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The Genomics of Cardiovascular Disorders

Therapeutic Implications

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Abstract

Cardiovascular disease (CVD) is a complicated series of disorders that result from the interaction between genetic predisposing mechanisms and environmental factors. Over the last few years substantial progress has been made in defining the molecular basis of several genetically transmitted non-atherosclerotic CVD such as hypertrophic and dilated cardiomyopathies, long-QT syndrome and essential hypertension. This review represents a summary of the current knowledge about the major gene polymorphisms found to be associated with these CVDs. Moreover, we will discuss how the discovery of disease-associated genes will greatly enhance the ability to formulate advanced diagnoses, to define prophylactic therapeutic strategies to prevent or reduce the progression of the disease and, finally, to proceed to the development of new drugs tailored for the specific cellular or molecular functions altered as consequence of the predisposing genes.

Cardiovascular disease (CVD) is the primary cause of death in developed countries. The prevalence and incidence of cardiovascular disorders have dramatically increased in the last century resulting not only in premature death but also in a huge cost to society. People experiencing debilitating symptoms need extensive care and medication, both of which are extremely costly.

CVD is a complicated series of disorders that can be divided into 2 categories: atherosclerotic and non-atherosclerotic syndromes. These categories include diseases such as atheroma, essential hypertension, myocardial infarction (MI), congestive heart failure (CHF) and cardiac arrhythmias, whose causes are a result of the combination of two factors. The first is a genetic predisposition to developing heart disease and the second is environmental, such as lifestyle. Cardiac inflammatory diseases that are primarily caused by bacterial or viral infections, such as rheumatic fever and viral endocarditis, may constitute a third group. However, a genetic predisposition towards developing heart disease cannot be completely ruled out in this group either. In this review, we will deal with those cardiovascular disorders for which a clear genetic cause or predisposition has already been established. For reasons of space, we will limit the discussion in this review to the non-atherosclerotic syndromes represented by hypertrophic and dilated cardiomyopathies, long-QT syndrome and essential hypertension.

Over the last few years, substantial progress has been made in defining the molecular basis of several genetically transmitted non-atherosclerotic CVD.[1] Genes have been discovered which are associated with CVD, and which may influence the

risk of developing a particular disease or even affect its progress. The discovery of such genes will greatly enhance the chance of diagnosing CVD early, even in those individuals who do not as yet show conventional clinical symptoms. It will also help to formulate prophylactic therapeutic treatments that are designed to prevent or reduce the progression of the disease. In addition, it should be possible to develop new drugs that could be targeted at specific cellular or molecular function or functions that have been altered by the predisposing gene or genes.

1. Strategies Used to Study the Genetics of Cardiovascular Disease (CVD)

When complex multigenic and multifactorial diseases are considered, the most frequently used approach adopted to identify the predisposing or causal genes is that of the candidate gene. This is based on mechanistic hypotheses that are in turn based on physiology. This approach looks at mutations or polymorphisms of those genes encoding for the proteins which are directly involved in the altered organ function. It also verifies through linkage or case-control studies, whether they are associated with the phenotypic expressions of the disease. An alternative approach, less frequently used in the past but now more widely used as consequence of the Human Genome Project,[2] is that of random genome scanning or positional cloning.[3] This identifies quantitative trait loci (QTLs) with a high lod score, that are associated with phenotypic traits of the disease in affected individuals. These QTLs, identified by positional cloning, may be used to detect the loci of interest throughout a very costly and time-consuming approach. To date, no gene involved in polygenic diseases has been detected by this approach and it is doubtful whether it will give meaningful results in the future. Such a study involving this latter approach would also investigate the patient's relatives who may possess unknown or apparently unrelated genes whose polymorphism may account for the genetic defect. The identification of the genes involved in CVD such as hypertrophic and dilated cardiomyopathies followed a candidate gene approach, while the investigation of the genomics of cardiac arrhythmias (long-QT syndrome) and essential hypertension are currently being studied by using both strategies.

Some of the strategies adopted to discover predisposing or causal genes of CVD can also be applied to develop a pharmacogenomic approach to therapy. Indeed, case-control association studies can be useful not only to verify if a given polymorphism has an higher frequency in the affected than in control individuals (and thus may be responsible for the disease), but also may indicate if patients carrying the 'affected-allele' respond better to a therapy able to correct the altered genetic mechanism than patients carrying the 'normal-allele'. Pedigree-based or paired siblings linked analysis are not readily applicable to pharmacogenomics. Moreover, although these approaches are less susceptible to stratification errors than case-control studies, they require a large sample size since the number of genes involved in a cardiovascular disorder is usually high and their effect is small, and this represents a practical obstacle to their systematic application. Finally, population-based epidemiological studies are necessary to try to identify and characterise genetic, social and environmental factors that favour the maintenance of a disease in the population at large. This approach allows us to look at the 'context dependency' of a given polygenic disease, that is, at the synergistic influence that either genetic or environmental factors exert on the aetiology of a given disease.[4] Since the response and the tolerance to a therapy depend not only on the genetic predisposition but also on the gene-environment interactions (e.g. smoking habits, diet, stress), genetic epidemiology may help in orienting the therapeutic strategies in a given population.

2. Genes Involved in Human CVD

We will summarise the most recent advances in the identification of those genes that are associated with these diseases and that we have listed in tables I, II, III and IV, for clarity. It should also lead us to

understand the molecular basis of the progression of the disease.

2.1 Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is characterised by idiopathic myocardial hypertrophy and occurs in most cases as a familial disease with an autosomal dominant pattern of inheritance.[5,6] Common morphological features of this syndrome are left and/or right ventricular hypertrophy (VH) that is usually asymmetric, enlargement of the intraventricular septum, and myocyte hypertrophy and disarray around areas of connective deposition. HCM leads to a reduction of cardiac performance accompanied by arrhythmias and premature sudden deaths.[5,6] However, the clinical expression of this disease is highly variable and providing an accurate prognosis for affected individuals is difficult. In fact, one of the main problems is that the severity of common clinical indicators (VH, arrhythmias or blood pressure response to exercise) is not sufficient to predict if sudden death will occur.

Genotype-phenotype linkage studies have been performed during the last decade on affected families to try to identify on a molecular level, reliable genetic markers that can predict the outcome of HCM. These studies have led to the identification of several mutations all found in genes coding for sarcomeric proteins[7] (see table I). These are the mutation areas in the genes of affected proteins: the β-myosin heavy chain (β-MYHC),[8] the ventricular myosin light chain 1 (MLC-1s/v),[9] the ventricular myosin regulatory light chain 2 (MLC-2s/v),[9,10] cardiac troponin T (TnT),[11] cardiac troponin I (TnI),[12] α-tropomyosin (αTM),[13] and the cardiac myosin binding protein C (cMYBP-C).[14-16] As well as these genes, an additional locus has been described on chromosome 7,[17] whose gene is not known, and others will certainly be reported in the future. For each of these genes several polymorphisms have been identified, but not all the mutations have been associated with a negative prognosis. This is particularly true for the β-MyHC gene (*MYH7*). At least 50 different mutations of this gene were found in unrelated families with HCM,[7] but the most malignant were missense mutations located at codons 403, 719 and 741.[18,19] Recently a Gly716Atg polymorphism was described in a Korean family that is associated with a malignant prognosis, 100%

Table I. Genes involved in human hypertrophic cardiomyopathies

Gene	Protein	Category	Function	Locus
Causative genes				
MYH7	β-Myosin HC	Sarcomeric protein	Energy and mechanical force transduction	14q11.2-q.12
MYL3	Myosin LC-1s/v	Sarcomeric protein	Energy transduction/structure regulation	3p21.2-3p21.5
MYL2	Myosin LC-2s/v	Sarcomeric protein	Energy transduction/structure regulation	12q23-q24.3
TnnT2	cTnT	Sarcomeric protein	Calcium-dependent contraction	1q3
TNNI3	CTnI	Sarcomeric protein	Calcium-dependent contraction	19p13.2-q13.2
TPM1	α-TM	Sarcomeric protein	Calcium-dependent contraction	15q22
MYBPC3	CmyBP-C	Sarcomeric protein	Energy transduction/structure regulation	11p11.2
?	?	?	?	7q3
ACTC	Actin	Cytoskeletal protein	Mechanical force transduction	15q14
Disease associated risk factor genes				
AGTR1	AT1-RC	Receptor	Fluid volume and BP regulation Cell proliferation	3q21-q25
DCP1	ACE	Enzyme	Fluid volume and BP regulation Cell proliferation	17q23

ACE = angiotensin converting enzyme; **ATI-RC** = angiotensin I receptor; **BP** = blood pressure; **CmyBP-C** = myosin binding protein; **CTnI** = troponin inhibitory protein; **cTnT** = tropomyosin binding protein; **HC** = heavy chain; **LC-1s/v** = ventricular light chain 1; **LC-2s/v** = ventricular regulatory light chain 2; **αTM** = αtropomyosin; **?** = unknown.

penetrance and early disease expression.[20] On the other hand, mutations at codons 908 or 606 were associated with a near-normal life expectancy.[8,18]

These observations point to the need to understand which protein function is altered by the mutations in *MYH7*. It has been observed that all the mutations associated with the worst prognoses are located either in the head or in the head-rod junction of the molecule and induce a change of charge on the protein.[7] This leads to a 'poisoned' polypeptide that is incorporated into the sarcomere and this changes either the function of the wild-type protein or its ability to associate normally with other sarcomeric filaments.[7] This hypothesis has been supported by results obtained by incorporating the mutant protein in skeletal muscle *in vivo*.[21] This results in an alteration of the function of the skeletal muscle. Additional support for the hypothesis has come from developing animal models of HCM. Two mouse models have been produced by the introduction of the R403Q mutation of the human *MYH7* gene into the mouse *MYH6* gene.[22,23] This encodes the α-MHC that is the only myosin form present in this species. In one model the mutation was introduced by homologous recombination[22] and in the other by transgenic expression.[23] Both models, although representing the heterologous genotype, developed forms of HCM very similar to the human one. Homozygous mice died within 7 days of birth while heterozygous mice survived, although they developed a cardiac dysfunction from the 5th week of age which was characterised by functional and histological alterations similar to those in human HCM.[22,23] Further *in vitro* functional studies showed that the HCM mutants of *MYH7* alter sarcomere function either by decreasing the translocating filament activity and/or force, leading to a hypocontractile phenotype.[24] It can therefore be proposed, that in this case, HCM develops as a compensatory mechanism to overcome the decreased sarcomeric contractile function.

A different situation involves other sarcomeric proteins such as the cardiac troponin T (cTnT) or αTM. In both these proteins, either missense mutations[25] or mutations leading to abnormal splicing and thus to truncated polypeptides,[26] have been described. In these cases a hypercontractile state underlies the phenotypic characteristics of HCM and it may be hypothesised that hypertrophy develops not as a compensatory process but as the direct consequence of an increase in contractility. This hypothesis was recently substantiated by results obtained by Sweeney et al.[11] They produced transfected quail myotubes that expressed 3 different variants of the human TnT protein associated with HCM. All three mutants decreased the force production triggered by calcium sensitivity and two of these mutations increased the unloaded shortening velocity 2-fold.[11] Therefore, these data suggest that TnT mutations may cause the disease by increasing the energetic load of the heart.

Recently, mutations in the cardiac myosin binding protein C gene (*MYBPC3*) have been found in unrelated families with HCM.[14,27,28] Most of these mutations lead to a truncated protein that lacks the myosin-binding domain.[14,15,27] However, the real sequence of molecular mechanism leading from truncated sarcomeric proteins to final functional alterations needs to be further studied. However, it is interesting to note that when patients affected by HCM were studied by looking at the presence of mutations in the *MYBPC3* gene or in the β-*MHC*, the former have a delayed onset of CVD and a better prognosis than the latter.[29] Finally, α-cardiac actin gene (*ACTC*) has been found to be associated with HMC by a linkage study.[30] As described in section 2.2, this gene has also been implicated in the pathogenesis of dilated cardiomyopathy (DCM) and it has been suggested that the different *ACTC* polymorphism described may affect sarcomeric function leading to altered contraction in the case of HCM or reduced force transmission in DCM.[30]

Another crucial aspect that can be elucidated by genetic analysis of CVD is to identify the genetic markers that are not strictly associated with the cause of the disease but which can be used to predict how it will progress and which factors may affect its phenotypic expression. In this respect, particular attention has been given to the role of the renin-angiotensin system as the modulator of cardiac hy-

pertrophy (table I). In a recent study, Osterop at al.[31] analysed the influence of angiotensin II receptor A/C1166 and ACE insertion/deletion (I/D) polymorphisms on the degree of VH in unrelated individuals with HCM. It was concluded that only the AT1-RC1166 allele is associated with a higher degree of VH and septal thickness, and that it works independently from the ACE I/D polymorphism and plasma renin activity (PRA).

2.2 Dilated Cardiomyopathy

DCM is a heterogeneous disease characterised by ventricular dilatation and systolic contractile dysfunction. In around 30% of patients, it has a genetically inherited origin and in the remaining 70% it is acquired (i.e. secondary to myocarditis, ischaemic heart disease, etc.).[32] Polymorphisms of several genes have been identified as associated to DCM (see table II). Familial DCM can be transmitted as an autosomal dominant or recessive trait,[33-37] as a mitochondrial DNA[38] or as an X-linked trait.[39,40]

The genes for 2X-linked cardiomyopathy have been identified as the *dystrophin* gene that is also responsible for Duchenne muscular dystrophy and the *G4.5* gene in Barth syndrome.[41] Multiple mutations have been described in both genes.[39-41] Mutations in the *dystrophin* gene lead to alterations of the structural integrity of the cardiac contractile apparatus. The normal function of this protein is to facilitate the link between cardiac muscle cytoskeleton and the extracellular matrix. This ability is lost in the mutated variants. Alterations of dystrophin and dystrophin-associated glycoproteins are also involved in the molecular process underlying the acquired forms DCM. Recently, Badorff et al.[42] demonstrated that Coxsackievirus B3 can cause DCM through the activity of a viral protease 2A that cleaves dystrophin both *in vitro* and *in vivo* leading to a myocyte dysfunction.

Six other gene loci associated with familial DCM have been mapped on different chromosomes (1q32, 2p31, 9q13, 10q21-q23, 1p1-1q1, 3p22 and 3p25)[43] but the genes responsible for the disease have not yet been identified. Recently, mutations in the *ACTC* gene located on chromosome 15q14, have been described in two unrelated families.[44] These mutations affect the domain of actin that binds to Z bands and intercalated discs, thereby altering the transmission of force in cardiac myocytes.[30]

Although 70% of DCM is thought to be non-familial, the potential contribution of genetic defects to these acquired forms cannot be excluded. Some studies recently indicated that gene poly-

Table II. Genes involved in human dilated cardiomyopathies

Gene	Protein	Category	Function	Locus
Causative genes				
DMD	Dystrophin	Cytoskeletal protein	Mechanical force transduction	Xp.21.2
G4.5	Tafazzin	Cytoskeletal protein	Unknown	Xq28
ACTC	Actin	Cytoskeletal protein	Mechanical force transduction	15q14
?	?	?	?	1q32
?	?	?	?	2p31
?	?	?	?	9q13-q21
?	?	?	?	10q21-q23
?	?	?	?	322-p25
Disease associated risk factor genes				
PAFAH	PAF acetylhydrolase	Enzyme	PAF catabolism	6p12-p21.1
DCP1	ACE	Enzyme	Fluid volume and BP regulation Cell proliferation	17q23
AMPD1	Adenosine monophosphate deaminase	Enzyme	Adenosine catabolism	1p13

ACE = angiotensin converting enzyme; **BP** = blood pressure; **PAF** = platelet activating factor; **?** = unknown.

morphisms can be associated with idiopathic or ischaemic DCM (table II). Ichihara et al.[45] found an association of a missense mutation in the plasma platelet activating factor (PAF) acetylhydrolase gene (*G994-T*) with non-familial DCM in a Japanese population. Although is not likely to be causative, it may contribute to the genetic susceptibility to, or the progression towards, CHF in these patients, since abnormalities in the function of this enzyme may reduce the tissue defence from PAF-dependent oxidative stress. Polymorphism in the ACE gene (I/D) has been demonstrated to be an independent risk factor for the development of MI and treatment with ACE inhibitors reduces both the mortality and the rate of re-infarction.[46] Raynolds et al.[47] found that the DD allele frequency is significantly higher in patients with idiopathic and ischaemic DCM than in a control population. This suggests that this ACE gene variant (and the concomitant higher circulating ACE levels) may contribute to the pathogenesis of these forms of cardiomyopathies. Two other more recent studies failed to find a difference in the frequency distribution of the I/D ACE alleles between patients affected by non-familial DCM and the general population.[48,49] However, in one study a worse ejection fraction and increased left ventricular diameters were observed in patients with the DD allele.[48] In the other study,[49] the frequency distribution of the three genotypes (II, I/D and DD) did not follow the Hardy-Weinberg equilibrium in the affected population because there was a significantly lower frequency of the I/D genotype. The meaning of this finding is unclear, in fact reduction of the frequency of a given genotype can be interpreted either as a protective effect of heterozygosity on the development of the disease that reduces the frequency of affected individuals or, *vice versa*, as a detrimental factor that increases mortality in this group of patients thus reducing their number.

Another recent study was conducted by Loh et al.[50] with the aim of identifying the gene or genes that may be associated with improved clinical outcome in patients with CHF caused by non-familial DCM of different aetiologies (coronary artery disease, idiopathic CM and 'other'). The adenosine monophosphate deaminase locus (*AMPDI*) was selected for this study[50] because a nonsense mutation in the *AMPDI* gene leads to a loss of the protein function which would be expected to increase the cardiac levels of adenosine, a potent cardioprotective agent.[50] Indeed, the authors found that individuals homozygous (-/-) or heterozygous (-/+) for the mutation have a significantly longer duration of CHF symptoms before needing to undergo transplantation than those individuals homozygous for the wild-type allele (+/+). It was suggested that the increased adenosine levels, as a result of a reduction in AMPD activity caused by the mutation, may play a protective cardiac role delaying the need for transplantation.[50]

2.3 Long-QT Syndrome

Long-QT syndrome (LQTS) is a cardiac disease that causes ventricular arrhythmias and sudden death as consequence of abnormalities in cardiac action potential repolarisation, which can be seen as a prolonged QT interval. Two familial forms of LQTS have been described: a common autosomal dominant form called Romano-Ward[51] and a rarer autosomal recessive form called Jervell and Lange-Nielsen, which is also characterised by the presence of congenital deafness.[51] Five loci have been identified for the Romano-Ward form and two of these account also for the Jervell and Lange-Nielsen form[52] (table III). Four of these loci encode ion channel proteins: *SCN5A* on chromosome 3 encodes for the cardiac sodium channel, *HERG* on chromosome 7 for the I_Kr potassium channel, *KvLQT1* on chromosome 11 for the α subunit of the I_{ks} potassium channel and *Mink* on chromosome 21 encodes for ancillary subunit of the I_{ks}. The last 2 loci are associated with both LQTS.[43] A fifth gene located on chromosome 4 has not yet been identified. Since many families with hereditary LQTS have been shown not to have any of these genes, other genes are probably involved.[43]

Most of the mutations described associated with LQTS are missense mutations and they generally account for an altered function of the encoded ion

Table III. Genes involved in human long-QT syndrome

Gene	Protein	Category	Function	Locus
Causative genes				
SCN5A	Cardiac sodium channel	Ion transporter	Cell ion gradient	3p21-p23
HERG	Potassium channel	Ion transporter	Cell ion gradient	7q35-q36
KvLQT1	α-Subunit potassium channel	Ion transporter	Cell ion gradient	11p15.5
MinK	Subunit potassium channel	Ion transporter	Cell ion gradient	21q22.1-p22
?	?	?	?	4q25-q27

? = unknown.

channel. In particular, mutations in *KvLQT1* and *HERG* generally induce a 'loss of function' of the channel.[53,54] If we look at the physical effects, a reduction in the outward K^+ current should lead to a delayed shortening of the action potential with consequent adrenergic hyperactivation and increased incidence of arrhythmic events, especially during exercise. In contrast to the effect of mutations on potassium channels, those caused by mutations on *SCN5A* which encode for the sodium channel, lead to an increased activity of this channel as result of a defective inactivation.[55] The functional consequence of this defective inactivation is a prolonged and increased inward Na^+ current, which once again results in a prolonged QT interval with the occurrence of arrhythmias, especially when the patient is resting.

3. Therapeutic Implications

All the advances achieved during the last few years in defining the genetic-molecular basis of several genetically transmitted cardiovascular disorders, have two main practical applications. The first is to develop precise molecular diagnostic tests that are able to identify patients at risk for a given disorder before clinical symptoms and overt organ failure can develop. The second application is in developing a therapeutic or prophylactic treatment that is able to interact specifically with and/or correct the consequences of a particular molecular defect. This should prevent the disease or slow its progression or its development.

As described in section 2, continuous progress has been made in defining the genotype-phenotype associations of many hereditary cardiovascular disorders and this has already let us use molecular diagnosis techniques for non-affected members of affected families in clinical practice. Unfortunately, progress in developing tailored therapies aimed at curing a specific molecular defect underlying a particular disease, is still in its infancy.

Treatment of cardiomyopathies, either dilative or hypertrophic, is currently aimed at relieving the symptoms that accompany heart failure as the disease progresses; namely, altered cardiac contractility, hypertrophy, arrhythmias and increased sympathetic drive. A wide range of drugs is available for such treatment and these are currently being used in different combinations. At the moment, the rationale for using them is exclusively based on pathophysiological considerations. Diuretics promote the loss of sodium and water thereby reducing ventricular filling pressure and increasing cardiac output. β-blockers reduce heart rate and have an antiarrythmic effect. In addition, β1-selective blockers, such as metoprolol and bisoprolol, and also a non-selective β-blocker, carvedilol, have recently been shown to reduce the symptoms and improve the prognosis in patients with DCM.[56-58] These β-blockers probably work by antagonising the neuroendocrine activation that occurs in heart failure. Digitalis inhibits the cardiac Na^+/K^+ pump, thus increasing cardiac contractility. A recent National Institutes of Health (NIH)-sponsored trial [the Digitalis Investigation Group (DIG) study],[59] showed that digoxin does not affect survival rate in patients with CHF when added to other conventional therapy, but reduces hospitalisation as a result of worsening of symptoms.[59] Conversely, other inotropic agents (phosphodiesterase inhibitors, partial β blockers, dopa-

mine antagonists, calcium sensitisers, etc.) reduce the symptoms of CHF but lower the survival rate. ACE inhibitors and angiotensin (AT) II receptor antagonists inhibit the cardiac formation or activity of AT II, and thus exert a beneficial effect on cardiac remodelling and hypertrophy, which reduces the risk of overt CHF and increases survival rates. The use of the latter class of drugs in cardiomyopathies may also be amenable to a classical pharmacogenomic approach. In fact, the two fundamental tools necessary for this approach are available: (i) genetic polymorphism, which may modulate the activities of the renin-angiotensin-system (RAS), have been identified, and (ii) drugs, such as ACE inhibitors and ATII receptor antagonists, which block the cardiac RAS effects (VH and heart failure) are currently used.

The recent definition of genetic-molecular alterations in the ion channels associated with the LQTS means that it might be possible to treat these forms by developing drugs which are able either to reactivate the defective current through the potassium channels or to inhibit the hyperactive sodium channel. The former approach has not yet been pursued, even though results obtained by inducing an overexpression of the human potassium channel *HERG* in rabbit cardiomyocytes indicate that this strategy is effective in suppressing delayed repolarisation and post depolarisation arrhythmias.[60] Indeed, the latter approach seems more feasible since recent results indicate that both sodium channel inhibitors, such as lidocaine or mexiletine,[54,61] and free fatty acids[62] are able to normalise the altered I_{Na} current associated with the *SCN5A* mutation. Moreover, the observation that in some circumstances antiarrhythmic drugs able to prolong repolarisation (such as cisapride) are also able to induce torsade de pointes[63] has been recently explained by the presence of 'silent' mutations in the potassium channel genes[64] that become symptomatic only when interacting with external factors, such as these kinds of drugs.

4. Essential Hypertension

Essential or primary hypertension could be defined as a complex trait, affecting around 20% of human population, determined by multifactorial components, whose clinical outcome is an increased risk of myocardial infarction, stroke and end-stage renal failure. This definition implies that essential hypertension is a heterogeneous syndrome produced by the interaction of multiple genetic mechanisms and environmental factors. Whereas most of the environmental factors have been identified (diet, socio-economic status, smoking habits etc.), the genetic ones are poorly known, although it is accepted that many genes could be involved. The mechanisms that underlie the phenotypic expression of a polygenic disease like essential hypertension are enormously complex. This disease results from the interaction between some major genes with a polygenic background leading to certain intermediate (organ and cell functions, enzyme activities) and final phenotypes (blood pressure). Furthermore, these intermediate phenotypes are independently modulated by the environment.[65-67] This interaction is also time-dependent since compensatory mechanisms come into play to counteract the original alteration.[68] The time dependent variation of pressor mechanisms has been discussed in detail by some authors.[69,70] It is due to the progressive readjustment of the feedback mechanisms connecting all the physiological pathways that control blood pressure. Of course each of these pathways is controlled by peculiar gene polymorphisms which modulate their response. Whatever is the genetic major cause(s) of hypertension, such a feedback mechanism must be involved in one way or another to reset the final level of blood pressure.

In the last 10 years, the use of molecular genetics to study essential hypertension has allowed us to identify some QTLs or genes that influence blood pressure, especially in rat models of genetic hypertension.[71] In particular, through both the candidate gene approach or random genome scanning, some specific genes have been identified for the following proteins angiotensinogen,[72] ACE,[73]

α-adducin,[74] SA,[75] β2-adrenergic receptor,[76] G-protein β3 subunit[77] and β subunit of the sodium channel[78] (see table IV). In humans, the problem of the association between a given genetic trait and hypertension has so far been approached with both case-control and linkage studies. Few genes, *ADD1*[79-81] and *AGT*,[65,72] turn out to be involved in human essential hypertension when both types of study are considered,[82] even though negative findings have also been published. ACE gene polymorphism, although associated with MI[83] and cardiac hypertrophy,[84] seems less implicated in the genetic mechanisms of essential hypertension[85] with the available studies yielding more controversial results. These findings are especially relevant for the therapeutic impact that ACE inhibitors and ATII receptor antagonists may have on patients with hypertension for whom I/D polymorphism of the ACE gene is most likely associated with an increased risk of VH and CHF.[83,84]

The reasons for the large number of conflicting results regarding the genetic association between a given gene polymorphism and hypertension can be related to the following considerations: (i) the polymorphism is not primarily associated with the disease but is in linkage disequilibrium with another polymorphism that is causally related to the disease, (ii) errors in population stratification can affect the association study, (iii) the effect of the polymorphism of interest may be influenced and masked by other genetic polymorphisms (epistatic interaction) or nongenetic factors (environment, lifestyle, diet), making difficult any comparison among studies carried out in different populations or with different protocols.

4.1 Pharmacogenomics of Essential Hypertension

A variety of antihypertensive drugs are now available, including calcium antagonists, ACE inhibitors, β-blockers, diuretics, α-blockers, centrally acting anti-hypertensives and more recently, ATII receptor antagonists. This last class of drugs is effective in lowering blood pressure through a different mode of action with respect to ACE inhibitors and seems to have an ever-increasing degree of tolerance. However, this large choice of antihypertensive medication may also reflect the difficulty in finding a single drug that effectively lowers blood pressure without producing adverse effects that affect the patient's tolerance. The management of essential hypertension has certainly been successful in reducing the risk of fatal and nonfatal strokes,[86] but its impact on coronary heart disease is still not clear.[87] It is a common misconception that the available antihypertensive drugs are not only effective but also well tolerated. However, it is unfortunately true that there is a large proportion of patients with hypertension whose blood pressure is not adequately controlled or who stop their treatment because of the undesirable adverse effects and poor levels of tolerance. The situation could be improved by having a better knowledge of the mechanisms which influence the efficiency of the

Table IV. Genes involved in human essential hypertension

Causative gene	Protein	Category	Function	Locus
DCP1	ACE	Enzyme	Fluid volume and BP regulation Cell proliferation	17q23
AGT	Angiotensinogen	Substrate enzyme	Fluid volume and BP regulation Cell proliferation	1q42.3
ADRB2	β_2RC	Receptor	Adrenergic control	5q31-q32
ADD1	α-Adducin	Cytoskeletal protein	Signal transduction Cell structure regulation	4p16.3
SCNN1B	β subunit sodium channel	Ion transporter	Cell ion gradient	16p12.2-p12.1
SA	?	?	?	16p13.11
GNB3	G-protein β3 subunit	Receptor	Signal transduction	12p13

ACE = angiotensin converting enzyme; **BP** = blood pressure; **RC** = receptor; **?** = unknown.

drug in different individuals and by understanding why some patients can tolerate the drug while others can not.

The variation of the individual's response to antihypertensive drugs may be caused by either the heterogeneity of the mechanisms underlying hypertension[88] or to interindividual variations of pharmacokinetics of the drugs,[89] or both. In all 3 cases genetic heterogeneity is involved. The observation, for instance, that race influences the response to particular classes of antihypertensive drugs is in keeping with this notion.[90] It is now possible to approach this problem through a new branch of pharmacology: the discipline of pharmacogenomics, which is aimed at explaining individual variation of blood pressure response to drugs with genetic polymorphism.[91,92] As far as we know only a few studies have addressed the problem of an association between a given locus and the blood pressure response to a specific antihypertensive agent.

Some studies approached the problem of a possible association between treatments and the ACE/angiotensin genetic variants. However, these studies have produced contradictory results. Dudley et al.[93] investigated whether the M235T polymorphism of the *AGT* gene and the I/D polymorphism of the *ACE* gene can predict blood pressure responses to a β-blocker, ACE inhibitor and a calcium antagonist in patients with essential hypertension. They recorded a large variability between individuals in blood pressure responses to these agents that cannot be associated with the analysed polymorphisms.

In another study, Hingorami et al.[94] found an association between the *AGT* genetic variants and the magnitude of the decrease in blood pressure after ACE inhibition. Very recently, O'Toole et al.[95] studied the effect of the ACE I/D genotype on the response to ACE inhibitor therapy in patients with CHF, carrying out a double blind, crossover study comparing captopril with lisinopril. They found a significant relation between ACE genotype and the change in blood pressure with captopril, with the II genotype being more sensitive to the treatment than the I/D and DD. However, this was not the case with lisinopril.[95] Although this study has many limitations and its results cannot be considered as conclusive, it is of particular interest in view of the protective role of ACE inhibitors on mortality, recurrent MI and the progression of CHF in patients with left ventricular dysfunction.[46,96] Recently, Hunt et al.[97] demonstrated that the 235T allele of the *AGT* gene is associated with a greater blood pressure decrease than the 235M allele after sodium restriction. This finding supports the hypotheses that variation within the *AGT* gene may contribute to alteration of salt sensitivity in patients with essential hypertension and, thus, it implies that these patients may benefit from salt restriction as a nontherapeutic intervention to reduce their blood pressure.

A different pharmacogenomic approach was used by Vincent et al.[98] They used genetic hypertensive rats of the Lyon strain to investigate the cosegregation of genetic loci with acute blood pressure responses to drugs acting on the renin-angiotensin system (losartan), the sympathetic system (trimetaphan) and calcium metabolism [L-type calcium antagonist, darodipine (PY-108068)]. By using microsatellite markers, they identified a QTL on rat chromosome 2 that specifically influenced the blood pressure responses to the dihydropiridine calcium antagonist. The same locus had no effect on either basal blood pressure or on responses to the ganglion blocking agent or ATII receptor antagonist.[98] Therefore, this study represents one of the first examples in which a specific locus has been demonstrated to influence the response to a given antihypertensive therapy.

Recently, an association of the *Gs*α gene with essential hypertension has been described.[99] The *Gs*α gene polymorphism consists of the presence or absence of a restriction site for FokI. The influence of the FokI allele on blood pressure response to β-blockade was examined in a treated group of patients showing that the frequency of the FokI+ allele was significantly higher in the good responders to this therapy than in the poor responders, thus suggesting that *Gs*α genotype may influence the blood pressure response to β-blocker therapy.[99]

Our group has been involved for many years in attempting to identify at least one of the genetic mechanisms causing hypertension both in a rat model and in humans. These results could be used to identify new pharmacological targets for innovative hypertension treatments and for the characterisation of patients with essential hypertension who are likely to be treated successfully with such compounds.

We previously observed that genetic hypertension in rats of the Milan strain (MHS) is primarily due to a renal abnormality in the ability to excrete salt.[100] This abnormality is primarily determined, since hypertension can be transplanted with a MHS kidney in Milan normotensive strain (MNS) controls.[101,102] The enhanced tubular sodium reabsorption of MHS rats is due to a faster transepithelial sodium transport, mediated both by the apical (Na^+/H^+ exchange, Na^+/K^+ Cl^- cotransport)[103] and basolateral (Na^+/K^+ pump) ion transport systems.[104] Parallel observations carried out on rats and patients with essential hypertension, or in individuals prone to develop essential hypertension because of a positive familiarity for this disease, revealed that there is a subgroup of patients who share many functional, hormonal and biochemical characteristics with MHS rats.[103] MHS rats, therefore, can be considered to be a valid model for studying the genetic mechanisms of human hypertension.

Further studies aimed at identifying the cellular and biochemical origins of the ion transport alterations in MHS rats have led to the identification of an immunological difference of the cytoskeletal protein adducin between MHS and MNS rats.[74] Adducin is an α/β heterodimeric protein which is involved in the assembly of the spectrin-actin cytoskeleton.[105] It also modulates the actin polymerisation,[105] binds calmodulin,[106] is phosphorylated by protein kinase C and tyrosin kinase,[107] and regulates cell-signal transduction.[108] We further demonstrated that in both MHS rats[74] and humans,[79,80] point mutations in the α-adducin gene are genetically associated with hypertension. Transfection of the 'hypertensive' and 'normotensive' α-adducin variants in rat kidney cells (NRK) showed that the former increases the surface expression and maximal rate of the Na^+/K^+ pump.[109] Moreover, in a cell-free system, the 'hypertensive' variant of α-adducin directly stimulates the isolated Na^+/K^+ pump at significantly lower concentrations than the 'normotensive' one.[110] This finding, therefore, provides the genetic-molecular basis for a constitutive increase in tubular sodium reabsorption in MHS rats. The cellular and biochemical alterations caused by α-adducin polymorphism in MHS rats are also associated with an increased production of an endogenous Na^+/K^+ pump inhibitor, the so called ouabain-like factor (OLF).[111,112] This is involved in the Na^+/K^+ pump modulation and consequently in the cell ion handling.

In humans, the polymorphism of α-adducin at position 460, consists of the substitution of tryptophan (Tpr) for glycine (Gly). A positive association between 460Tpr adducin allele and hypertension has been found in different populations.[80,81,113] Moreover, patients carrying this allele show a less steeper pressure-natriuresis curve,[114] a greater fall in blood pressure after low salt diet or diuretic treatment,[80] and lower PRA[80] than patients carrying the 460Gly variant. However, others failed to confirm a positive association between the 460Tpr adducin allele and hypertension.[115,116] These last 2 studies referred to the Japanese population for which the allelic frequency of the 460Tpr variant is much higher (54 to 60%) than in Caucasians (13 to 23%). However, a recent study[117] comparing two Italian populations, one from Milan and the other from Sardinia, showed that basal PRA was lower and blood pressure fall after a diuretic therapy more pronounced in patients with hypertension carrying at least one 460Trp allele than in Gly460Gly homozigotes, irrespectively of the presence (Milan population) or the absence (Sardinian population) of the association between the 460Tpr variant and hypertension.[117] Therefore, the α-adducin polymorphism may be used in humans to identify patients in whom a genetic abnormality in renal sodium handling underlies a salt-sensitive form of essential hypertension.

Moreover, increased plasma OLF levels in a subgroup of patients with hypertension have been found which are positively correlated with left VH.[118] The link among α-adducin polymorphism, increased renal Na^+/K^+ pump activity and expression, tubular sodium re-absorption, OLF levels and hypertension, has not been fully elucidated. However, the following working hypotheses may be proposed: the primary genetic molecular defect of α-adducin may affect the actin-cytoskeleton structure and function leading to an increase of Na^+/K^+ pump units on the cell membrane with a consequent enhancement of the overall sodium transport across renal tubular cells. This, in turn, causes an increase of renal sodium re-absorption and increase in blood pressure.

Moreover, increased OLF levels seem to affect, *per se*, the expression and activity of the renal Na^+/K^+ pump. Indeed, we demonstrated that long term infusion of very low doses of ouabain in normotensive rats,[119] which is considered very similar if not identical to endogenous OLF, and the long term incubation of cultured renal cells with nanomolar concentrations of ouabain,[119] led to an up-regulation in the maximal rate of the Na^+/K^+ pump. Therefore, a biphasic action of ouabain on the Na^+/K^+ pump activity may be proposed: (i) at high concentrations it inhibits the pump and in the long term an up-regulation of pump expression takes place as a positive feed-back mechanism by which the cell can re-establish the equilibrium of the Na^+/K^+ ion gradient altered by pump inhibition;[120,121] (ii) at very low concentrations (nanomolar range) a prolonged exposure to ouabain (or OLF), either in the whole rat[119] or cultured rat renal cells,[119] may lead to an increased pump pool size as consequence either of a positive feed back mechanism, which responds to a subliminal and technically undetectable pump inhibition, or it may also be due to a novel cellular effect of ouabain that stimulates the transcription machinery of the Na^+/K^+ pump either through the activation of other transport systems or by a direct mechanism. This suggests that long term exposure to low concentrations of OLF may participate in the pump overexpression primarily induced by α-adducin.[122]

Based on these findings, it can be postulated that any pharmacological intervention that is able to interrupt this vicious cycle by normalising the hyperactivation of the renal Na^+/K^+ pump, may be effective in lowering or preventing hypertension in those forms which are caused by alterations of the Na^+/K^+ pump and OLF levels.

A new compound, PST-2238, was able to antagonise, at nanomolar concentrations, the Na^+/K^+ pump overexpression caused in cultured rat kidney cells either by α-adducin polymorphism or nanomolar ouabain concentrations. It has been demonstrated to be effective *in vivo* by lowering blood pressure and normalising the increased Na^+/K^+ pump both in MHS[123] and ouabain-hypertensive rats,[119] at oral doses of 1 to 10 μg/kg.

The ability of PST-2238 to antagonise the effect of α-adducin mutations and ouabain (or OLF) on renal Na^+/K^+ ATPase activity and expression has been also demonstrated in cultured NRK. In NRK cells transfected with the 'hypertensive' variant of α-adducin, the increased Na^+/K^+ pump activity at maximum rate of activity (Vmax) is lowered to the level of normal wild type cells by incubating the cells with PST-2238 at a concentration of 10^{-10}, 10^{-9} mol/L for 5 days.[123] In wild type NRK cells incubated with ouabain (10^{-9} mol/L) for 5 days, the simultaneous presence of PST-2238 (10^{-14} to 10^{-9} mol/L) normalises the ouabain-dependent increase of the Na^+/K^+ pump at Vmax (+35%), while the compound does not show any intrinsic activity on the Na^+/K^+ pump rate at concentrations up to 10^{-5} mol/L. [119]

From the pharmacogenomic point of view, it is interesting to stress that this compound does not lower blood pressure in spontaneously hypertensive rats (SHR),[123] in which both α-adducin polymorphism,[124] the renal Na^+/K^+ ATPase[125] and OLF levels do not seem to be involved in causing the difference between SHR and their control Wistar-Kyoto (WKY) rats.[126]

The ability of PST-2238 to normalise renal Na^+/K^+ ATPase up-regulation in MHS rats, ouabain-induced

hypertensive rats, NRK cells transfected with 'hypertensive' α-adducin, and NRK cells in which Na^+/K^+ ATPase activity is increased as a consequence of long term ouabain incubation, raises the question of whether both mutated α-adducin and ouabain (or OLF) affect Na^+/K^+ ATPase expression and activity (and consequently cause hypertension) through a common mechanism which is corrected by PST-2238. As ouabain affects the half-life of cell membrane Na^+/K^+ pumps[127] by a combined mechanism of a transient increase in the rate of synthesis[128] and decrease in the pump degradation rate,[127] an alteration in cell membrane Na^+/K^+ ATPase cycling may be proposed as the common mechanism triggered either by α-adducin or ouabain that is corrected by PST-2238. Research on this topic is currently ongoing.

Although PST-2238 is a digitossigenin derivative, it is devoid of any cardiac and hormonal effects typical of digitalis or antimineralcorticoid drugs.[122] It does not bind to other receptors involved in blood pressure regulation or hormonal homeostasis,[119] has a good toxicological profile and is currently undergoing clinical trials.[122]

According to its pharmacological profile, PST-2238 may represent the prototype of a new class of antihypertensive agents that control blood pressure. It specifically corrects a biochemical alteration (up-regulation of the Na^+/K^+ pump) that is primarily determined by a genetic defect (α adducin polymorphism) and further aggravated by a hormonal deregulation (OLF increase). When these findings are considered as part of a pharmacogenomic approach to the therapy of essential hypertension, it appears that compounds like PST-2238 are candidates for use in patients in whom, like MHS rats, alterations of renal sodium handling and OLF levels can be associated with a specific genetic polymorphism. This genetic defect is one which affects the biochemical machinery involved with intracellular sodium transport.

5. Future Development

Drug discovery is now entering a new era, an era led by the new sciences of genomics, proteomics and bioinformatics. By these approaches it is reasonable to forecast that in the next 5 to 10 years many of the new targets for drug discovery will be identified. Identification of these new targets will benefit from new methods available now, and others which are improving all the time, which can be used to rapidly genotype large numbers of genetic variants in hundreds of patients. Two basic genomic approaches can be followed to this end. Firstly, the expressed sequence tag (EST) that allows identification of those genes undergoing expression in a given tissue, by identifying messenger RNA fragments that 'tag' for the full-length gene. The advantage of this approach is a quite rapid identification of a large number of genes, but it does not allow detection of polymorphisms in the non-coding regions (which are involved in the gene expression regulation) and to focus *a priori* on genes directly involved in a given disease. Differentially expressed genes can be also detected by differential display techniques.[129] Secondly, disease-based gene identification can overcame the weakness of the EST approach since this method allows direct identification of those genes associated with a target disease by a full sequencing which also takes into consideration the noncoding regions. Moreover, it is suitable for studying genes even if they are not expressed in all tissues. Its major disadvantage is the slowness of the entire sequencing process, but this is expected to be expedited by the availability of genome-wide single-nucleotide polymorphism (SNP) marker map. Using this approach the loci that influence the individual patient's response to therapy and consequently, those that may represent new pharmacological targets for the development of novel drugs, may be detected.[130] SNP detection can be achieved by techniques that allow rapid sequence analysis such as DNA microarray procedure.[131] These techniques will permit the simultaneous determination of thousands of multiple polymorphic gene variants from a single drop of blood.[132]

A new field that will give valuable information for determining the proteic target to which new drugs should be addressed is represented by proteomics.[133]

This technology allows identification of the gene product (protein) whose function is affected by the disease gene alteration or that is 'switched on or off' by a specific therapeutical manoeuvre. Proteomics is based on the possibility of recognising unknown proteins by coupling the 2-dimensional electrophoresis technique, which separates polypeptides on the basis of size and isoletric point, with mass spectrometry, which furnishes the amino acid sequence of a given protein. By this method a 'peptide-mass fingerprint' is obtained and comparison of the protein with those stored in protein databases is possible.

Finally, functional genomics, applied to the study of the function of genes involved in CVD pathogenesis, can now take advantage of the development of proteomics and bioinformatic approaches. These try to determine the gene function by comparing its sequence with that of other genes whose function is already known or by searching for regions in its sequence that are known to have given functions (e.g. protein binding). The impact of the Human Genome Project in this field is and will be more and more relevant, since in 2 or 3 years from now the sequences of all the human genes will be known, stored into gene banks and available for genetic analysis through the use of automated softwares.

6. Conclusions

Scientists are now in the position to have the theoretical background and the practical tools to improve our approach to the individual therapy of CVD. We may hypothesise that a better understanding of their genetic mechanisms will not only encourage the development of new pharmacological approaches to the discovery of novel drugs, but also will furnish a powerful tool for a better and more appropriate use of the available ones. As stressed in section 4, in complex, multi-factorial diseases like hypertension, the pathogenetic role of an individual gene polymorphism must be evaluated in the context of its overall genetic and environmental background. Therefore, understanding the interactions among genes, and between genes and the environment, which underlie a given pathophysiological alteration are of paramount importance. It is only when they are understood that the implementation of any pharmacogenomic approach will be possible. In this respect, the influence of epistatic interactions between different polymorphisms represent one of the most crucial aspects to consider for the correct application of pharmacogenomics. In essential hypertension, for instance, we are studying the interaction between α-adducin and ACE polymorphism on blood pressure response to acute changes in sodium balance. The result of this study[134] showed that these two polymorphisms interact epistatically in determining this response. Therefore, it is likely that similar interactions are also relevant to understanding the genetic basis of the response to drugs of an individual's blood pressure.

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