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Invited critical review

Cardiovascular diseases and genome-wide association studies

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ABSTRACT

Genome-Wide Association Studies (GWAS) on cardiovascular diseases and related quantitative traits revealed numerous genetic variants, which however have been partially replicated, probably due to the heterogeneity of the clinical phenotypes and the populations studied. Even if novel biological pathways have been identified through these studies, there is still a long way until the validation of causal variants and their use in clinical practice as factors for prevention, risk assessment and as targets for the development of new medications. GWAS methodologies should, in the following years, integrate gene–gene and gene–environment interaction analyses in a global research strategy and also involve subsequent transcriptomic and proteomic investigations. The GWAS era is very promising but it is just at the beginning.

Contents

1. Introduction: cardiovascular diseases: common disorders with important genetic component

Cardiovascular diseases (CVDs) are the leading cause of death in the world. Based on World Health Organization, 17.1 million people died from CVDs in 2004 and it is estimated that by 2030, approximately 23.6 million deaths will be recorded due to CVDs, mainly from heart disease and stroke [1]. The aetiology of CVDs is multifactorial, where a complex combination of environmental, genetic and clinical risk factors seems to play determinant role [2]. In fact, the pathogenesis of coronary heart disease (CHD) is known to be influenced by smoking, diabetes, hypertension, obesity, physical inactivity, alcohol intake and psychosocial conditions [3–5]. Genetic elements contribute to the development of CAD [6,7]. This complex architecture and its genetic background [8,9] are still poorly understood and substantial discrepancies remain in estimating the heritability of numerous CVDs-related quantitative traits (QTs) [10]. Risk algorithms such as the Framingham Risk Score [11] have traditionally incorporated classical clinical and environmental risk factors such as age, gender, blood lipid concentrations, blood pressure (BP), body mass index, family history and

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smoking habit for primary prevention strategies purposes. The incorporation of genetic risk factors in the risk assessment algorithms can be beneficial for the improvement of preventive public health strategies and could open new landscapes for more effective treatment (personalised medicine) and reduced complications [12]. The identification and validation of genetic variants that predispose to CVDs and related QTs may lead to better understanding of the fundamental pathogenetic pathways and to the establishment of new biological pathways and novel risk factors.

Over the last 2 decades, genetics of chronic diseases and related QTs were assessed by candidate gene approaches [13]. Twins and families studies were used to estimate heritability, while population-based studies were used to identify variants that are associated with a phenotype and to explore interactions between genetic and environmental factors. Candidate genes are those considered to influence complex phenotypes due to their participation in biological pathways or due to their location close to a region of interest [13]. This "hypothesis-driven" research has provided the foundation for the development of genetic CVDs risk profiles [12]. However, since 2007, genome-wide association studies (GWAS) have brought a revolution in the field of genetic background of chronic diseases like CVDs and their associated QTs [14–16]. The recent technological advances are enabling the rapid and accurate assessment of millions of single-nucleotide polymorphisms (SNPs) in large populations, leading to an increased number of GWAS for different CVDs phenotypes and related traits. These studies resulted in the identification of hundreds of genetic factors, some robustly replicated, however with small individual effects [17,18]. Moreover, GWAS revealed previously unsuspected pathological pathways [8,19]. Nevertheless, the identification of causal variants explaining the high heritability of the different CVDs phenotypes, which could be used for prevention, diagnosis, treatment and prognosis of CVD patients, has not yet been achieved with satisfactory results through the GWAS [20].

In this work we review GWAS findings concerning CVDs and their related QTs, the reasons for missing heritability and future challenges.

2. Methods

2.1. Literature search

The GWAS investigator of HuGENavigator engine [21] and the NHGRI Catalog of published GWAS (http://www.genome.gov/gwasstudies, assessed May 2011) [22] were used in order to assess GWAS published between 2007 and 2011, using the keywords of "cardiovascular diseases", "coronary heart disease", "coronary artery disease", "diabetes", "obesity", "hypertension", "blood pressure", "anthropometric measurements", "response to medication" and "lipids". In order to confirm the retrieved findings, we have checked the related articles and their reference lists in the MEDLINE engine using the same previous keywords along with 'GWAS' or 'single nucleotide polymorphism (SNPs)'. In addition, we restricted the search results to articles published in English language and conducted on humans.

2.2. Data extraction

Two authors independently extracted the following information from each study: CVDs traits, variants and their near gene(s), genomic region, corresponding trait(s), reference(s), P-values, number of subjects in each study (discovery and replication), the variant risk allele and the effect size. Genome-wide significance was considered when P-value $<5\times10^{-8}$.

3. Brief overview of GWAS on cardiovascular diseases and their related quantitative traits

To date, a vast number of GWAS focusing on about 150 distinct diseases and QTs have reported several hundreds significant SNP-trait associations. GWAS have been conducted on cardiovascular events (CHD) and specifically to myocardial infraction (MI) and they have reported the association of at least 16 genetic variants found in 15 genes (Supplementary Table 1). They have identified SNPs on chromosomal regions 1p13, 1q41, 2q36, 3q22, 6p24, 6q25, 7q22, 9p21, 10q11, 10q23.2, 10p11.23, 11q22.3, 12q24, 15q25 and 15q22 that are associated with risk of CHD or its main complication, MI [23–28]. The associations between SNPs on 9p21 and CHD or MI were the most strongly replicated findings in the majority of the assessed ethnic groups [29–31].

However, caution is needed when interpreting data found in GWAS for CVDs, given the weak sample size of the majority of these studies. The genetic variants are rarely robustly linked to the cardiovascular events. Similarly, the lack of suggestive associations stressed out the value of using accurate clinical subtype classification for the phenotypes and standardised methodology for multi-centre studies.

GWAS of CVDs-related QTs could provide an alternative approach for better comprehending the phenotypic diversity of a given CVD as stated by the Wellcome Trust Case Control Consortium [32].

A significant proportion of GWAS has been focused on obesity. The identified genetic variants, including some paediatric studies, have been replicated in less than 2/3 of them. Interestingly, the *FTO* gene was identified as the first locus harbouring common variants with an unequivocal impact on obesity predisposition, diabetes and fat mass in population studies. The most important reports are summarised in Supplementary Table 1.

More than 1/4 of all GWAS for CVDs related QTs have studied fasting plasma glucose and diabetes (Supplementary Table 1). Up to 90% of them have been replicated in European, American and Asian populations. In two distinct studies, Zeggini et al. [33,34] reported the association of type 2 diabetes with 22 SNPs close to the following genes: *CDKN2A, CDKN2B, FTO, HHEX, IGF2BP2, KCNJ11, CDKAL1, TCF7L2, THADA, TSPAN8, LGR5, VEGF, NOTCH2, ADAM30, PPARG, SLC30A8, JAZF1, SYN2, CDC, ADAMTS9, CDC123, CAMK1D*. Of these, 2 SNPS in the vicinity of *TCF7L2* had the greatest association with type 2 diabetes (Supplementary Table 1).

Also, lipid profile and BP studies represent a considerable part of CVD GWAS. About 2/3 of SNPs associated with lipid levels have been successfully replicated, notably 3 in the vicinity of *CETP* (Supplementary Table 1). It is important to mention that, although rs3764261 in *CETP* was strongly associated with total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) (P-value $=7\times10^{-380}$) [35], it was not associated with CHD. Interestingly, a number of GWAS have achieved to find some loci which were associated with both lipid levels and CHD risk. For example, there are some replicated SNPs in *CETP, APOC1, APOB, APOA1* genes that are associated with more than one related trait in CVD risk (Supplementary Table 1).

It should be noted also that the meta-analyses on hyperlipidaemic and diabetic individuals [25,35,36], have included some common subsamples. Therefore, similar association results will not be surprising [37].

In addition to lipid profiles, replication studies have been relatively successful in confirming BP and hypertension (HTN) key loci [38–40]. In the largest joint meta-analysis published in May 2009, by CHARGE [41] and GlobakBPgen [8] consortia, researchers discovered 14 loci associated with systolic BP, diastolic BP and/or HTN. Recently, Takeuchi et al. [38] succeeded in replicating 7 loci in Japanese subjects, whereas Liu et al. [39] and Hong et al. [40] have confirmed some of them in Chinese Hans and Koreans respectively. These main

findings constitute a milestone in bringing genome knowledge into BP regulation in diverse populations, as it will be discussed below [42].

In 2009, a GWAS assessed the possible associations between several hundred thousand SNPs and warfarin dose in about 1000 Swedish patients. The results showed 3 significant SNPs around *CYP2C9* and *VKORC1*. After removing the effects of those SNPs through multiple regression adjustment, an additional signal was observed, implicating another SNP around the cytochrome P450 gene (*CYP4F2*) [43]. Similar results were presented in Japanese population [44]. These findings raise the probability that testing patients for variations in the two mentioned genes might provide information that could improve clinical algorithms currently used to guide the administration of warfarin.

Also, the role of genetic factors in differences of lipid-lowering response to statin treatment seems to be influenced by several loci with small individual contributions that are compounded when simultaneously inherited. Although several loci have been investigated in relationship with efficacy of statin using the candidate gene approach, the combined contribution of these genotypes explains a relatively small proportion of the variation in statin lipid-lowering efficacy [45– 47]. GWA analysis of lipid-lowering response to statin treatment has previously failed to identify novel loci. In order to find eventual genetic variants, Barber MJ et al. [48] have done a combined analysis on 3 statin GWA studies. They have eventually found that the most provocation is the association of rs8014194 in *CLMN* gene with changes in total cholesterol in response to statin treatment. They have observed strongest association of this SNP in one of three populations. However, this genetic variation was less strongly associated with LDL-C reduction.

4. GWAS strengths and limitations

4.1. Current GWAS designs

GWAS involve various tiered designs including case–control and cohort studies for the identification of trait/disease-SNP associations. These strategies allow affordable procedures to be carried out by conducting a genome-wide scan in a discovery set in order to select a core of significantly associated SNPs that will be subsequently genotyped in larger replication sets for confirmation [49]. These intricate designs involve more and more tiers according to the complexity and originality of the trait of interest [50] and allow the elimination of previous spurious associations (false positives) [18]. Traditionally, genetic associations having causal functionality and appearing in multiple populations and studies are considered reliable [18,49].

Testing the association of millions of SNPs with a specific trait or disease implies a large number of statistical tests (at least one per SNP) making nominal significance (0.05) inappropriate for originally selecting associated variants. Such designs also require large populations [51] and this need led to the recruitment of large international consortia, in which substantial pooled GWAS have been conducted. Indeed, significant progress in the management of the methodological weaknesses of GWAS has been made under the auspices of large consortia providing numerous independent populations for pooling analyses and replications in specific fields, such as the Glucose and Insulin Related Traits Consortium (MAGIC) [52]. These pooling strategies required controls for differences in allele frequencies [13] to minimise false positive findings and reinforce the validity of genetic variants found to be associated with specific traits or pathologies.

Only 12% of SNPs discovered by all the GWAS performed to date and associated with traits are located in the vicinity of protein-coding regions of genes, although SNPs in protein-coding regions are heavily over-represented on genotyping arrays [22]. Forty percent falls in intergenic regions, and another 40% is located in introns. The repeated replication of signals falling consecutively in the so-called “gene deserts”, although it initially raised concern in the scientific community, has sharpened the focus on the potential roles of intronic and particularly intergenic regions in regulating gene expression [14].

4.2. Future challenges

Over the past few years, GWAS have reported a large number of novel genetic variants associated with CVDs [53,54]; nevertheless, many challenges remain.

There is a need to perform GWAS on populations with diverse geographic ancestries, which have undergone more mutations and greater recombination events. This type of studies could give greater degrees of genetic variation and shorter stretches of linkage disequilibrium allowing better localisation of genome-wide association signals [20,32]. Several different ethnicities such as Japanese [38], African Americans [55] and mainly Europeans [8,41] have been examined. European populations are closely related [42], and their genome has undergone less recombination events than for example those of Africans [56], an advantage that has increased the number of studies concerning this specific descendent. Will the initial findings be replicated in non-European populations? Recent GWAS do not support this probability. The researchers believe that different populations have each one a unique population structure (different modifier genes and different gene-gene and gene-environmental interactions), different lifestyles and different environmental factors, which may expose or mask the risk of a SNP, for example rs6903956, in CAD [28]. Moreover, based on GWAS report in 2009 [57] there is a significant association between rs1252453 in *PHACTR1* on chromosome 6p24 and early onset MI in some European and American populations. However, according to HapMap data, not only the minor allele frequency of rs1252453 in Chinese population is 0%, but also none of the 113 SNPs in or near *PHACTR1* was associated with CAD in Chinese population [28]. Therefore, the public health policies and strategies, if they will include genetic factors in preventive medicine, should depend on ethnic group and may not be applicable worldwide [58].

Another interesting question is why numerous worldwide replication studies confirmed loci but not SNPs. Genetic variants having high occurrence in Europeans may have different frequencies across different populations. Also, many SNPs are rare in non-Europeans [59]. Moreover, allelic frequencies may be different even if the same SNP is found in diverse populations [60].

In this context, GWAS involving African populations are potentially of interest. Low linkage disequilibrium between genetic variants is one of their main characteristics, due in part to a long history of recombination and mutations. When SNPs extend over short genomic regions, further sequencing may be very effective in defining the true risk variant [42].

What will be the application of these findings in clinical practice and for predicting CVDs risk?

Based on the success of GWAS, some commercial companies are already offering to the public i*n vitro* diagnostic genotyping assay to assess for example the CHD risk, although the risk attributable to any individual variant has been modest to date [61]. We believe that commercial assays do not give complete genomic coverage, as certain regions are not covered. Therefore, the influence of variation at these missing regions in determining CHD risks is very likely to have been underestimated, and some novel loci may have been missed completely. Nevertheless, even if valid genetic variants with modest effects explain a substantial population-attributable risk fraction they do not necessarily provide clinically useful prediction for individuals or specific population, groups [62]. However, combining multiple SNPs having modest effect into a global genetic risk score could improve the identification of high-risk populations and improve individual's risk assessment. Anderson et al. [61], have demonstrated the feasibility and the potential utility of simultaneously considering the joint effects of 5 different SNPs (rs599839 in *CELSR2*, rs2383206 in

9p21.3, rs289715 in *CETP*, rs78739461 in *ApoF* and rs1799963 in *F2)* retrieved from candidate gene studies and GWAS in not only improving the net risk classification of intermediate-risk individuals but in also predicting the risk of premature CHD [61].

Similarly to Anderson et al. [61], Kathiresan et al. [63] integrated several SNPs highly associated in GWAS with LDL-cholesterol (rs7575840 in ApoB, rs4420638 in APOE cluster, rs12654264 in HMGCR, and rs1529729 in LDLR) and HDL-cholesterol levels (rs3890182 in *ABCA1*) into a cardiovascular risk score [63,64]. However, in the study of Kathiresan et al. [63] the clinical risk prediction was not improved by genotype scores, nevertheless, there was a significant improvement in risk classification.

From a pharmacogenomics point of view, more studies taking into account the GWAS results are needed to prouve the clinical usefulness of genotyping to medication.

4.3. Missing heritability in cardiovascular diseases, an Achilles' heel

Although recent GWAS have an enormous sample size reaching up to 200,000 participants and revealing hundreds of associations with extremely impressive P-values, much of the genetic risk remains unexplained, and this represents the so-called 'dark matter' of genetic risk.

A large "hidden heritability" of unknown nature that may be explained by both low minor frequency alleles (MAF) and rare variants exists. However, the role of rare variants in population screening for CVDs has not been illustrated until now. GWAS have predominantly focused on common variants (allelic frequency >5% in the general population) [56] and ignored variants with low MAF (0.5%<MAF<5% in the general population) and rare frequency (MAF<0.5%), which are believed to have a greater risk effect [56]. In contrast to common variants, which are thought to be old in the history of humanity, rare variants are more recent and therefore not geographically extended [42]. Recently, deep sequencing revealed a myriad of rare, deleterious variants [65] and assessing their associations with increased risk will be the first step to better understand their role in CVDs. When focusing on rare genetic variants, GWAS require very large populations and different statistical methods that jointly analyse variants in a locus instead of testing each variant individually. The next generation of high resolution arrays with more genome-wide coverage is making this goal feasible.

Structural large variants (>500 Kb) such as deletions and duplications (Copy number variants, CNV) represent rare variants that may affect gene expression and molecular pathways in humans [9]. These genomic imbalances occur at an allele frequency of <0.05% and are present in about 8% of the total population [9], making them rare but collectively common. Results available suggest that CNVs strongly affect gene expression, thereby affecting mRNA splicing and transcriptional activity [66].

Gene–environment interactions (GxE) are also issues that might blind many GWAS. Integrating GxE could help in highlighting different loci effects according to modifiable risk factors, particularly in CVDs. This strategy needs large consortia, methodological efforts and stratified designs in order to improve the existing tools of GWAS that do not yet incorporate complex statistics. Although, environmental pathways acting through epigenetic mechanisms to modify gene expression [67] and DNA methylation [66] have given some fruitful results in other chronic diseases [68] and exposures [69], they are yet to be studied in CVDs. In addition to the usual challenges reported for genetic association studies, a successful GxE study should take into account sample size, exposure assessment and heterogeneity, described in full elsewhere [67]. GxE in GWAS requires enormous populations [67]. Smith and Day [70] explained that detecting an interaction needed a sample size at least 4 times greater than that required for detecting a main effect of comparable magnitude. Although difficult, studying GxE interactions could help in understanding discrepancies due to heterogeneous exposure.

Gene–gene (epistatic, GxG) interactions may also play an important role in discovering genes that have not yet been found by the consensual single-locus approach. This statement has been extensively reviewed [15,71,72] and both parametric and non-parametric multi-locus methods have been developed to detect such interactions [73] in the last years. Epistatic interactions have been documented for susceptibility to cancer [74], morphology [75] and autoimmune conditions [76]. However, current epistatic designs do not have genome-wide coverage, so that their application to high-dimensional genome-wide data including all imaginable SNPs remains a crucial challenge [77].

Furthermore, we advise that all loci associated with CVDs and related QTs should be replicated in paediatric populations, as a common variant could have a continuous impact throughout life. Epidemiological and pathophysiological evidence suggests that the precursors of CVDs originate in childhood [78,79]. The atherosclerotic process starts in childhood [80] and numerous studies have shown that CVDs risk factors during childhood can affect the risk in adulthood. For example, increased BP levels during childhood strongly predict HTN in adults [81]. Isolating genetic variations that may influence a given risk factor for CVDs at childhood, where many environmental factors such as alcohol intake, stress and smoking are absent, might have major implications for public health and could be a challenge in designing primary preventive strategies.

5. Concluding remarks and perspectives

GWAS have revealed many novel genetic risk variants in CVDs, making it an auspicious era for the better understanding of genetics. For the time being, we need to grasp the unexpected involvement of certain functional and mechanistic pathways identified in CVDs processes involving many QTs. These have to be extensively investigated in parallel with the pathology itself. Future GWAS must also detect low MAF and rare variants, and include GxG, GxE and well-designed candidate gene studies (in term of statistical power, homogeneity and exposure assessment) and involve subsequent transcriptomic and proteomic investigations. It is time to pause and think, instead of digging further down for more genetic loci with even smaller phenotypic effect.

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Disclosures

No conflicts of interest.

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