Genomic copy number variation and its potential role in lipoprotein and metabolic phenotypes

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Purpose of review

This review examines the role of copy number variation in the human genome as a newly recognized determinant of lipoprotein and metabolic phenotypes.

Recent findings

Much of the recent progress defining the molecular basis of lipoprotein disorders has been the result of studying genomic DNA at the single nucleotide level, for instance with nucleotide sequence analysis or genotyping to detect single nucleotide polymorphisms. Focus on single nucleotides, however, fails to capture the complete spectrum of potential genetic variability. Recent genome-wide mapping studies have demonstrated the surprising ubiquity of large-scale copy number variations in apparently healthy people, adding to the complexity of the 'normal' genome, but also emphasizing this form of genetic variation as a potential disease mechanism. The application of this understanding to the genetics of lipoprotein disorders has been rapid. For instance, the use of novel techniques to detect copy number variations, such as multiplex ligation-dependent probe amplification, has revealed many additional causative mutations in the low-density lipoprotein receptor gene in patients with familial hypercholesterolemia.

Summary

Copy number variations thus represent a new level of genomic variation that is both an important mechanism of monogenic lipoprotein disorders and a potential contributor to common complex lipoprotein and metabolic phenotypes in the general population.

Keywords

atherosclerosis, DNA analysis, genetics, molecular diagnosis, monogenic disorders

Curr Opin Lipidol 18:174–180. © 2007 Lippincott Williams & Wilkins.

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Current Opinion in Lipidology 2007, 18:174–180

Abbreviations

CNV	copy number variation
EBESA	exon-by-exon sequence analysis
HeFH	heterozygous familial hypercholesterolemia
LDLR	low-density lipoprotein receptor
MLPA	multiplex ligation-dependent probe amplification
PCR	polymerase chain reaction
PCSK9	proprotein convertase subtilisin/kexin-type 9
SNP	single nucleotide polymorphism

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Introduction

Genetic variation exerts a sizeable, complex influence on numerous lipoprotein and metabolic phenotypes. For example, heritability estimates of lipid parameters are up to 60% for total and HDL cholesterol, and up to 80% for total triglycerides [1]. Genome-wide scans have failed to identify loci accounting for a large fraction of the variation, suggesting the involvement of many genes as determinants of these traits. In fact, present evidence suggests that hundreds of genes together with environmental factors are involved in susceptibility to cardiovascular disease and its risk factors [1]. The quest to identify genetic components began in the 1980s with evaluation of large-scale genomic DNA rearrangements, followed in the 1990s by sequence analysis of genomic DNA at the level of individual nucleotides. The recent recognition of large-scale copy number variations (CNVs) as a ubiquitous source of genomic variation in the general population [2••], however, has rekindled interest in this form of genomic variation as a contributor to the genetic landscape of monogenic disorders and complex phenotypes.

Technology drives discovery of specific mutation types

Technology is an important determinant of the mutation type detected. For instance, before the invention of the polymerase chain reaction (PCR) in the mid-1980s, the most prevalent type of human genomic DNA mutation was large-scale genomic DNA rearrangement, such as large insertions and deletions, as detected by Southern blotting. Southern blots were ideal for detecting large genomic DNA rearrangements involving over 200 nt of target genomic DNA. Examples of mutations detected by Southern blotting include those discovered in the low-density lipoprotein receptor (LDLR) [3] gene and lipoprotein lipase (LPL) gene [4], associated with familial

hypercholesterolemia and primary LPL deficiency, respectively. In fact, from a state of the art review on familial hypercholesterolemia from that era, over 50% of reported *LDLR* gene mutations were of the insertion/deletion variety [5].

Detection of small mutations, however, was beyond the Southern blots' resolving capacity, unless a mutation altered a recognition site for a restriction endonuclease. The invention of PCR combined with automated DNA sequencing enabled the quick detection of small mutations at the single nucleotide level using relatively few resources, with less expense, effort and radiation requirements than Southern blotting. Thus, the overwhelming majority of mutations in recent databases are of the single nucleotide variety discovered by PCR-based exon-by-exon sequence analysis (EBESA), such as the numerous *LDLR* single nucleotide mutations identified in familial hypercholesterolemia subjects [6]. Furthermore, genes encoding several apolipoproteins, lipoprotein receptors, and key enzymes in the lipoprotein metabolism pathways have been discovered to harbour common single nucleotide polymorphisms (SNPs) associated with inter-individual variation in plasma concentrations of lipoproteins and lipids. For example, HDL cholesterol levels have been associated with genetic variation in ATP-binding cassette, sub-family A, member 1 (ABCA1) [7,8] and also in hepatic lipase [9]. Similarly, depressed LDL cholesterol concentrations have been associated with genetic variation in proprotein convertase subtilisin/kexin-type 9 (PCSK9) [10•]. SNPs contribute to disease causation and susceptibility through a variety of mechanisms (Fig. 1).

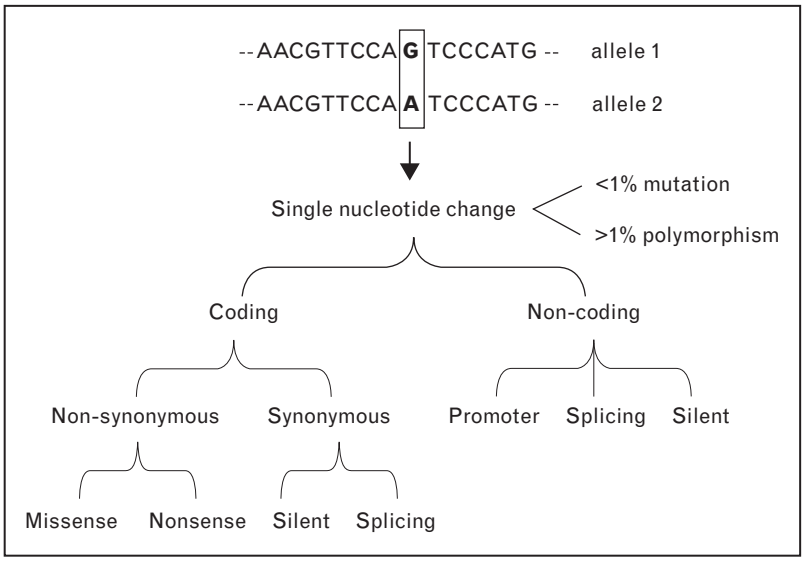
PCR-based methods of interrogating genomic DNA are practically limited to individual nucleotides within a region of 200–1000 bp. Thus, a large deletion that affects one allele in a heterozygote may never be detected by PCR and sequence analysis, since there is no target sequence upon which amplification primers can anneal. Only the normal allele is amplified, creating effective hemizygosity for that region of genomic DNA. Search for a DNA sequence change will yield a false negative result. Multiplex ligation-dependent probe amplification (MLPA) is one of several new analytical methods [11] to detect CNVs – specifically medium or larger genomic DNA deletions or insertions involving whole exons – that would otherwise be overlooked by EBESA. As a semi-quantitative method, internal standards and controls create an additional burden for each MLPA analysis, but this is circumvented by the high throughput afforded by rapid, automated fragment detection.

Copy number variations: from perceived rarity to common form of genomic variation

In contrast to SNPs, which have been considered to be the predominant form of genomic DNA variation in quantitative terms [12], large chromosomal alterations in the germ line have always been thought to be relatively rare. Recent work, however, has shown that submicroscopic large-scale chromosomal alterations, collectively referred to as CNVs, are more ubiquitous and common than was previously appreciated [13••,14]. Most recently, the construction of the first genome-wide CNV map has clearly revealed the dramatic, encompassing nature of CNVs. Based on subjects from the HapMap collection, the first-generation CNV map identified close to 1500

Figure 1 Single nucleotide genomic changes

The 'swap' of a single nucleotide with another, such as the replacement of the wild-type guanine (allele 1) with adenine (allele 2), is referred to as either a mutation, if present in under 1% of the general population, or as a single nucleotide polymorphism (SNP), if present at a frequency over 1%. SNPs are common and span the human genome. Most single nucleotide changes are found outside of coding regions (noncoding) and have no impact on the biological function of a protein (silent), though they may impact gene expression or splicing. Variants found within the coding region, however, may code for functional changes in amino acid structure (missense) or predict premature protein truncation (nonsense), thus having a possible direct association with disease.



discrete copy number variable regions, covering 12% (approximately 360 Mb) of the human genome [2••]. The completion of this project signals a new chapter in human genomic research.

CNVs include variants involving 1 kb or more of genomic DNA [13••,14] such as structural changes that are qualitatively analogous to those that previously were resolved cytogenetically, including duplications, deletions and translocations; and nonquantitative variants that primarily affect qualitative genomic attributes of large chromosomal segments, including inversions with no net change in copy number. While CNVs have long been appreciated to exist within abnormal somatic tissues such as tumours, CNVs occurring within the germ line have been less well studied and considered to be rare. With the recent findings, however, it is now relatively common to observe that one of a homologous pair of chromosomes can be up to a million nucleotides and up to 20 genes shorter than the other. While this understanding is nascent, the biological and medical implications of these dramatic variations could affect our conventional concepts of 'common', 'normal', 'healthy' and 'diseased'.

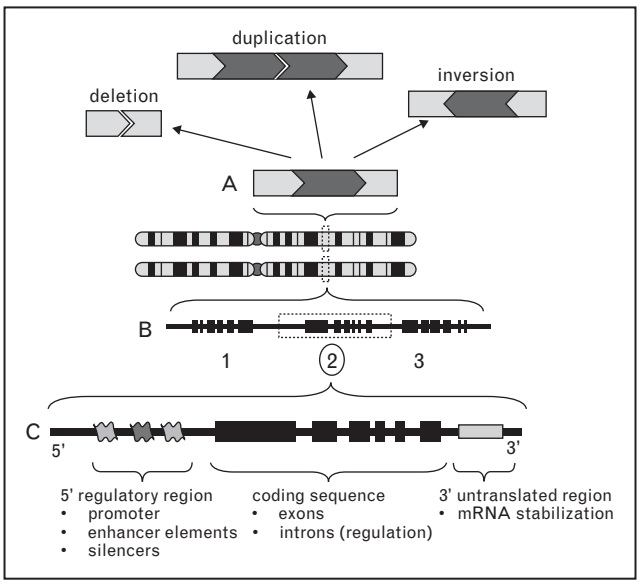
Copy number variations and their potential to explain genetic susceptibility to common diseases

The size and ubiquity of CNVs suggests a potential role for susceptibility to common complex, polygenic diseases such as cardiovascular disease, if only for the simplistic reason that certain CNVs span regions containing many genes. While promising as a hypothesis for human genetic research, the ultimate proof of the involvement of CNVs in common disease phenotypes will require large-scale studies comprising well phenotyped cohorts and comprehensive, robust methods to classify individuals according to their CNV status.

Quantitative mechanisms for copy number variations in disease

The potential etiologic mechanisms for CNV in disease have both a quantitative and a qualitative dimension (Fig. 2). CNVs can result in phenotypic diversity by altering transcriptional, and presumably translational, levels of genes and their products. For instance, haploinsufficiency occurs when one copy of a dosage-sensitive gene has been deleted, producing deficient function that cannot be rescued. Haploinsufficiency also applies to genomic deletions that do not result in a monogenic disease, being instead present in healthy and apparently normal individuals, but requiring additional genetic or environmental factors to also be present for the abnormal phenotype to be expressed later in life. This has already been demonstrated for certain CNV genes both at the transcriptional [15,16] and translational [17] levels. Also, gene dosage increases can be pathological in humans [18]. Correlation of CNV with mRNA and protein levels and phenotypic effects might further be modulated by tissue-specificity or developmental factors.

Figure 2 Large-scale genomic DNA copy number variation



The top of the figure shows some of the types of copy number variations (CNVs), while the bottom of the figure shows some of the consequences of CNVs at the level of the gene. Segment A represents the normal structure of a region of one of a homologous pair of chromosomes. The type of genomic alterations of the normal structure that lead to deletion, duplication and inversion are shown at the top. Segment B represents the normal structure of a chromosomal locus that contains a cluster of three genes (1, 2 and 3). Segment C shows the detailed normal structure of gene 2, including key functional elements, such as the 5′ regulatory region, with promoter, enhancer and silencer elements, the 3′ untranslated regions that can regulate message stability, and also the intron–exon structure of the gene. Any of these structural and functional elements can become involved in a CNV, with a range of functional consequences depending on the size of the variant and the affected functional domains.

Qualitative aspects of disease-causing copy number variations

The mechanisms by which CNVs produce disease also depend on the nature of the genomic region affected [13••,14] and include the following: involvement of several genes at a gene cluster, with the potential for multiple effects on intermediate pathways and possibly phenotypes; involvement of a single gene with increased gene dosage or allelic loss leading to increased or decreased gene expression, respectively; involvement of a single gene in the presence of a risk allele, with loss of wild-type allele leading to hemizygosity for risk allele, while gain of a wild-type allele perhaps decreasing risk; and various consequences from involvement of noncoding regulatory regions, for example loss of a cis-enhancer element or binding site leading to decreased gene expression, loss of a silencer binding site leading to

increased gene expression, or loss of 3′-untranslated sequence leading to decreased mRNA stability.

Potential clinical implications of copy number variations

The chance of finding normal genomic variants in screened samples is now markedly increased, given the sensitivity of the new methods and the ubiquity of CNVs over 1 kb in the human genome. We have already shown that testing for CNVs expands the range of mutations in familial hypercholesterolemia [19••]. In a clinical research setting, how will the management and counseling of a patient and his or her family unfold with, and without, taking CNVs into account? Will ethical issues arising from analysis of CNVs simply mirror past issues encountered when using cytogenetic methods? Could past medical genetic diagnosis be revised in light of new knowledge afforded by CNVs? Should archived specimens be re-evaluated in light of the CNV map? These questions and others [20] will require attention over the coming months and years.

Application of copy number variation detection methodology in familial hypercholesterolemia

Heterozygous familial hypercholesterolemia (HeFH; MIM 143890), an autosomal dominant disease that affects approximately 1 : 500 people, is characterized by markedly elevated plasma concentrations of LDL cholesterol, typically above the 95th percentile for age and sex [21]. Without timely diagnosis and intervention, HeFH patients have an increased risk of fatal coronary heart disease that is up to 100-fold higher than the general population [22]. HeFH most commonly results from a mutation in the LDL receptor gene (*LDLR*, MIM 606945), which is located on chromosome 19p13 and comprises 18 exons [21]. The LDL receptor is a cell-surface glycoprotein that binds the apolipoprotein B moiety on the LDL particle, initiating receptor-mediated endocytosis [21]. The catabolic defect in HeFH patients results in a doubling of plasma LDL cholesterol concentration. At the molecular level, HeFH is now commonly diagnosed using EBESA of *LDLR* from genomic DNA [6,19••].

Molecular heterogeneity of heterozygous familial hypercholesterolemia

Genetic studies over the past 20 years, primarily using EBESA discovery platforms, have shown that various *LDLR* mutations, of a total of approximately 800, are found in most HeFH patients [23]. This method, however, seems to find mutations in only a portion of clinically diagnosed familial hypercholesterolemia patents [6,19••]. Part of the gap in detecting mutations in HeFH is related to its genetic heterogeneity [24]. For instance, a phenocopy of HeFH is called HCHOLAD2 (MIM 144010), which results from a missense mutation in *APOB* affecting the LDL receptor-binding domain of apolipoprotein B-100 (MIM 107730) [24], and accounts for 5–10% of the HeFH phenotype. A rare HeFH subtype called HCHOLAD3 (MIM 603776) results from gain-of-function mutations in *PCSK9* (MIM 607786), which encodes a protease that degrades the LDL receptor intracellularly [24]. A similarly rare familial hypercholesterolemia phenotype with autosomal recessive inheritance is called HCHOLAR1 (MIM 603813), which results from mutations in *ARH* (MIM 605747) encoding a putative adaptor for the LDL receptor [24]. Even after accounting for genetic heterogeneity, however, many clinically diagnosed familial hypercholesterolemia patients have no *LDLR* mutation with EBESA.

EBESA mutations in Ontario heterozygous familial hypercholesterolemia patients

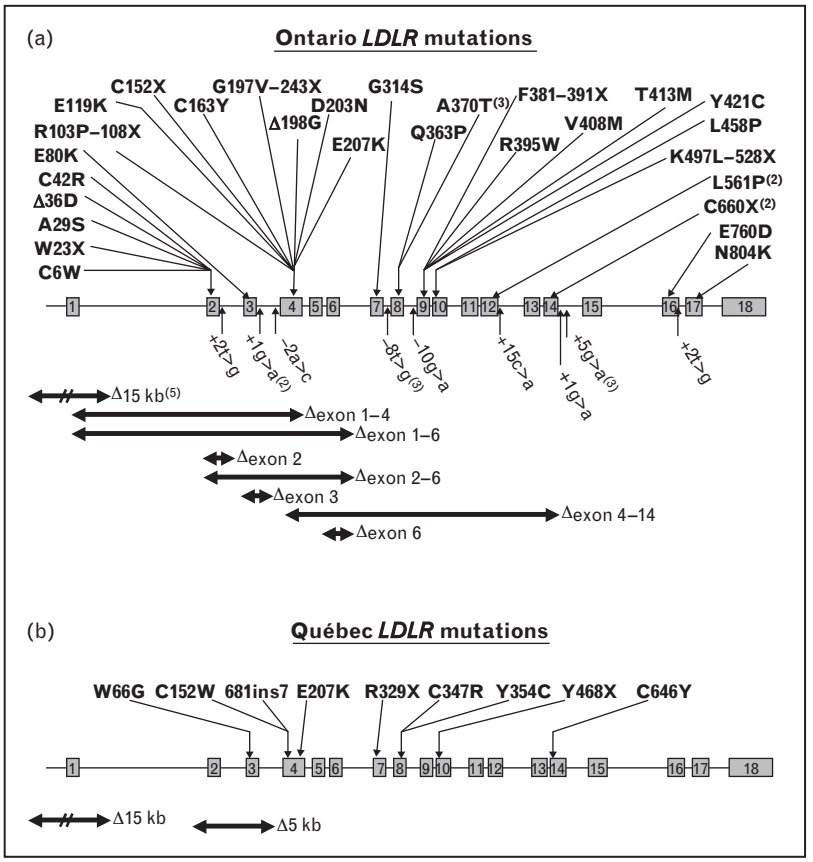
In 80 HeFH patients from Ontario, Canada, we used EBESA of *LDLR* and showed that only approximately 50% of patients had mutations at the single base level, including single nucleotide mutations that caused single amino acid changes, premature termination codons and RNA splice site changes. In addition, a few small insertions and deletions were found. The spectrum of mutations in Ontario HeFH patients found by EBESA is shown above the genomic map in the top half of Fig. 3. Sequencing of *APOB* showed that an additional approximately 7% of HeFH patients were heterozygous for the R3500Q receptor-binding domain mutation, which is consistent with other study samples [22]. No mutations were found in either *PCSK9* or *ARH*. Thus, about one-third of Ontario HeFH patients had no mutation in any gene detectable with the EBESA genomic DNA screening platform.

Heterogeneity of genomic mutation types in heterozygous familial hypercholesterolemia

Another possible explanation for the gap in familial hypercholesterolemia molecular diagnosis is inadequate sensitivity of EBESA to detect certain mutation types, such as CNVs. For instance, a large genomic DNA deletion that removes a region of one chromosome that spans several *LDLR* exons would effectively create hemizygosity for the normal-sequence single exons present on the unaffected allele. Since only one allele from an affected individual would be amplified and sequenced, the resulting EBESA profile is indistinguishable from homozygosity for normal *LDLR* alleles.

We applied the MLPA method in the approximately 35% of HeFH patients who did not have mutations detected by EBESA. We found that about two-thirds of HeFH patients with no *LDLR* mutation detected by EBESA had

Figure 3 Spectrum of *LDLR* mutations in Canadian subjects



Linear map showing the genomic structure of the *LDLR* gene in the centre of each panel. Filled numbered boxes represent exons. Arrows point to the mutation position. Above the map are single nucleotide and small insertion–deletion (indel) mutations. Single letter amino acid codes are shown, along with mutated codon number. Splicing mutations are indicated directly under the linear map, on the diagonal. The position relative to the intron–exon boundary is shown numerically and the mutated nucleotide is shown in lower case. Larger deletions are shown as thick bidirectional arrows further below the linear map, with the span of the deleted region indicated by text and delimited by the arrow heads. (a) Ontario *LDLR* mutations. In the 80 Ontario heterozygous familial hypercholesterolemia (HeFH) patients studied, 58 had mutations in the *LDLR* gene, five had mutations in the *APOB* gene and among the 58 Ontario HeFH patients with *LDLR* mutations, 40 had single nucleotide mutations, and 18 had indel mutations. There were 45 unique *LDLR* gene mutations in total, with no predominant recurring mutation. When mutations occurred in more than one unrelated proband, the number of recurrences is indicated within superscripted parentheses. Among the 31 single nucleotide mutations 19 were missense mutations [C6W, A29S, C42R, E80K, E119K, C163Y, D203N, E207K, G314S, Q363P, A370T (three probands), R395W, V408M, T413M, Y421C, L458P, L561P (two probands), E760D and N804K]; three were nonsense mutations [W23X, C152X and C660X (two probands)]; and nine were splicing mutations [intron 2 +2t>g, intron 3 +1g>a (two probands) and −2a>c, intron 7 −8t>g (three probands), intron 8 −10g>a, intron 12 +15c>a, intron 14 +1g>a and +5g>a (three probands), and intron 16 +2t>g]. Among the 14 unique indel mutations two were inframe deletions detected by nucleotide sequence analysis (Δ36D and Δ198G); four were small frameshift deletions causing premature truncations (R103P-108X; G197V-243X; F381-391X and K497L-528X); and eight were larger deletions of approximately 300–15 000 nucleotide base pairs detected only by multiplex ligation-dependent probe amplification (MLPA) analysis, with the span of the deleted region shown by bidirectional arrows [Δ15 kb including exon 1 (five times; also very common in Quebec HeFH, see lower panel), Δexon1-4, Δexon1-6, Δexon2, Δexon2-6, Δexon3, Δexon4-14 and Δexon 6]. Each mutation was clearly heterozygous by DNA sequencing or MLPA; and absent from the genomes of 100 normolipidemic subjects. (b) Quebec *LDLR* mutations. Reports of large clinical cohorts consistently showed that over 90% of HeFH patients in Quebec have one of 11 *LDLR* mutations, most of which are highly recurrent in the population. Among the eight Quebec mutations involving single nucleotides six were missense mutations [W66G, C152W, E207K (also found in Ontario), C347R, Y354C and C646Y]; and two were nonsense mutations (R329X and Y468X). Among the three indel mutations one was a small frameshift deletion with premature truncation (681ins7); and two were larger deletions [Δ15 kb including exon 1 (also found in Ontario patients) and Δ5 kb]. Reprinted with permission [22]. © 2006 Canadian Medical Association.

an abnormal *LDLR* MLPA pattern [19••]. We then showed using genomic DNA sequence analysis of the deletion break point that the abnormal MLPA pattern in five patients corresponded to a specific deletion, namely the French Canadian *LDLR* Δ15 kb, involving the 5′-upstream translated region of *LDLR*. While *LDLR* missense mutations are most common in HeFH, MLPA abnormalities, mainly deletions, appear also to be very prevalent in Ontario [19••]. The spectrum of mutations in Ontario and Quebec HeFH patients found by MLPA is shown in Fig. 3 below the genomic maps of the *LDLR* gene; the results suggested that MLPA might help to diagnose HeFH by detecting *LDLR* CNVs. More recently, abnormal *LDLR* MLPA patterns have also been

reported for familial hypercholesterolemia patients from Norway [25••], which similarly indicated that CNVs were present in a large proportion of affected individuals, and Denmark [26••] in which CNVs represented a very minor component of the total number of *LDLR* mutations. Thus, it seems likely that molecular diagnostic strategies for HeFH will vary between regions. In some areas such as Quebec, it will be possible to diagnose most individuals using just a few mutational detection methods, while a broader-based mutational detection strategy will be required to molecularly diagnose HeFH patients from Ontario.

Biochemical attributes according to molecular diagnosis

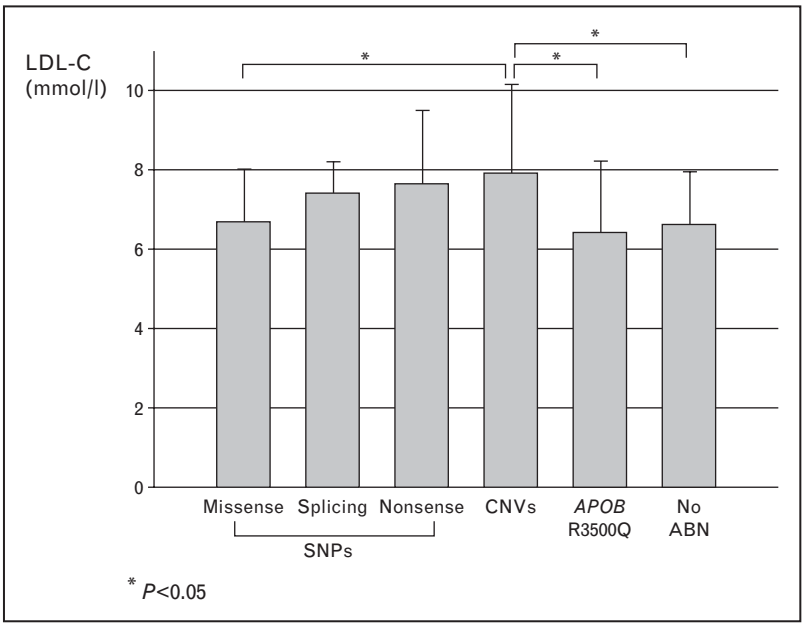
To evaluate genotype–phenotype correlations, we stratified patients into molecular sub-groups: heterozygotes for missense mutations; heterozygotes for splicing mutations; heterozygotes for either nonsense mutations or in-frame deletions; abnormal MLPA pattern; heterozygotes for *APOB* R3500Q and no *LDLR* mutation and normal *APOB* receptor binding domain sequence (Fig. 4). Patients with abnormal *LDLR* MLPA patterns had significantly higher mean untreated plasma LDL cholesterol than the other subgroups. Patients who had no mutation after analysis had a similar mean untreated plasma LDL cholesterol to patients with missense mutations and patients with *APOB* R3500Q (Fig. 4). This is an example of an apparently systematic genotype–phenotype correlation and indicates that heterozygous *LDLR* CNVs are associated with a more severe biochemical phenotype than the other types of mutations.

Conclusion

The ability to screen for pathological CNVs has markedly increased the number of *LDLR* mutations detected in HeFH subjects. The findings indicate that the molecular diagnosis of a relatively common genetic disease such as HeFH will be complicated by molecular heterogeneity in some jurisdictions. Furthermore, the CNV map in healthy 'normal' individuals adds a new dimension to the study of the human genome with immediate implications for biology and perhaps longer term implications for medicine. Future genomic mapping experiments and genome-wide association analyses – and their respective detection technologies – will need to account for the presence of CNVs, as it is likely that CNVs will be shown to be a similarly important mechanism of mutation. Current platforms to study the genome may need to be redesigned either to maximize detection of CNVs or minimize their interference with detecting other forms of genomic variation. As the 'personal genome' moves closer to becoming a reality, it will be important to account for CNVs along with other types of mutations and variants, and then to interpret their biological meaning for any individual. Finally, the new CNV discoveries should be placed within the wider context of contemporary discourse on genomic variation studies and their biological, health and societal implications. This could wait, however, until more extensive characterization is completed in order to fully understand

Figure 4 Genotype-phenotype correlation of *LDLR* mutation in Ontario familial hypercholesterolemia subjects

The figure shows mean ± standard deviation (SD) of plasma LDL cholesterol (LDL-C) in Ontario heterozygous familial hypercholesterolemia (HeFH) subjects classified according to genotype, including most of the mutations shown in Fig. 3. The lowest mean LDL cholesterol was seen in subjects with heterozygous *LDLR* missense mutations, but also the *APOB* R3500Q mutation and individuals with HeFH but no genomic DNA mutation detected in any candidate gene screened using any method (No ABN). HeFH individuals with *LDLR* splicing mutations and nonsense mutations had sequentially higher mean plasma LDL cholesterol concentrations, while subjects with CNVs involving the *LDLR* locus had the highest mean LDL cholesterol concentrations. SNP, single nucleotide polymorphism.



the implications and potential applications of human genomic CNVs.

Acknowledgements

Supported by the Jacob J. Wolfe Chair in Functional Genomics, the Edith Schulich Vinet Canada Research Chair (Tier I) in Human Genetics, a Career Investigator award from the Heart and Stroke Foundation of Ontario, and operating grants from the Canadian Institutes for Health Research, the Heart and Stroke Foundation of Ontario and Genome Canada through the Ontario Genomics Institute.

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- • of special interest
- •• of outstanding interest

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