDL-cholesterol, and HOMA index) and non-traditional (PAI-1 and serum uric acid) metabolic syndrome-related phenotypes. A general metabolic syndrome factor was derived from maximum likelihood based factor analysis using the data from the NHLBI Family Heart Study. A significant signal was observed on 2q with additional lower signals observed on chromosomes 7, 12, 14 and 15. Most of these QTLs were not replicated in the present study. However, the suggestive linkage observed on 1p (57 cM) and 19q (50 cM) could represent possible replication of the suggestive linkage observed in Blacks (1p, 56 cM) and in Whites (19q, 60 cM), respectively, for principal component 2 in the HERITAGE Family Study (13). The identification of different QTLs among studies may be due to differences in study populations, the nature and number of variables used to define the metabolic syndrome and the number of factors extracted. The lack of replication for the QTL reported on 2q36 by Tang et al. (30) can also be explained by the lack of diabetic subjects in the present study. In fact, their linkage signal on 2q36 was attenuated after the exclusion of diabetic subjects suggesting that these individuals contribute substantially to the linkage. It is also worth mentioning that the suggestive linkage observed on 3q overlapped with the QTL reported by Kissebah et al. (51) for six individual traits of the metabolic syndrome.

The signal observed on 15q25 represented a novel QTL for the metabolic syndrome. Arya et al. (23) have reported a suggestive linkage (LOD = 2.0) for a blood pressure factor near the same location. No QTL for any individual component of the metabolic syndrome have been identified in that region except for a significant linkage signal for blood pressure in a Chinese population (52). However, the locus has been linked to LDL-C (53), familial hypercholesterolemia (54) and insulin-dependent diabetes mellitus (55; 56). We have also reported in the QFS cohort a QTL for fat-free mass on 15q25-q26 (48). Whether or not the linkage signal is cause by the same gene is unknown at that time. Several candidate genes are located under the peak signal, including among others, perilipin (PLIN), neuromedin B (NMB), hepatic lipase (LIPC) and insulin-like growth factor-1 receptor (IGF1R). Further studies will be required to test these putative candidate genes.

In conclusion, the present study revealed the presence of a genetic locus on chromosome 15q linked to a general metabolic syndrome factor accounting for about 42% of the variance shared by WC, BMI, fasting insulin, glucose, HDL-cholesterol and triglyceride levels, as well as SBP and DBP. Suggestive evidence of linkage was also found on chromosomes 1p, 3p, 3q, 6q, 7p, 19q and 21q. These QTLs may contain genes influencing the metabolic syndrome-related phenotypes.

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Table 1. Phenotypic characteristics of study participants by sex and generation groups.

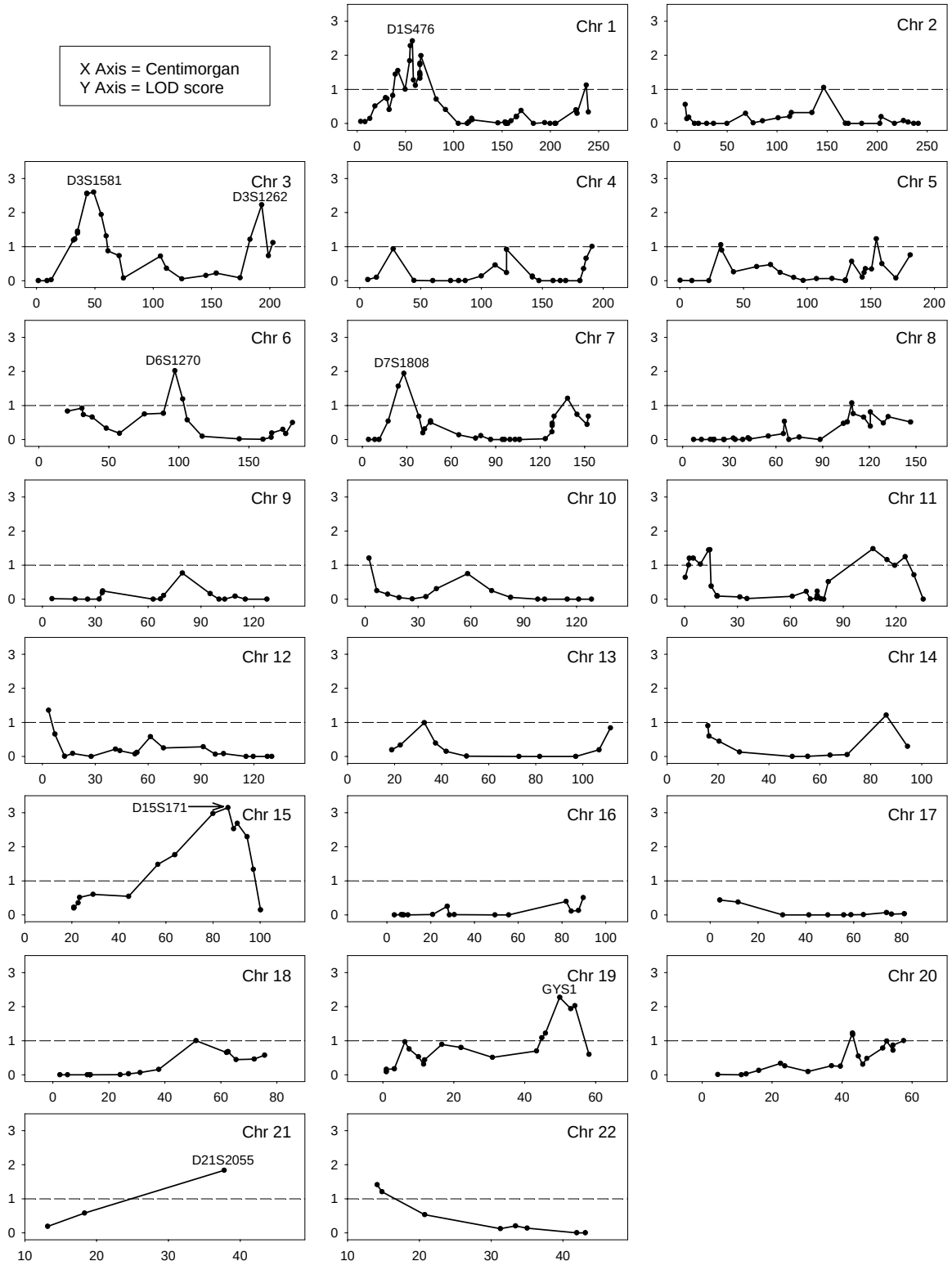
Variables	Fathers (134)	Mothers (184)	Sons (171)	Daughters (218)
Waist circumference (cm)	98.94 ± 14.46	87.35 ± 17.26	89.68 ± 19.04	82.28 ± 17.94
Body mass index (Kg/m^2)	28.83 ± 6.01	28.63 ± 7.46	26.78 ± 7.61	27.28 ± 8.29
Insulin (pmol/L)	71.12 ± 49.22	64.27 ± 49.88	78.25 ± 60.20	80.62 ± 58.65
Glycemia (mmol/L)	5.38 ± 0.59	5.15 ± 0.56	5.13 ± 0.45	4.97 ± 0.47
Triglyceride (mmol/L)	1.88 ± 0.99	1.67 ± 2.43	1.34 ± 0.71	1.25 ± 0.57
HDL-cholesterol (mmol/L)	1.07 ± 0.27	1.40 ± 0.36	1.10 ± 0.24	1.27 ± 0.30
Systolic blood pressure (mm Hg)	124.14 ± 18.53	124.14 ± 18.98	114.66 ± 13.19	109.84 ± 11.76
Diastolic blood pressure (mm Hg)	77.40 ± 10.19	75.09 ± 9.06	69.47 ± 9.80	67.28 ± 8.34

Values are means ± SD.

Table 2. Results of factor analysis.

Variables	Factor-loadings pattern			Communality
	Factor 1	**Factor 2**	**Factor 3**	
Waist circumference	0.97	-0.14	0.03	0.95
Body mass index	0.93	-0.19	0.11	0.91
Insulin	0.62	-0.21	-0.06	0.43
Glycemia	0.44	0.00	-0.01	0.19
Triglyceride	0.50	0.08	-0.27	0.33
HDL-cholesterol	-0.41	0.23	0.73	0.76
Systolic blood pressure	0.51	0.56	0.04	0.57
Diastolic blood pressure	0.56	0.77	-0.05	0.91
Common variance	3.36	1.06	0.63	5.05
Common variance (%)	66.53	20.99	12.48	100.00
Total variance (%)	42.00	13.25	7.88	63.13

Figure 1. Genome-wide linkage results on the metabolic syndrome factor for autosomal chromosomes (Chr). Logarithm of the odds (LOD) scores are presented on the *y* axis, and genetic distances are presented on the *x* axis in centimorgans. The horizontal dashed line represents a LOD score of 1.00. The markers with the highest LOD score for every genomic region with LOD score above 1.75 are shown. GYS1, glycogen synthase 1.


X Axis = Centimorgan
Y Axis = LOD score
Chr 1
D1S476
Chr 2
Chr 3
D3S1581
D3S1262
Chr 4
Chr 5
Chr 6
D6S1270
Chr 7
D7S1808
Chr 8
Chr 9
Chr 10
Chr 11
Chr 12
Chr 13
Chr 14
Chr 15
D15S171
Chr 16
Chr 17
Chr 18
Chr 19
GYS1
Chr 20
Chr 21
D21S2055
Chr 22

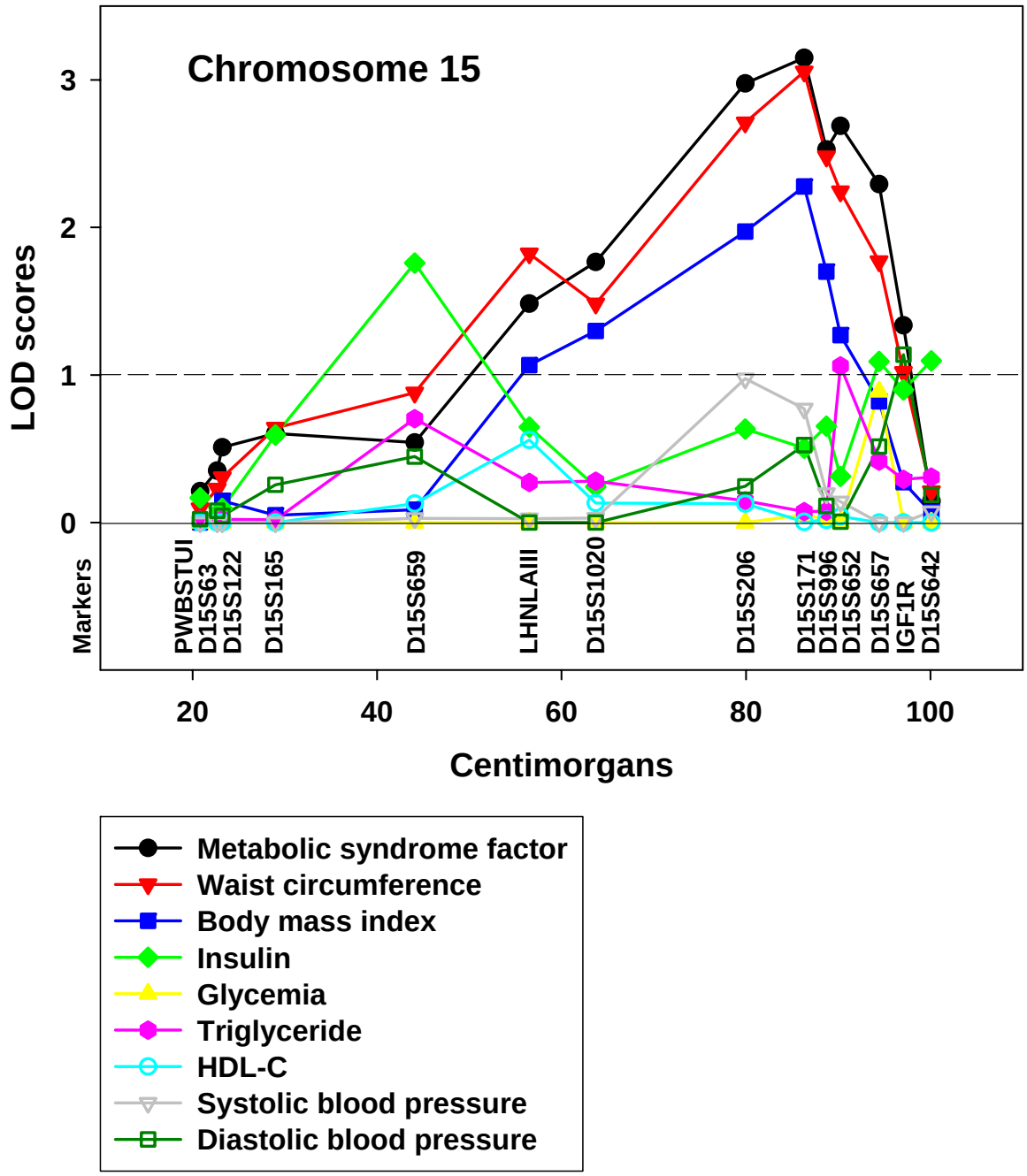


Figure 2. Results of linkage analysis on chromosome 15 for the metabolic syndrome factor and the eight original variables. Genetics markers used for linkage are indicated under the *x* axis. The horizontal dashed line represents a LOD score of 1.00.

Conclusion

The prevalence of the metabolic syndrome has risen tremendously over the past decades[7]. People with this syndrome are at increased risk to develop diabetes mellitus[17] and CVD[19] as well as at increased mortality from CVD and all causes[213]. Understanding the genetic contribution and the genetic determinants of this condition is particularly relevant considering its progressive economic burden on medical health care. However, this task is particularly challenging for geneticists knowing that multiple genes and environmental factors as well as their interactions contribute to the expression of the metabolic syndrome. In this thesis, we have attempted to shed some light on this question using both candidate gene and genome-wide scan approaches on the metabolic syndrome and its individual components. A total of four candidate genes have been investigated and nine phenotypes underwent genome-wide searches. Throughout this work, some polymorphisms located within the candidate genes have been associated with specific phenotypes and many genomic regions containing susceptibility genes have been identified.

In chapters 1 through 3, we used a candidate gene approach to investigate the effect of a common genetic variation, L162V, in the PPARα gene. PPARα is responsible for the translation of nutritional and metabolic stimuli into changes in gene expression[214] and is thus an excellent candidate gene in the context of the metabolic syndrome. In addition, PPARα mediates the effects of fibrates, a class of drug recognized to regulate lipoprotein-lipid metabolism[215]. In chapter 1, we have documented a part of the interindividual variation in the response to fibrate therapy depending on the presence or the absence of the PPARα L162V mutation. After the 6-month intervention therapy with gemfibrozil, carriers of the V162 allele had a greater increased in HDL_2-cholesterol compared to non-carriers. These results and others[216] suggest potentially greater benefits of fibrate treatment among individuals carrying the PPARα V162 allele. In chapter 2, we have reported that the same mutation was associated with lower values of adiposity phenotypes in participants of QFS. This observation makes biological sense considering the functional differences between the leucine- and the valine-containing protein products[216,217]. Rodent experiments have demonstrated that deactivation of PPARα increases body fatness[218], whereas its activation by fibrate treatment prevents weight gain and reduces adipose tissue[219,220]. Accordingly, it is possible that the reduced adiposity values observed in subjects carrying the PPARα V162 allele are explained by a greater activity of the valine-containing protein. In

chapter 3, it has been shown that the PPARα L162V mutation acts individually and in interaction with the PPARγ P12A mutation to modulate glucose/insulin parameters following an oral glucose tolerance test. This chapter has demonstrated that genetic polymorphisms in candidate genes encoding proteins with overlapping functions can interact and account for a significant contribution to the final manifestation of the trait. Chapters 1, 2 and 3 constitute a perfect example of the complex genetic architecture underlying components of the metabolic syndrome. Indeed, taken together, these chapters suggest that the same mutation acts individually, interacts with other genetic variants and also influences the response to a treatment. The multifaceted effects of a single mutation demonstrate again the complex puzzle that geneticists must face.

In chapter 4, we have investigated the association between the PLTP gene and obesity-related phenotypes. This study was motivated by genome-wide scans in human and mouse pointing to the PLTP locus as a candidate region for obesity[221-223]. In addition, important functions governing lipid metabolism have been ascribed to PLTP[224,225]. Two intronic SNPs were genotyped and tested for their association with several indices of adiposity in QFS. Both single locus and haplotype association tests for family-based study revealed significant associations. Accordingly, this thesis reports two genes associated with obesity, namely PLTP and PPARα. Of course, independent replications for both of these genes will be required to confirm these significant associations.

Chapters 5 and 6 concern genome-wide scans performed on plasma lipid and lipoprotein concentrations. These two studies revealed the existence of multiple loci influencing blood lipids and lipoproteins. Indeed, evidence of linkage has been found on chromosome regions 1q43, 11q13-q24, 15q26.1, and 19q13.32 for LDL-cholesterol, 12q14.1 for HDL-cholesterol, 2p14, 11p13, and 11q24.1 for triglycerides, 18q21.32 for LDL-apoB, and 3p25.2 for apoAI. Some of these regions have been linked to lipid-related traits before, whereas others represent new findings. Other studies will be required to identify the causal genes within these regions. Chapter 6 also summarizes the loci providing evidence of linkage from all the previous published genome-wide scans carried out on blood lipid-related phenotypes. This exercise has been undertaken to make an update in the field and help investigators in positioning new findings without having to digest the heavy literature anew. A cumulative of 152 linkage signals have been gathered in this exercise. Although it may ease the interpretation of the next genome-wide

scans on blood lipid-related phenotypes, displaying all these loci on the same map also revealed that a large portion of the genome is now covered with a least suggestive evidence of linkage. With this work, we were unable to achieve a coherent and comprehensive picture of the loci contributing to blood lipids and lipoproteins. This, again, demonstrates the difficulty of finding genes influencing complex traits.

The introduction synthesizes the accumulating evidence of the complex genetic etiology underlying LDL particle heterogeneity. Genetic epidemiology studies have clearly shown a genetic contribution to the LDL subclass phenotypes. The results from chapter 7 have confirmed this finding by showing high familial resemblance for LDL-PPD in 681 individuals participating in QFS[226]. Indeed, an ANOVA comparing between- versus within-family variance indicated that there was about two times more variance between families than within families. Thus, results from the QFS suggested that the family lines accounted for close the 50% (47-49% depending on covariates adjustment) of the variance in LDL-PPD phenotype. In addition, the pattern of familial correlations revealed no spouse correlation but significant parent-offspring and sibling correlations for the LDL-PPD phenotypes, suggesting that genetic factors are the major determinants of the familial aggregation.

Heritability studies from previous reports (see Table 4) have shown that at least 30% to 60% of the variation in LDL size is attributable to genetic factors. Heritability estimates for LDL-PPD in the QFS study fall within this range[226]. In chapter 7, three LDL-PPD phenotypes based on three different adjustment procedures have been constructed: LDL-PPD1 adjusted for age, LDL-PPD2 adjusted for age and BMI, and LDL-PPD3 adjusted for age, BMI and triglyceride levels. Heritability estimates for the three phenotypes were 58.8, 58.4 and 52.0%, respectively. The high heritabilities obtained may be explained by the design of the study. Indeed, in this case, heritability is defined as the proportion of variance due to additive familial effects, including both genetic and nongenetic sources of variance. Although, the pattern of familial correlations in the QFS study suggested that the familial resemblance is mostly attributable to genetic factors, heritability estimates derived from this cohort may be considered as upper bound estimates for LDL-PPD.

Complex segregation analyses have consistently demonstrated the existence of a single gene with major effect (see Table 5). Again this finding has been confirmed in the QFS (Chapter 9). All

hypotheses of no familial resemblance, no major effect, and no multifactorial effect have been clearly rejected in that study, suggesting that both the major and the multifactorial effects were significant. Tests on the transmission probabilities have been also carried out, and the environmental hypothesis (equal τ's) has been rejected whereas the Mendelian τ's was not. The putative gene accounted for 24%, 24% and 52% of the phenotypic variance of the age-adjusted, age-BMI-adjusted and age-BMI-triglyceride-adjusted LDL-PPD, respectively. In addition, another 22-34% of the variance was attributable to residual polygenic and familial environmental factors.

Taken together, it seems clear from a genetic epidemiology perspective that LDL size is under the influence of genetic factors. The results obtained from the QFS have simply reinforced this fact by demonstrating: 1-high familial aggregation, 2-significant heritability, and 3-the existence of a major gene effect. This consistency observed between studies has clearly stimulated the search for the causal genetic variants.

However, searching the DNA-based variations responsible has proved to be a difficult task owing to inconsistency and lack of replications among studies (see Tables 6 and 7). Indeed, linkage and association studies with candidate genes have produced some of the expected results, but in general the effect of positive hits does not seem to be uniform in all populations and environmental backgrounds. Genome-wide linkage scans have been undertaken to fill the gap and have produced interesting leads that need to be followed-up. In chapter 8, a genome-wide scan has been carried out in 681 subjects from 236 nuclear families participating in the QFS[227]. The strongest evidence of linkage was found on chromosome 17q23, with a LOD score of 6.76 for the phenotype adjusted for age, body mass index and triglyceride levels. Other chromosomal regions provided LOD > 2.0, including 1p33-p31, 2q33-q36, 4p15-q13, 5q13-q14 and 14q23-q32. Thus, this genome scan gives strong evidence for the presence of a major quantitative trait locus (QTL) located on 17q, but also demonstrated the multilocus nature of LDL size.

The APOH gene is a particularly interesting candidate gene in the 17q area. In chapter 9, the promoter, the exons ant the exon-intron splicing boundaries have been sequenced in subjects of the QFS cohort. Five genetic variations have been identified, including three missenses mutations. The entire cohort has been then genotyped for genetic association testing. An

haplotype family-based association test revealed the existence of an haplotype significantly associated with greater LDL size. This result suggests that the APOH gene is responsible for the genome-wide linkage signal observed on chromosome 17q. However, considering the limitations of association studies (see introduction), independent replication of this finding is essential before reaching conclusions.

Figure 11 summarizes the contribution of the present work in identifying the genes responsible for the large and consistent genetic influences observed on LDL size. Although the genome-wide linkage scan performed in the QFS cohort has been very fruitful in finding quantitative trait loci (QTL) for LDL size, the rate of replication with the previous published scans[130,131,151,209] is low. Indeed, the only evidence of replication is observed on chromosome 5 with a QTL observed for LDL median diameter of baboon exposed to a low-cholesterol, high-fat diet[209]. This genomic region contains the HMG CoA reductase gene which constitutes an interesting candidate gene to test in the near future.

It is becoming obvious that several different genetic loci contributed to the expression of small, dense LDL. This observation suggests that different genetically determined metabolic mechanisms may give rise to the phenotype. For most of the loci identified so far, it is unclear whether the effect is direct or mediated through the interrelationship with other metabolic parameters such as glucose/insulin homeostasis and triglyceride metabolism. The number of false positives reported is difficult to assess but may be important due to publication bias toward positive findings. Accordingly, this summary should be interpreted with caution and awareness since some of the positive loci may eventually prove to be false positives.

Understanding the genetic etiology of small, dense LDL will help to elucidate the complex multifactorial networks involved in the progression of atherosclerosis and its ultimate consequence—CHD. Although searching the genes has been and continues to be a demanding adventure, the challenge may still be ahead in order to identify the combination of genes and environmental circumstances predisposing to small, dense LDL. It should be emphasized, however, that the nongenetic factors influencing the expression of small, dense LDL can be taken to our advantage by treating genetically susceptible individuals with appropriate lifestyle modifications.

Figure 11. Ideogram of human karyotype showing chromosomal locations of genes and QTLs potentially involved in LDL size/density. Results from QFS are in red, while all the other results presented in Figure 10 are in gray. Genes and QTLs are placed on the hybrid map showing the sequence and the cytogenetic locations. Information to construct the ideogram has been obtained from the UCSC Genome Browser (http://genome.ucsc.edu). The alternated black and white colors on the chromosomes have been used to distinguish a cytogenic band from the adjacent ones and do not correspond to the band colors observed on Giemsa-stained chromosomes. ABCA1, ATP-binding cassette, sub-family A, member 1; ADRB3, β3-adrenergic receptor; APO, apolipoprotein; CYBA, p22 phox; CETP, cholesteryl ester transfer protein; LDLR, low-density lipoprotein receptor; LIPC, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; SOD2, manganese superoxide dismutase; SRB1, scavenger receptor class B type 1.


Chr 1
Chr 2
Chr 3
Chr 4
Chr 5
Chr 6
Chr 7
Chr 8
Chr 9
Chr 10
Chr 11
Chr 12
Chr 13
Chr 14
Chr 15
Chr 16
Chr17
Chr 18
Chr 19
Chr 20
Chr 21
Chr 22
Mega-basepairs
APOB
MTP
SOD2
LPL
ADRB3
ABCA1
APOA1/
C3/A4/A5
SRB1
LIPC
CETP
LCAT
CYBA
APOH
LDLR
APOE

Finally, chapter 10 was an attempt to summarize this thesis by identifying chromosomal regions harbouring genes contributing to the clustering of the metabolic syndrome-related phenotypes. Factor analysis has been used for that purpose to create a quantitative metabolic syndrome variable representing the common variance among the individual components of the syndrome. Factor analysis has revealed one underlying factor (the metabolic syndrome factor) with high loading for all the metabolic syndrome-related phenotypes, suggesting that common factors account for the observed risk variables clustering. A genome-wide scan on the metabolic syndrome factor revealed the existence of a QTL on chromosome 15q indicating the presence of a gene located in the area contributing to the shared variance among components of the metabolic syndrome. Again, further studies will be required to locate the causal gene.

Many questions arise and have remained unsolved throughout this work. For example, many chromosomal regions containing genes affecting components of the metabolic syndrome have been identified. However, the ultimate goal of QTL mapping is to identify the genes underlying these polygenic traits and to gain a better understanding of them. Except for the 17q region identified for LDL-PPD, no attempt was made in this work to locate the genes causing the linkage signals. In addition, all the association studies reported in this thesis require replication before reaching conclusions. The limitations of association studies are highlighted in the introduction and leave some uncertainties about the results presented. Accordingly, a substantial amount of work has emerged from this thesis and hopefully it will generate a lot of follow-up studies.

The genetic dissection of the metabolic syndrome is a tremendous challenge. The present thesis shed some lights on different aspects of the genetics of the metabolic syndrome, but above all underscores the difficulty of the task. Without new development in finding genes involved in complex human diseases, a long adventure is anticipated before reaching the finish line. However, the recent years have witnessed the development of novel methods and strategies for the genetic dissection of complex human diseases. These emerging new methods and ideas are clearly welcomed to tackle the challenge and fulfill the promise hold by the field of genetics, that is better understanding the pathogenesis of complex diseases and consequently improve prevention strategies, diagnostic tools and therapies.

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