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Editorial Board

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Editorial

Genetics of Hypertension and Cardiovascular Disease

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With the force something like a tsunami wave, a surge of genome-wide association studies (GWASs) in common complex diseases has flooded the recent literature and left us awash with data regarding the identification of causative genetic risk factors for cardiovascular disease such as hypertension. Nevertheless, the more slowly moving tide of the classical candidate gene approach also makes steady progress to clarify the interaction between gene function and pathogenesis of hypertension. Like most other common conditions the estimated effects of each genetic variant on blood pressure and the predisposition to hypertension is quite small, yet the delineation of genetic contribution could reveal new targets for risk reduction in cardiovascular disease.

The main focus of this special issue is to highlight the genetic basis of hypertension and cardiovascular disease and outline recent progress in genomics. In addition, several successful investigations in common diseases are introduced as good examples of the potential for better understanding of hypertension using genomic approaches.

In the first part of this issue, two papers provide clear reviews of the recent progress in the GWAS approach for hypertension and coronary artery disease. These papers elucidate not only the common findings in studies that, to a certain extent, disparate but also interprete these findings in the context of the advantages and limitations of GWAS. This context includes the contrast between the very strong (genome-wide) significance of associations and the relatively small phenotypic effects of each SNP. In terms of discovery potential, unexpected candidate genes obtained from GWAS provide fresh perspectives in hypertension research.

But the genomic picture is for more complex than isolated SNPs. It involves the ways in which the sophisticated organization of the operational elements of the genome interdigitates to provide tissue and development stage-specific regulation and integration of gene expression. It also aligns these factors with phenotypic changes that can result. Two subsequent review articles illustrate these principles by showcasing a new cutting edge of cardiovascular research in the form of microRNAs (miRNAs) and scavenger receptors. Both papers disclose the potentials of these newcomers as therapeutic targets of cardiovascular disease.

In a more focused review of candidate genes, the next 2 papers focus on gene polymorphisms of adrenergic receptors. K. Masuo examines the critical role of beta2- and beta3-adrenoceptors gene polymorphisms on hypertension or metabolic syndrome unequivocally. K. B. Boström et al. report that a polymorphism (Arg389Gly) of beta1-adrenoceptor gene is significantly associated with increased risk of obstructive sleep apnea (OSA) with hypertension.

In the research using candidate gene approach, precise and appropriate phenotyping is important to identify small but significant genetic effect. G. D. Kitsios and E. Zintzaras reveal the protective effect of a haplotype of endothelial nitric oxide synthase gene (NOS3) for predisposition to hypertension. K. Sugimoto et al. point out that a polymorphism in the promoter region of regulator of G protein signaling-2 (RGS2) gene affects the difference of antihypertensive medication in a clinical trial.

The last two papers extend the consideration of novel and unexpected concepts for cardiovascular research. A recent topic is the awareness of shared physiological mechanisms between osteoporosis and cardiovascular disease. F. Marini

and M. L. Brandi elegantly review the genetic aspects of osteoporosis and touch on the shared mechanisms. The last manuscript by G. Mertens offers a unique perspective on these issues by zooming out to take an evolutionary view. To consider the underlying mechanisms of atherosclerosis, we have to take a larger view without becoming lost in details of the immediate.

We hope that this special issue is useful for your research and is conducive to the incorporation of new concepts and methods in your own laboratory.

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Review Article

Genome-Wide Association Studies of Hypertension: Light at the End of the Tunnel

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Despite its significant genetic component, the study of hypertension by genome-wide association presents more challenges than other common complex diseases. Its high prevalence, heterogeneity, and somewhat unclear definition are the challenges that need to be overcome on one hand. On the other hand, there are issues of small effect sizes and pleiotropism that are not specific to hypertension alone but nonetheless magnify the problems of genetic dissection when coupled with phenotypic misclassification. We discuss issues of study design and summarise published genome-wide association studies (GWASs) of hypertension and blood pressure. With careful study design and analysis success is possible, as demonstrated by the recent large-scale studies. Following these, there is still further scope to advance the field through high fidelity phenotyping and deep sequencing.

1. Introduction

Hypertension is the major factor responsible for the most deaths worldwide (around 7 million or 12.8% per year) and this is 46% more than tobacco usage, the next major risk factor [1]. From an epidemiological and clinical perspective, blood pressure at the higher end of the normal population distribution is associated with an increased risk of cardiovascular mortality and morbidity. But clinical risk assessment is based on a predefined threshold at which the quantitative blood pressure phenotype in converted into a binary trait (hypertension) and management strategies are directed towards blood pressure reduction below this threshold at which the risk of excess cardiovascular events is abolished. Hence large-scale efforts to dissect the genetic underpinnings of hypertension are justified. While the causation of hypertension is multifactorial with both genetic and environmental components, the purpose of this paper is to discuss the genetic determinants of high blood pressure and GWAS study design.

For any genetic study, the key initial step is phenotypic specification. Here whether studying blood pressure as a quantitative trait or the qualitative hypertension phenotype depends on whether one follows the Pickering argument that blood pressure is inherited as a "graded character," and hence a complex non-Mendelian trait, or the Platt suggestion that hypertension is a qualitative abnormality and a simple Mendelian disease [2]. The observed normal unimodal distribution of blood pressure and its complex multifactorial aetiology support studies of blood pressure as a quantitative phenotype. According to the latter argument, if hypertension is a dichotomous risk trait, one would expect a bimodal distribution and this is not observed. However both approaches are useful and complementary in the genetic dissection of this trait which has so far been extremely resistant to the GWAS approach.

2. Evidence for a Genetic Component in Hypertension

There are multiple strands of evidence showing that genetic factors contribute to blood pressure and hypertension. Firstly, the normal distribution of blood pressure in the general population indicates the presence of multiple environmental and genetic factors and thus a polygenic aetiology. Secondly, rare monogenic forms of hypertension associated with major defects in renal salt handling prove that gene

mutations can cause hypertension leading to a hypothesis that minor variations in these genes may contribute to the common essential hypertension. Finally from a population perspective, there is considerable evidence from twins and family aggregation studies indicating the presence of a heritable component.

It is estimated that around 30% of variation in blood pressure is due to genetic factors [3]. Hypertension is about twice as common in individuals who have one or two hypertensive parents, and blood pressure is more closely correlated in monozygotic than dizygotic twins [4, 5]. In the Montreal Adoption Study investigators compared blood pressure correlation between biological sibling pairs and adoptive sibling pairs (as well as parent-child correlations). SBP correlation coefficients were 0.38 and 0.16 for biological and adopted siblings, respectively, and DBP coefficients 0.53 compared with 0.29 respectively [6].

Two measures that are commonly used to assess the genetic component of a trait are heritability (h^2) which is the fraction of variation in disease susceptibility due to genetic factors, and sibling recurrent risk (λs) which is the degree of elevated risk of disease for a sibling of an affected individual compared with a member of the general population. The heritability of clinic systolic blood pressure is around 15–40% and 15–30% for clinic diastolic blood pressure [7, 8]; whereas for ambulatory night-time systolic and diastolic BP the heritabilities are 69% and 51% [7]. It is pertinent to point out that though the heritability estimates are considerable, this does not equate to magnitude of genetic effect. This is because the denominator in the estimate of heritability comprises measurement error and variances attributable to genes, shared environment, unshared environment and unmeasured determinants. This is illustrated by the example above where minimizing measurement errors by using ambulatory night-time values inflates the heritability estimates. Heritability is also a property of the population studied and low heritability estimates would suggest that genetic mapping would be difficult for that phenotype. The sibling recurrent risk of hypertension is around 1.2–1.5 [9], indicating a phenotype with modest genetic effect.

3. Strategies for Gene Mapping of Complex Diseases

The field of common complex disease genetics has in recent years moved from linkage to association study design because association analysis has far greater power to detect variants of modest effect and of lower frequency. The initial successes of hypertension gene mapping have come from studies of monogenic syndromes. The monogenic diseases are examples of diseases which are under very strong negative selection primarily because they affect fitness and are less likely to be transmitted to the next generation. The monogenic Mendelian forms of hypertension are thus rare, and responsible gene mutations are highly penetrant, and under very strong selection which keeps them at low frequencies with high levels of allelic heterogeneity. Thus these are highly amenable to linkage analysis. In contrast, susceptibility variants involved in essential hypertension are likely to have low or medium penetrance and are probably not subject to such strong selection resulting in lower allelic heterogeneity and greater prevalence of the trait. Thus linkage analysis as expected has not really provided robust validated loci for hypertension. To detect loci conferring a genotypic relative risk of 1.5 (minor allele frequency (MAF) = 0.1) by linkage analysis requires an estimated 67,816 affected sibling pairs (ASPs) whereas detection is possible through association with just 2218 singletons [10]. Moreover it is easier to recruit participants from the general population (than families as required for linkage), and there are fewer sampling restrictions in some disease categories such as late onset.

Association studies are typically performed in unrelated population samples (although it is possible to conduct them on related individuals). For qualitative traits they measure statistical association between a disease (phenotype) and genetic marker (genotype) directly by comparing allele frequencies of cases and controls. The aim is to establish whether a particular allele occurs in cases, compared with controls, more frequently than expected by chance. Quantitative traits, for example, blood pressure, cholesterol, and glucose, are assessed for association using linear regression. GWAS is typically an indirect or map-based approach. This measures the association between a phenotype and a marker allele (or "tag" SNP), which is correlated with the true causal allele due to linkage disequilibrium (LD). Linkage disequilibrium is defined as "the statistical association, within gametes in a population, of the alleles at two loci" [11] (on the same chromosome). It is assumed that typically a causal variant will not have been typed in GWAS which take advantage of LD to genotype a set of tag SNPs as proxies for the entire set of SNPs. The amount of LD between two loci is summarised by the metric r^2 (squared correlation coefficient for each SNP) which varies between 0 and 1. The maximum r^2 can be used to translate coverage (measure of how well the SNPs that are part of a genotyping set capture all known variants) to the sample size that is required for an indirect association study. To cover unobserved loci well an r^2 value of ≥ 0.8 with typed loci is considered sufficient [12]. In general SNPs in LD are more likely to be inherited together because they are physically close to each other on the chromosome. But this is not necessarily the case; studies have shown that levels of local LD vary, with some adjacent SNPs being independent despite their proximity and others of $\geq$100 kb apart being in a high or a significant LD [13]. Patterns of LD are affected by many factors such as population growth, population structure, admixture, natural selection, genetic drift, rate of recombination and mutation, and gene conversion [14].

Traditionally association studies tested hypotheses based on candidate genes, for which there was prior evidence (of known physiological pathways that affect the phenotype in question) that a genetic variant influenced disease risk. Unsurprisingly many of the candidate genes for hypertension are involved in sodium balance. No candidate gene study has yet demonstrated a reproducible association with hypertension [15]. There are several potential reasons for this, which

highlight the drawbacks of candidate gene studies:

(i) the wrong genes may have been selected;

(ii) the causative genes may be upstream or downstream from the genes studied;

(iii) discovery of genetic variants in novel pathways is not possible as candidate gene studies rely on a priori information regarding disease mechanisms.

In addition to the above there are the possibilities of population stratification, phenotypic and locus heterogeneity, and insufficient sample size: problems common to candidate gene studies and GWAS. Finally the SNPs studied might not provide complete coverage of the variants within the genes.

4. The GWAS Strategy

The chance of detecting genetic variants that influence common disease depends on the underlying genetic architecture. That is to say, the number of susceptibility alleles, whether they are common or rare, their frequency, and whether their action is neutral or deleterious. Allelic spectra vary greatly between disease genes. Common alleles and those of high frequency are of course easier to detect, as are deleterious alleles. The entire GWAS strategy is based on the Common Disease Common Variant (CDCV) hypothesis, that the causative genes for common diseases have relatively simple allelic spectra, that is, one or a few predisposing alleles of relatively high frequency. For GWAS it has been suggested that, as a rough guide, SNPs should meet a threshold of MAF $\geq$ 1% [16] or MAF $\geq$ 5% [11] to be considered common. As yet there is insufficient empirical evidence to determine the validity of the CDCV hypothesis, and arguments for and against have been put forward. These are crucial to research using SNP mapping to predict common disease risk, which assumes that the theory is by and large accurate (linkage studies of families or ASPs, by contrast, are robust to allelic heterogeneity).

A key part of the argument against the CDCV hypothesis rests on the fact that the risk of common disease depends on the interaction of many genes and environmental factors. In particular late-onset disorders of high prevalence in modern western society have been heavily influenced by changes in lifestyle factors such as diet and physical activity, and not by common disease-predisposing alleles. The risk conferred by any one factor, whether genetic or environmental, is weak and most cases are not predominantly determined by genetic variance. On the other hand, the relative risks observed in family members (a more rapid than linear decline in risk with increasing distance of relationship) support the conclusion that the majority of risk is due to a modest number of loci with common disease-predisposing alleles. The existing evidence suggests that alleles of both high and low frequency play a part in common disease [17–22].

Wang et al. have argued against making a distinction between rare and common disease-predisposing alleles [12]. Instead they propose that the allelic spectrum of disease associated variants be considered in the context of all variants in the human genome. In this framework the neutral model is that the allelic spectrum of disease variants and that of all variants are the same. Most susceptibility variants would be rare (MAF $<$ 1%); however SNPs with MAF $>$ 1% would still account for more than 90% of genetic differences between individuals and therefore make a significant contribution to phenotypic variation [23]. This lies somewhere between the two opposing views regarding the CDCV hypothesis. Common diseases vary in their allelic spectra depending on the evolutionary forces exerted upon them; nevertheless it is estimated that each will likely have hundreds of common and rare variants contributing to their familial clustering [12].

The International HapMap Project is a global consortium mapping all common SNPs in different populations across the world. The availability of SNP maps from HapMap led to a revolution in the dissection of common diseases and traits based on the common disease common variant hypothesis using the GWAS approach. This is a hypothesis-generating approach where no assumptions are made regarding the location or function of the causal variant. The dense genotyping chips that are now available contain hundreds of thousands of tag SNPs and offer increasingly better coverage of the human genome (whether within or outside genes). Adequate coverage requires $\geq$300 000 SNPs with more needed for African samples due to greater genetic diversity in those populations [11] and thus less LD [24, 25]. Though there is some concern that a set of tag SNPs that were selected in one population may not perform well in another [11, 14], the availability of dense SNP arrays will overcome this. There is also evidence of good tag SNP transference across populations [26, 27]. This is especially true for different populations within the same continent; the greatest disparities are between African and non-African samples.

While GWASs scan the genome for association signals without selecting for gene regions, Jorgenson and Witte have argued for a gene-centric approach to GWAS [28]. The reasons they outline are the following: genic variants are more likely to be functionally important than nongenic; variants in many genes are in lower LD than those outside genes so may be difficult to capture through indirect association. By focusing solely on genes and not the whole genome there is potential to increase coverage of genes and decrease the genotyping burden. The genic approach has greater power to detect variants within genes but suffers from a loss of power for nongenic variants. Despite this Jorgenson and Witte demonstrated empirically using HapMap data that it is more efficient in detecting causal variants than the indirect whole-genome approach when related to genotyping burden. Their suggestion of the best overall GWAS approach is to combine indirect genotyping data with gene-based SNPs in high priority regions, or alternatively to use a more stringent LD threshold in genic regions to "over-capture" genic SNPs.

A limitation of GWAS is that they are very expensive, especially with the large sample sizes that are required for small effects. However, technological advancements are rapidly reducing the cost of genotyping. In a bid to further reduce costs, some researchers have adopted a two-stage

GWAS study approach. In stage 1 a proportion of the samples are genotyped on all markers, and then in stage 2 a proportion of these markers are genotyped in the remaining samples [29]. Another approach to make studies more economical is the use of common controls for several groups of different disease cases. This was recently demonstrated by the Wellcome Trust Case Control Consortium (WTCCC) [30]. In 2008 Donnelly summarised all GWAS recorded at that time in the National Human Genome Research Institute catalogue [31], which amounted to more than 300 replicated associations for more than 70 common diseases and quantitative traits [32]. As of September 2009, there were more than 500 published GWAS at $P \leq 5 \times 10^{-8}$ (see Figure 1).

5. Study Design Issues in GWAS

5.1. Significance Thresholds. By convention statistical significance using frequentist methods is determined using the *P*-value threshold of .05, with values below this considered significant (i.e., there is evidence to reject the null hypothesis of no effect). This is not appropriate for GWAS because the large number of tests performed increases the chance of type I error. An alternative threshold, proposed by Risch and Merikangas and now widely adopted, is $P < 5 \times 10^{-8}$, which corresponds to an equivalent false positive rate of 5% for 1 000 000 independent tests of association [10]. This is calculated using the simple Bonferroni correction for multiple testing, which calculates a new significance threshold by dividing .05 by the number of tests performed. In practice this is conservative as it does not take levels of LD into account; the use of tagging SNPs means the genome can be covered sufficiently with around half this number of SNPs (i.e., ~500 000). This threshold is preferable to the traditional .05 but it has been argued that *P* value alone is not adequate for assessing significance. In addition to the possibility of false positives within a study, the issue of multiple testing can be viewed in the context of replication across studies. If several groups publish the same nominally significant association and these are combined, then the problem arises [11].

5.2. Statistical Power. Statistical power to detect a phenotype-genotype association is dependent upon the magnitude of effect, the frequency of causal allele(s), and the sample size. Moreover, for indirect association it is not only the disease predisposing allele frequency that matters, but also the marker allele (or tag SNP) frequency, and the power to detect an association is the greatest when these frequencies match [33]. The extent of LD also influences the likelihood of observing an association. However if the effect size is large this is less important with power being high even at low to moderate LD. Effect size for case control studies is measured as an odds ratio (OR), which estimates the odds of an individual in a given exposure group (i.e., with a certain genetic variant) being a case versus being a control. If the OR is significantly greater than 1 then the variant confers susceptibility to the disease, if it is significantly less than 1 then its effect is protective. Unfortunately large effects are usually rare.

For common complex diseases most published genetic effects have to date been modest (OR ~ 1.1–1.5) [17, 34], so a reasonable level of LD is necessary as well as the disease allele being common and close to the marker allele frequency. These conditions translate to a feasible sample size of several thousands of cases and controls. An exception, which no doubt increased expectations of similar findings, is the association between APOE4 and late-onset Alzheimer disease [35] for which the allelic OR is 3.3 [33]. As yet only a small percentage of the human genome has been subject to well-designed association study so it is unknown whether the published effect sizes are representative of the genome overall [12]. The effect sizes observed are expected due to the multifactorial nature of the diseases concerned and individually translate to only a small increase in population absolute risk. Multiple common risk variants of small effect have been combined theoretically, however, to construct risk scores of greater practical significance. Studies of the distribution of genetic effect sizes in other species such as rodents, *Drosophila melanogaster*, crops, and livestock suggest that there will be few genetic loci of large effect and many loci of small effect [36–42]. This view is now widely accepted in the field of common disease genetics [43].

5.3. Population Stratification. Population stratification acts as a confounder and can result in artefactual evidence of association [44–46]. It occurs when there are two or more strata in a population, and both the risk of disease and the frequency of marker alleles differ between strata. It therefore may appear that the risk of disease is related to the marker alleles when in fact it is not. A similar concept is admixture, which refers to "the mixture of two or more genetically distinct populations" [12]. The International HapMap Project has demonstrated clear genetic differences between geographically separated populations [47].

The effect of stratification on analysis increases with increasing sample size because even modest levels of underlying population structure are amplified [48, 49]. This has particular relevance to GWAS as they are employing larger and larger samples. A final important point is that confounding by population stratification tends to actually decrease (counter intuitively) with increasing number of ethnic groups [50]. This is because the direction of bias may differ between groups so that the overall combined effect is diluted. Whether population stratification is a real concern or not, to avoid any possibility of bias it is now commonplace in studies of unrelated cases and controls to employ stratification detection and correction methods [46].

Aside from matching cases and controls for genetic background and relevant environmental factors as well as possible and straightforward adjustment for ethnic group, a number of possible solutions to population stratification have been proposed. One is genomic control (GC) which employs detection and correction methods, for example, by using a bank of randomly selected markers (preferably > 100 [11]) that are unrelated to the question of interest to assess

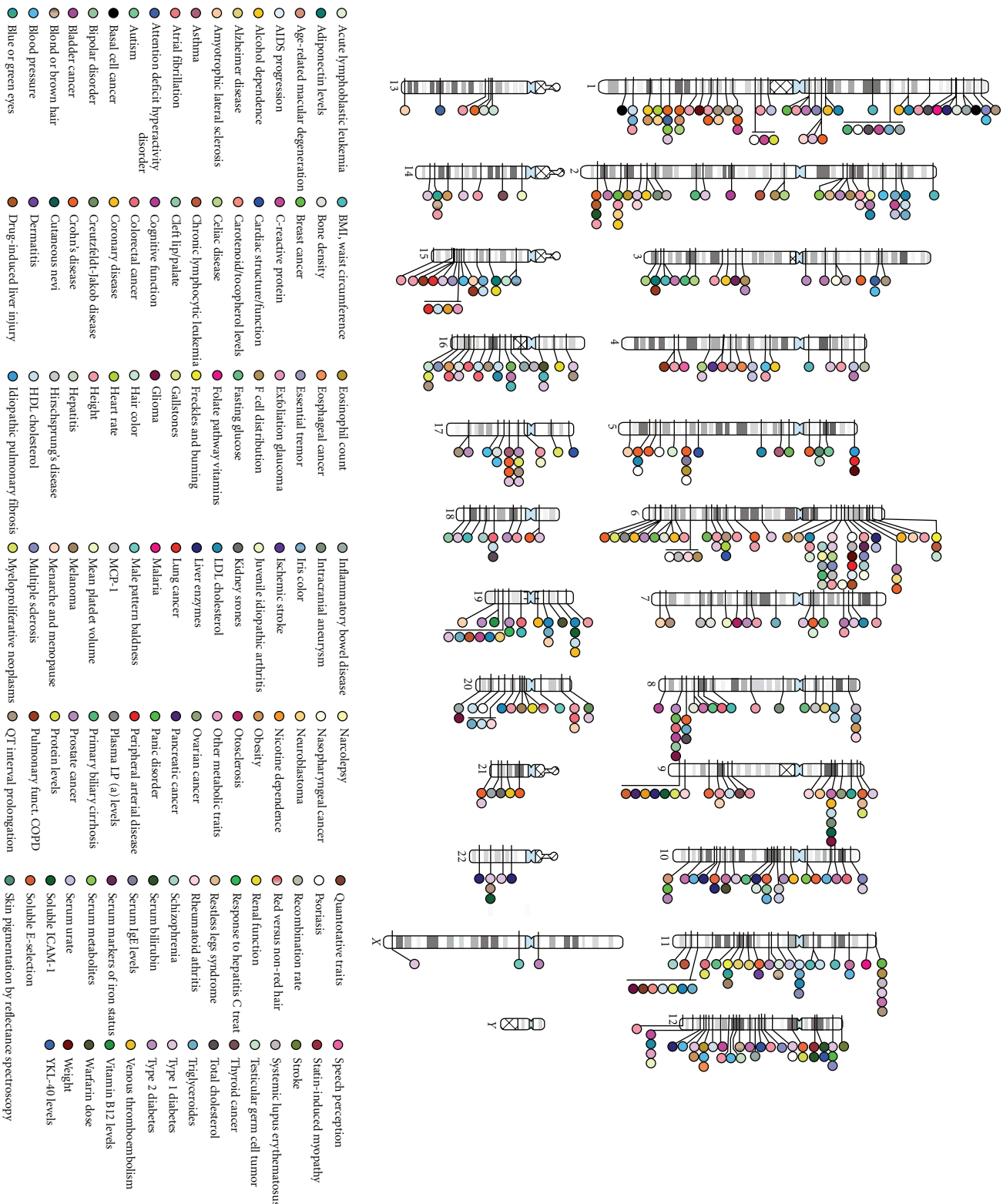

Figure 1: Published genome-wide associations until Sept. 2009. 536 published GWAS at $P \leq 5 \times 10^{-8}$ (reproduced with permission from [31]).

artefactual association [48, 51–53]. A scaling factor is then applied to the association results to adjust for the level of ethnic variation observed. A similar approach is structure assessment (SA), which too uses unlinked genetic markers for detection but then attempts to match homogeneous subgroups of the sample for association analysis within these subpopulations [54–57]. It is assumed that any significant association observed within a subpopulation cannot be due to population structure; there is an issue, however, about how many subpopulations to apply since they are a theoretical concept [11]. Explicit detection/correction methods by principal components analysis have also been employed [58, 59]. Due to the large number of markers genotyped in GWAS it is possible to detect low levels of stratification. However, a caveat to all of these detection methods is that with a large enough sample size even small biases will be statistically significant and may lead to overcorrection.

5.4. Replication. Many published association findings have failed to be replicated. This is partly due to the so-called "winner's curse," which is a bias whereby genetic effect size estimates are overestimated in initial discovery studies of disease-predisposing variants. The degree of effect size inflation can be reduced or eliminated by increasing sample size, and several correction methods have been proposed (e.g., [60, 61]). Technical bias can occur if cases and controls are not genotyped and analysed together in the same way. It is also thought that poor choice of controls and population stratification affect data quality and therefore play a part in replication failure. Meta-analyses that have examined replication failure indicate that in the majority of instances false positives are to blame [17, 62].

Replication of GWAS findings is as important as replication of candidate gene associations, if not more so. The large number of SNPs studied in GWAS and resulting volume of statistical tests performed increases the likelihood of observing type I errors, that is, false positives. In 2007 an NCI-NHGRI (National Cancer Institute and the National Human Genome Research Institute in the US) Working Group on Replication in Association Studies published an excellent summary of their recommendations on the reporting of initial association studies and criteria for replication [63]. There are certain situations in which there are insufficient participant numbers for replication, such as rare diseases or environmental exposures. These concerns do not affect most association studies of common diseases, though, so usually replication is advised. A strategy that increases power over that of individual studies and may be more cost effective than replication is meta-analysis of genome wide datasets [64, 65]. This collaborative way of working is increasingly common as investigators attempt to detect loci with smaller effects.

6. GWAS of Hypertension/Blood Pressure

To date there have been few GWAS of hypertension and/or blood pressure, with varying degrees of success in detecting associations with genetic variants. The WTCCC, made up of over 50 British research groups, conducted a GWAS study of 2000 cases each for 7 complex diseases of major public health importance; bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, type 1 diabetes, and type 2 diabetes. These were compared with 3000 shared common controls that came from two sources: 1500 from the 1958 British Birth Cohort and 1500 blood donors that were recruited for the project. The 2000 hypertension cases were unrelated participants from the British Genetics of Hypertension (BRIGHT) study [9]. Over the entire genome there were 21 SNPs identified with P values lower than the genome wide significance threshold of 5×10^{-7}. Of these, 10 were known associations. Unfortunately, of the 7 diseases of interest, hypertension could be described as the loser in that it was not associated with any SNPs at $P < 5 \times 10^{-7}$. Moreover there was no evidence for any of the variants previously associated with hypertension (at least partly due to some not being well tagged by the Affymetrix chip, for example, promoter of the WNK1 gene). There were, however, a similar number and distribution of marginal results (with P-values between 10^{-4} and 10^{-7}) to the other case groups.

It was speculated that the lack of a positive result for hypertension in the WTCCC study may have been due to poorly tagged variants or that hypertension may have few common risk alleles with larger effect sizes. Furthermore, misclassification bias may have reduced the power of detecting effects. The common controls were not specifically phenotyped for blood pressure. Due to the high prevalence of hypertension and its existence on the continuum of blood pressure some of the controls may have been misclassified cases. It was estimated that the misclassification of 5% of controls (i.e., if 5% of controls were in fact undiagnosed cases) would translate to a loss of power equivalent to a 10% reduction in sample size [30]. This is because of the dilution of any observable genetic difference, caused by the blurring of the distinction between cases and controls. Considering the expense of genome-wide association analysis and the anticipated relatively small effect sizes any reduction in power poses a serious problem. Moreover individuals with blood pressure in the mid-range of normotension (that is not considered to pose a risk clinically) may still be at increased risk in relation to individuals with low-blood pressure.

Two recent studies that have had considerably more success are the Global Blood Pressure Genetics (Global BPgen) Consortium study, conducted by Newton-Cheh et al. (2009) [71], and the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium study, conducted by Levy et al. (2009) [72]. But in contrast to the WTCCC, these were studies of blood pressure as a quantitative trait and on hypertension. Both had very large discovery samples, providing sufficient power to overcome variations in genotyping platform, participant ascertainment and method of blood pressure measurement between component studies. Each dealt with the confounding effect of antihypertensive medication by adding 15 mmHg to recorded SBP and 10 mmHg to DBP for those who were on such treatment.

In the Global BPgen discovery GWAS ($N = 34,433$) two SNPs achieved $P < 5 \times 10^{-8}$ (considered genome wide

Table 1: Summary of recent GWAS of hypertension and/or blood pressure.

	Publication date	Phenotype	Discovery sample			Discovery and replication meta-analysis		
			N	OR/beta	Lowest *P*-value	*N*	OR/beta	*P*-value
Levy et al. [66]	Sept-07	DBP	1233	—	3.31×10^{-6}	—	—	—
		SBP	1260	—	1.69×10^{-6}	—	—	—
*Wang et al. [67]	Jan-09	SBP	542	—	7.6×10^{-5}	7125	1.9	1.6×10^{-7}
Org et al. [68]	Mar-09	hypertension	364/596	0.49	2.34×10^{-6}	3808/4334	0.78	1.39×10^{-6}
Cho et al. [69]	May-09	SBP	8842	−1.309	9.1×10^{-7}	16703	−1.064	1.3×10^{-7}
Adeyemo et al. [70]	Jul-09	DBP	8842	−0.882	1.2×10^{-6}	16703	−0.63	3.0×10^{-6}
		SBP	1017	—	4.72×10^{-8}	—	—	—
		DBP	1017	—	.448	1997	—	.162
		hypertension	509/508	0.58	5.10×10^{-7}	875/1122	—	.009

* Observed an SNP with a lower *P*-value but did not report on it as situated in gene desert.
—Value not reported (or in the case of meta-analysis replication not attempted).

significance for this analysis). Once these and borderline significant findings were meta-analysed with validation data there were eight loci associated with either DBP or SBP genome-wide. Investigators also assessed whether these loci were associated with hypertension. The initial GWAS was not conducted for hypertension due to lack of power, so instead the significant loci were examined in planned secondary analysis (N range $= 57,410$–$99,802$). In the secondary samples all eight alleles showed association with hypertension in the same direction of effect as continuous blood pressure. It should be noted that all of the reported associations translate to a very small change in blood pressure, approximately 1 mmHg per allele SBP or 0.5 mmHg per allele DBP. However, the effects of multiple variants can be combined to produce a meaningful change in population cardiovascular risk.

Taking a genome wide significance threshold of $P < 4 \times 10^{-7}$, the CHARGE discovery GWAS ($N = 29,136$) identified 13 significant SNP associations for SBP, 20 for DBP, and ten for hypertension. There is quite a bit of overlap between phenotypes with many of the top hits attaining significance in the same direction of effect for more than one phenotype.

The top ten loci for SBP, DBP, and hypertension (30 in total) in the CHARGE cohort were checked for significance in the Global BPgen results. One SNP for SBP, four for DBP, and one for hypertension were assessed for independent replication in Global BPgen. Five SNPs out of this six attained $P < .008$, the threshold for external replication in Global BPgen. When the results for the same 30 SNPs in both studies were analysed together by meta-analysis, there were four associations of genome wide significance ($P < 5 \times 10^{-8}$) for SBP, six for DBP, and one for hypertension. Again effect sizes were very small, approximately 1 mmHg change in SBP per allele or 0.5 mmHg change in DBP per allele.

The remaining published studies have demonstrated far less success in identifying genetic variants that are associated with either hypertension, SBP, or DBP. Many have not observed any SNPs that reached genome-wide significance [66, 67, 73–75]. One possible reason for this failure in some studies is that hypertension and/or blood pressure were not the primary trait of interest [73], or not of a priori interest when the cohort was recruited [75] therefore phenotyping may not have been necessarily thorough. Another possibility is that there were not enough SNPs studied to provide sufficient coverage of the whole genome; in some studies fewer than 100 000 SNPs passed quality control measures [66, 67, 74].

Table 1 summarises the most significant hits from GWAS of hypertension and/or blood pressure published since 2007 (other than WTCCC, Global BPgen, and CHARGE), along with meta-analysed discovery and replication results if replication was attempted. One study conducted by Sabatti et al. [75] is omitted from the table because the authors did not publish any results for blood pressure, instead reporting that analysis of blood pressure did not produce any genome-wide significant results. However it was not the primary trait of interest; the authors also studied triglycerides, HDL, LDL, CRP, glucose, insulin, and BMI.

Levy et al. [66] and Wang et al. [67] failed to find any associations of genome-wide significance but had limited genomic coverage, studying just 70,897 and 79,447 SNPs, respectively. Furthermore the discovery sample employed by Wang et al. was small at 542 participants. Org et al. [68] and Cho et al. [69] also did not find any associations of genome-wide significance.

The vast majority of GWAS thus far have been conducted on samples of Caucasian individuals of European ancestry. One of the few studies to examine African Americans was that of Adeyemo et al. [70]. Their initial findings were promising; however replication was either not attempted (in the case of SBP) or the replication findings were in the opposite direction of effect (DBP and hypertension).

7. Future Directions

Following an initial dearth of positive results from GWAS of hypertension and blood pressure, the methodological advancement and achievements of recent studies provide

optimism and guidance for continued work in this area. Where to now in the search for further novel gene polymorphisms? One approach that has been proposed to increase the likelihood of detecting genetic effects is the recruitment of hypercontrols and individuals with severe hypertension [15]. This requires high fidelity phenotyping, with the overall study design comparing cases and controls at the extreme high and low ends, respectively, of the blood pressure distribution. This would markedly reduce the risk of misclassification bias and thus minimise any resultant loss of power.

Much of the unexplained variation in blood pressure may be due to rare variants that are undetectable through traditional GWAS case-control study design. However, intensively genotyped samples are becoming available from the 1000 Genomes Project, an open resource catalogue of human genetic variation [76]. The project is run by an international consortium and aims to describe over 90% of genetic variation down to 1% MAF. To date the genomes of more than 1000 individuals have been sequenced (with planned expansion to 2000 individuals in 2010) and around 10 million novel variants identified. The resource will enable the study of low frequency variants and aid fine mapping of regions of interest. Furthermore, next-generation deep sequencing technology is now available which will also increase the chances of rare variant detection [77]. All of this is carried out with the ambition to improve the diagnosis and treatment of this widespread condition.

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Review Article

Genome-Wide Association Study of Coronary Artery Disease

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Coronary artery disease (CAD) is a multifactorial disease with environmental and genetic determinants. The genetic determinants of CAD have previously been explored by the candidate gene approach. Recently, the data from the International HapMap Project and the development of dense genotyping chips have enabled us to perform genome-wide association studies (GWAS) on a large number of subjects without bias towards any particular candidate genes. In 2007, three chip-based GWAS simultaneously revealed the significant association between common variants on chromosome 9p21 and CAD. This association was replicated among other ethnic groups and also in a meta-analysis. Further investigations have detected several other candidate loci associated with CAD. The chip-based GWAS approach has identified novel and unbiased genetic determinants of CAD and these insights provide the important direction to better understand the pathogenesis of CAD and to develop new and improved preventive measures and treatments for CAD.

1. Introduction

Coronary artery disease (CAD) including myocardial infarction (MI) is a leading cause of death worldwide [1, 2]. The well-known conventional coronary risk factors include age, male sex, hypertension, diabetes mellitus, hypercholesterolemia, smoking and family history, which have been repeatedly demonstrated in multiple epidemiological studies [3–5]. Lifestyle and environmental factors play an important role in the pathogenesis; however, genetic predisposition is also thought to contribute to CAD/MI since these diseases cluster in families [6].

In the epidemiological studies using twins, the relative hazard of death among men from CAD when one's twin died of CAD before the age of 55 years, as compared with the hazard when one's twin did not die before 55, was 8.1 for monozygotic twins and 3.8 for dizygotic twins [7]. The recent epidemiological survey in the Framingham study showed that parental cardiovascular disease independently predicted future offspring events [8]. In this survey, participants with at least one parent with premature CAD had greater risk for events with age-adjusted odds ratios (ORs) of 2.6 for men and 2.3 for women compared with those with no parental CAD. These results support further research into genetic determinants of CAD risk. Elucidating the genetic determinants would improve risk assessment and provide better measures for prevention and treatment.

As the molecular biology and genetics had progressed, the genetic backgrounds were explored in the several genes which were thought to contribute to the pathogenesis of atherosclerosis and conventional coronary risk factors. Candidate gene studies tested the hypothesis that proteins known to be involved in the pathogenesis of atherosclerosis carry variants that affect their protein functions and the risk of developing CAD. In 1992, Cambien et al. [9] explored a possible association between CAD and a variation found in the gene encoding angiotensin-converting enzyme (ACE). The polymorphism ACE/insertion/deletion (ACE/ID) is strongly associated with the level of the circulating enzyme. They reported that the deletion homozygote (DD genotype),

which was associated with higher levels of circulating ACE, is significantly more frequent in patients with MI than in controls.

The representative variants associated with CAD/MI found by candidate gene approach are listed in Table 1. However, those candidate genes were not always reproducible in multiple studies later on. One of the reasons for poor reproducibility is that many of the study samples were not large enough with some exceptions to identify disease-associated genetic variants with odds ratio <2.0. In addition, candidate gene studies only tested a single to few variants for association with CAD and these approaches cannot discover unknown novel variants and also cannot evaluate how strong each variant contribute to the susceptibility to CAD. Therefore, the candidate gene approach resulted in only limited success in the elucidation of genetic risks for CAD.

In parallel with candidate gene studies, other strategies were carried out to interrogate the entire human genome without hypotheses on which genes may be responsible for disease risk. One of the strategies is genome-wide linkage analysis and it is based on the Mendelian cosegregation of a genetic marker within a family. However, great efforts had to be made in order to collect sufficient numbers of affected sibling pairs. Only a small number of studies were successfully performed and few genetic loci (2q21-22, Xq23-26 [10], myocyte enhancer factor-2 (*MEF2A*) [11], arachidonate 5-lipoxygenase-activating protein (*ALOX5AP*) [12], leukotriene A4 hydrolase (*LTA4H*) [13]) were detected to be associated with CAD (Table 2). These genes had never been suggested as causative genes before these family-based studies, suggesting the effectiveness of this approach in detection of novel genetic determinants. However, those associations were not always replicated. Such a family-based study has been frequently used to identify new loci in monogenic diseases, but the application of this strategy to multifactorial diseases is relatively limited.

In this situation, whole genome analysis in a case-control study design had gradually emerged around the beginning of the 21st century. Resources including the single nucleotide polymorphism (SNP) databases, major technological advances in high-throughput genotyping, and methods of data processing and statistical analysis allow researchers to confront limitations in previous approaches. Here, we introduce the representative genome-wide case-control association studies in the next two sections.

2. Establishment of J-SNP Database and Whole Genome Approach in Japan

Beginning in 2000, the Prime Minister's Millennium Project (J-SNP) was launched in Japan and about hundred thousand SNPs located in genes or in adjacent regions that might influence the coding sequence of the genes were identified in Japanese population. J-SNP established a web-based database and allowed researchers access to high quality SNP data [14]. Genome-wide association studies using this SNP database were performed in our country in many important

Table 1: The well-known genetic polymorphisms which are thought to be associated with myocardial infarction or coronary artery disease.

Location	Gene name/Polymorphisms
17q23	Angiotensin-Converting Enzyme insertion/deletion (intron 16)
1q42-q43	Angiotensinogen Met235Thr, −6G/A
3q21-q25	Angiotensin II type1 Receptor 1166A/C
8q21-q22	Aldosterone Synthase (CYP11B2) −344T/C, Lys173Arg
14q32.1-q32.2	Bradykinin B2 receptor gene −58T/C
6p24.1	Endothelin-1 Lys198Asn
7q36	eNOS Glu298Asp, −786T/C
17q21.32	Glycoprotein IIIa P1A1/A2
5q23-31	Glycoprotein Ia 807T/C
17pter-p12	Glycoprotein Ibα Thr145Met
4q28	βfibrinogen −455G/A
11p11-q12	Prothrombin 20210G/A
7q21.3-q22	PAI-1 4G/5G (promoter region)
7q21.3	Paraoxonase1 Arg192Gln, Leu54Met
8p12-p11.2	Werner Helicase Gene Cys1367Arg
1p36.3	Methylenetetrahydrofolate reductase 677C/T
16q24	NADH/NADPH oxidase p22phox 242C/T, 640A/G
5q31.1	CD14 Monocyte Receptor −260C/T
11q22.3	Stromelysin (MMP3) 5A/6A (promoter region)
20q11.2-q13.1	Gelatinase B (MMP9) −1562C/T
19q13.2	ApolipoproteinE E2/E3/E4
16q21	Cholesteryl Ester Transfer Protein (CETP) Ile405Val
9q31.1	ABCA1 gene Ile823Met
3p25	PPAR-gamma Pro12Ala, Pro115Gln
20q13.11-q13.13	Prostacyclin synthase gene
The number of 9-bp (CCGCCAGCC) repeats (promoter region)	
17q11.2-q21.1	MCP-1 −2518G/A

Table 2: Representative loci associated with CAD identified in the family-based studies.

Reporter (year)	Race	locus	LOD score	causative gene
Pajukanta et al. [10]	Finland	2q21-22	3.7	no gene identified
		Xq23-26	3.5	no gene identified
Wang et al. [11]	USA	15q26	4.19	MEF2
Helgadottir et al. [12]	Iceland	13q12-13	2.86	ALOX5AP
Helgadottir et al. [13]	Iceland	17q22	NA	LTA4H

NA = not available.

clinical fields including cardiovascular diseases, diabetes, renal dysfunction and autoimmune collagen diseases.

As the first genome-wide case-control association study in the world, Ozaki et al. [15] used 92,788 gene-based SNP markers and identified that the homozygosity in two SNPs in lymphotoxin A (*LTA*) at 6p21 was significantly associated with increased risk for MI in Japanese (odds ratio (OR) = 1.78). In vitro analyses showed that one functional SNP (Thr26Asn) caused a twofold increase in induction of several inflammation-related cell-adhesion molecules including vascular cell adhesion molecule 1 (VCAM1) in vascular smooth-muscle cells. Moreover, the SNP located in intron 1 of LTA enhanced the transcriptional level of LTA. These results indicated the variants in the LTA are risk factors for MI and implicated LTA as a novel pathogenic factor for MI. In the same year, LTA knockout mouse was shown to be resistant to atherosclerosis [16]. Double knockout mice of apolipoprotein E (ApoE) and LTA (ApoE−/−LTA−/− mice) showed less extent of atherosclerosis than ApoE−/−LTA+/+ mice, indicating LTA deteriorates atherosclerosis in vivo, consistent with the result of the genetic association study of LTA as a genetic risk for atherosclerotic disease.

Subsequently, they identified SNPs that were significantly associated with MI in lectin, galactoside-binding, soluble, 2 gene (*LGALS2*) [17], proteasome subunit alpha type 6 gene (*PSMA6*) [18], myocardial infarction associated transcript (*MIAT*) [19], inter-alpha (globulin) inhibitor 3 gene (*ITIH3*) [20] and BRCA1-associated protein gene (*BRAP*) [21] mainly by functional approaches. All the six causative genetic regions are related to inflammatory process in vasculature and are thought to contribute to the process in atherosclerotic changes through its inflammatory functions and thus increase the risk of MI.

3. Chip-Based GWAS and Novel Candidate Genetic Determinants for CAD

3.1. HapMap Project. Genome-wide association studies based on the J-SNP database (approximately 100,000 SNPs) have detected several SNPs which had significant association with myocardial infarction. However, the J-SNP database does not cover SNPs in intergene regions. In the meantime, the International HapMap Project was conducted to create a public genome-wide database of common SNPs and enable systematic studies of common SNPs for their potential role in human disease [22, 23]. The Project analyzed DNA samples from 90 people with European ancestry, 90 Yoruba people in Nigeria, 44 Japanese and 45 Han Chinese and has now genotyped over 3.1 million SNPs in each of these populations. However, testing all of these SNPs in a person's chromosomes would be extremely expensive. Adjacent SNPs across the genome are correlated each other, a phenomenon known as linkage disequilibrium and SNPs that are inherited together were compiled into "haplotypes". A haplotype block may contain many SNPs, but only a few "tag" SNPs can provide most of the information on the pattern of genetic variation in the block. The HapMap project identified these "tag" SNPs within haplotypes that uniquely identify these haplotypes. The HapMap data allowed efficient design of Chip-based genome association studies and allowed investigators to genotype far fewer SNPs while still retaining statistical power to find genetic variants related to common illness.

3.2. High throughput SNP Genotyping Platforms. The development of dense genotyping chips enables genotyping up to 1 million SNPs on a single small chip. This chip technology allowed genome-wide association studies (GWAS) to be performed on a large numbers of subjects. Chip-based GWAS typically involves genotyping approximately a few thousands cases with a disease and a few thousands of controls for about 500,000 tag SNPs. Since there are 500,000 comparisons per study, there is a high potential for false positive results. The proposed solution for that is applying the stringent P value using the Bonferroni correction for multiple tests. In that case, P value will be 0.05 divided by 500,000 and that is 0.0000001 (10^{-7}) and this stringent P value is often termed as 'genome-wide significance'. The most statistically significant variants identified in the initial case-control analysis are tested for replication in subsequent case-control studies. In GWAS method, associations between SNPs and the diseases are made free of bias of particular candidate genes. This makes the possibility of obtaining novel and unbiased information and provides the important direction to better understand the pathophysiology of the disease. For CAD, three chip-based GWAS were simultaneously reported in 2007 and all of them showed the significant association between CAD and SNPs on chromosome 9p21.

3.3. 9p21 and other Chromosomal Loci Associated with CAD/MI Detected in Chip-Based GWAS. Helgadottir et al. enrolled a total of 4587 MI cases and 12,767 controls and genotyped total 305953 SNPs using Illumina Hap300chip (Illumina) [24] (Table 3). All the participants were European descent. They identified disease association variant located in 9p21, adjacent to the tumor suppressor genes cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase inhibitor 2B (*CDKN2B*) with great statistical significance. This region had never been estimated to be associated with susceptibility to MI. They showed the allele G of the SNP rs10757278 (Figure 1) showed the strongest association with MI. The ORs for heterozygous and homozygous carriers of the risk allele G were 1.26 and 1.64, respectively. The ORs for early-onset MI (MI before the age of 50 for males and before the age of 60 for females) are 1.49 and 2.02 for heterozygous and homozygous carriers of the risk allele, respectively. They estimated the population attributable risk is 21% for MI in general and 31% for early-onset cases.

The SNPs on chromosome 9p21 associated with MI are located in the same disequilibrium block of the one which contains *CDKN2A* and *CDKN2B*. These genes encode two members of the inhibitors of CDK4 (Ink4) family of cyclin-dependent kinase inhibitors, $p16^{Ink4a}$ and $p15^{Ink4b}$, and a completely unrelated protein called ARF. The $p16^{Ink4a}$ and $p15^{Ink4b}$ which activates retinoblastoma (Rb) family members and ARF which activates p53 were shown to be upregulated in cancer cells. They play a critical role in cell proliferation and aging, senescence and apoptosis [25, 26]. However, sequencing 93 early-onset MI patients across these genes did

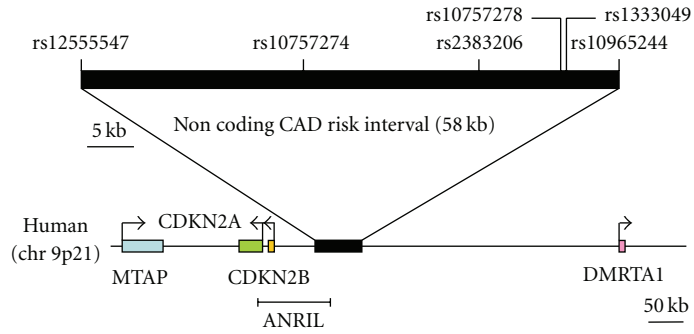

Figure 1

Figure 1: The 58 kb non-coding CAD risk interval on chromosome 9p21.

not reveal obvious causal functional variants or variants that could account for the correlation of rs10757278 to MI. The linkage disequilibrium block also contains two exons of the transcript hypothetical methylthioadenosine phosphorylase fusion protein mRNA, however the functional significance of the variants in this region remains to be elucidated.

McPherson et al. also identified a 58-kilobase region on chromosome 9p21 that was consistently associated with CAD in six independent samples consisted of 4306 cases and 20119 controls from four Caucasian populations [27]. They identified two SNPs rs10757274 and rs2383206 on 9p21 that are significantly associated with incident of CAD. The risk allele was associated with and ~15 to 20% increase risk of CAD in the 50% individuals who are heterozygous and ~30 to 40% increase in the 25% who are homozygous for the allele.

They further genotyped surrounding region of these SNPs in detail and found that eight additional SNPs at the locus spanning a 58-kb region were significantly associated with CAD. Again, the 58-kb region does not contain any annotated genes. however, the region overlaps a newly annotated noncoding RNA called noncoding RNA in the *INK* locus (*ANRIL*) [28]. *ANRIL* consists of 20 exons subjected to alternative splicing. The whole blood RNA expression levels of short variants of *ANRIL* are increased and the expression levels of the long variant are decreased in subjects homozygous for the risk alleles. There is also a positive correlation between transcript levels of the long variant of *ANRIL* and *CDKN2B* [29].

The study using genetically engineered mice showed that the deletion of orthologous 70-kb non-coding interval on mouse chromosome 4 (chr4$^{\triangle 70kb/\triangle 70kb}$mice) highly reduced cardiac expression of two neighboring genes *CDKN2A* and *CDKN2B*. Primary culture of smooth muscle cells from chr4$^{\triangle 70kb/\triangle 70kb}$mice showed increased proliferation and diminished senescence, the features relevant to atherosclerosis [30]. These findings indicated the noncoding interval is involved in the disease process via gene-regulatory effects on *CDKN2A* and *CDKN2B*.

The Wellcome Trust Case Control Consortium (WTCCC) study which enrolled 1926 case subjects with CAD and 2938 controls also reported the powerful association between the SNPs on chromosome 9p21 and CAD [31]. The strongest signal was seen at rs1333049, however the associations were seen for SNPs across >100 kb. Then, they further looked for replication in the German MI family study which involved 875 MI cases and 1644 controls using the GeneChip Human Mapping 500K Array Set (Affymetrix) [32]. The Same locus on chromosome 9p21 (rs1333049) had the strongest association with CAD in both studies with the risk increased by 36% per copy of the C allele. Of the nine loci which were shown to be strongly associated with CAD, two of these loci were able to replicate in the German MI study: chromosome 6q25.1 (rs6922269) and chromosome 2q36.3 (rs2943634). Further, the combined analysis of the two studies revealed four additional loci significantly associated with CAD: chromosome 1p13.3 (rs599839), 1q41 (rs17465637), 10q11.21 (rs501120) and 15q22.33 (rs17228212). In 2009, they performed another replication study with 11550 cases with CAD and 11205 controls from 9 European studies [33]. Other than the 9p21 locus, they confirmed significant association at 1p13.3 (rs599839), 1q41 (rs3008621) and 10q11.21 (rs501120). They were not able to show the significant association with 6q25.1 and 2q36.3 and there was no evidence for association with the locus at 15q22.33. The four loci (9p21, 1p13.3, 1q41 and 10q11.21) act independently and cumulatively increased the risk for CAD by 15% per additional risk allele. The genes located within or adjacent to the these four loci are listed in Table 4. The locus at chromosome 1p13.3 has been shown to be associated with increased plasma LDL cholesterol, and thus may contribute to CAD development [34–37].

The locus at 10q11.21 lies adjacent to the chemokine (C-X-C motif) ligand 12 gene (*CXCL12*) which encodes stromal cell-derived factor-1, a chemokine which plays a important role in stem-cell homing and regeneration of myocardial tissue in ischemic cardiomypopathy [38] and in promoting angiogenesis by recruiting endothelial progenitor cells from the bone marrow [39]. The SNPs at 1q41 locates within the melanoma inhibitory activity family, member 3 (*MIA3*) gene [40]. Underlying mechanism how these genetic loci affect the pathogenesis of CAD need to be further investigated.

The meta-analysis of aforementioned three studies [24, 27, 32] and 7 additional case-control studies successfully

Table 3: Representatives of chip-based GWAS of CAD/MI.

Author, year	Phenotype	No. of cases/controls	Chromosomal loci	OR
Helgadottir et al. [24]	MI	4587/12767	9p21	1.28
McPherson et al. [27]	CAD	3505/18745	9p21	1.26 (CCHS study)
			9p21	1.16 (CCHS study)
WTCCC [31]	CAD	2000/3000	9p21.3	1.47
Samani et al. [32]	CAD	2801/4582	9p21.3	1.28 (adjusted German)
			6q25.1	1.23 (adjusted German)
			2q36.3	1.08 (adjusted German)
			1p13.3	1.29
			1q41	1.2
			10q11.21	1.33
			15q22.33	1.21
CAD Consortium, [33]	CAD	11550/11205	9p21	1.2
			1p13.3	1.13
			1p41	1.1
			10q11.21	1.11
Clarke et al. [59]	CAD	7991/7946	6q26-27	1.51

Table 4: Genes located within or adjacent to the loci associated with CAD/MI.

Chromosome loci	Genes
1p33	PSRC1, CELRS2, MYBPHL, SORT1
1q41	MIA3
2q33	WDR12
2q36.3	no recognized genes
3q22.3	MRAS
6p24	PHACTR1
6q25.1	MTHFD1L
6q26-27	LPA
9p21	p16/CDKN2A, p15/CDKN2B, p14/ARF, MTAP, ANRIL
10q11.21	CXCL12
12q24	3SH2B3
21q22	MRPS6, SLC5A3, KCNE2

replicated the significant association between the risk allele (C) of the lead SNP, rs1333049 at chromosome 9p21 and risk of CAD (OR = 1.29) [41]. These study have analyzed primarily on European descent, however, since the allele frequency differs among the different ethnic groups, the risk of CAD related to the SNPs at 9p21 may differ among each ethnic group. Since then, the replicated results in other ethnics such as Chinese, Japanese and Pakistanis are published and the association of the SNPs at 9p21 with CAD seems to be consistent among various ethic groups [42–45].

It is noteworthy that the SNPs in 9p21 region are also found to be associated with variety of diseases such as ischemic stroke (OR = 1.01–1.21) [46, 47], abdominal aortic aneurysm (OR = 1.31), intracranial aneurysm (OR = 1.29) [48], peripheral artery disease (OR = 1.29) [49], incident heart failure (OR = 1.17) [50], perioperative myocardial injury after coronary artery bypass graft surgery [51], type 2 diabetes (OR = 1.20) [52, 53].

More recently, the loci other than 9p21 have been shown to be associated with CAD or MI. Erdmann et al. applied less stringent statistical thresholds on their GWAS for CAD to identify any dismissed SNPs with modest effects or low allele frequencies and they found one new locus on 3q22.3 in muscle RAS oncogene homolog (*MRAS*) (OR = 1.15), the gene thought to play an important role in inflammation [54, 55].

There is another GWAS which identified the SNPs in the gene related to inflammation to be associated with MI [56]. They found five SNPs which affect eosinophil counts in blood in Icelandic population and reported that a nonsynonymous SNP at 12q24 in SH2B adaptor protein gene (3*SH2B3*) was associated with MI significantly (OR = 1.13).

The GWAS of early-onset MI revealed 9 loci which have significant association [57]. Three of them were newly identified in the study: (i) an intergenic region between *MRPS6* (mitochondrial ribosomal protein S6), *SLC5A3* (solute carrier family 5 (sodium/myo-inositol cotransporter), member 3) and *KCNE2* (potassium voltage-gated channel, Isk-related family, member 2) on chromosome 21q22 (OR = 1.19), 6p24 in *PHACTR1* (phosphatase and actin regulator 1) (OR = 1.13) and 2q33 in *WDR12* (WD repeat domain 12) (OR = 1.17). The mechanism by which genes at these three regions increases the risk of MI needs to be elucidated. In addition to the common variant, copy number variations can be analyzed by SNP chip and there were no common or rare copy number variations associated with risk of early-onset MI in this study.

The group from the WTCCC/German MI study further conducted a genome-wide haplotype association study for

the first time and identified the *SLC22A3-LPAL2-LPA* gene cluster on 6q26-27 as a strong susceptibility locus for CAD (OR = 1.8) [58]. An increased level of Lp(a) lipoprotein is a classical hereditary risk for CAD. Clarke et al. identified three chromosomal regions (6q26-27, 9p21 and 1p13) were strongly associated with the risk of CAD using the Human CVD Bead Chip which included 48742 markers relevant to cardiovascular disease on 6500 subjects. Among them, the *LPA* locus on 6q26-27 encoding Lp(a) lipoprotein had the strongest association. They identified two *LPA* variants that were strongly associated with both an increased level of Lp(a) lipoprotein and an increased risk of CAD (OR = 1.70 and 1.92) [59]. Both variants were strongly associated with a reduced copy number in *LPA* kringle IV-type 2 repeats and an increased level of Lp(a) lipoprotein. After adjustment for the Lp(a) lipoprotein level, the association between the *LPA* genotype score and the risk of CAD was abolished. The importance of classical risk factor Lp(a) was reemphasized by the GWAS.

4. Limitation of GWAS

Initially, GWAS have been primarily assessed only on European descent and the results of these GWAS may not be applicable to other ethnics due to wide difference of distribution of SNPs and allele frequency. Further studies for various ethnicity need to be done with use of newer chips which contains 1 million SNPs to increase coverage.

The CAD associated loci have been found in regions without known gene-encoding loci. Therefore, further studies will be required to elucidate the exact functional mechanism by which these loci modulate CAD risk.

Utility of genotyping 9p21 for clinical risk assessment is controversial [60–62]. The odds ratios for CAD risk in each selected SNPs are small (around 1.2) and explain only a small proportion of the heritable, genetic component of susceptibility to the disease. Newer susceptibility loci for CAD need to be validated with replication studies and in the future, we should evaluate the genetic risks by combining multiple independent common variants susceptible for CAD.

The GWAS method is supported by the common disease-common variant hypothesis, which predicts that genetic variants causing common disease exist frequently, but each variant only have a small effect on disease susceptibility. Another hypothesis is the rare variant hypothesis. Rare variants have a minor allele frequency of less than 1%. The rare variant hypothesis postulates that common disease is caused by multiple rare variants which have a strong causative effect on disease and this hypothesis was confirmed in colorectal adenomas [63, 64]. Rare variants cannot be captured by GWAS and requires whole genome sequencing using next generation sequencing system.

In addition, other types of variants, such as insertion-deletion variant, block substitution and inversion variant, so called structural variants may account for important contributors to the diseases and are also hard to detect by the chip-based method. The next generation sequencing method is also helpful to find these structural variants.

5. Conclusion

(1) The SNPs Data from the HapMap project and development of new chip technology enabled genotyping large amount of common variants simultaneously and contributed to efficiently identify gene loci affecting susceptibility to common diseases including CAD.

(2) The region at 9p21 was shown to be significantly associated with CAD in 2007 and comprehensive replication across multiple studies provides unequivocal evidence that this locus is associated with CAD in European descent. This region is also associated with abdominal aneurysm, intracranial aneurysm and type 2 diabetes, and seems to be a very important region for various diseases.

(3) Since the odds ratios of the risk allele at 9p21 for CAD are small, screening for this risk allele probably affects little, if any, to the each individual's risk prediction. Using genomic tests to improve existing risk models would likely require combining the effects of multiple common genetic variants.

(4) Rare variants and structural variants which cannot be captured by GWAS need to be searched by whole genome sequencing.

(5) GWAS approach has identified novel and unbiased genetic contributors to CAD and these insights provide the important direction to better understand the pathogenesis of CAD and to develop new and improved preventive measures and treatments for CAD.

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Review Article

Scavenger Receptors and Their Potential as Therapeutic Targets in the Treatment of Cardiovascular Disease

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Scavenger receptors act as membrane-bound and soluble proteins that bind to macromolecular complexes and pathogens. This diverse supergroup of proteins mediates binding to modified lipoprotein particles which regulate the initiation and progression of atherosclerotic plaques. In vascular tissues, scavenger receptors are implicated in regulating intracellular signaling, lipid accumulation, foam cell development, and cellular apoptosis or necrosis linked to the pathophysiology of atherosclerosis. One approach is using gene therapy to modulate scavenger receptor function in atherosclerosis. Ectopic expression of membrane-bound scavenger receptors using viral vectors can modify lipid profiles and reduce the incidence of atherosclerosis. Alternatively, expression of soluble scavenger receptors can also block plaque initiation and progression. Inhibition of scavenger receptor expression using a combined gene therapy and RNA interference strategy also holds promise for long-term therapy. Here we review our current understanding of the gene delivery by viral vectors to cells and tissues in gene therapy strategies and its application to the modulation of scavenger receptor function in atherosclerosis.

1. Introduction

Scavenger receptors comprise a structurally diverse group of proteins [1]. Originally identified by Brown and Goldstein, they were defined by their ability to bind modified forms of low density lipoprotein (LDL) including acetylated LDL (AcLDL) and oxidized LDL (OxLDL) and were thus implicated as key regulators in initiation and progression of atherosclerosis [2]. This family of proteins has expanded to include eight different classes of membrane and soluble proteins (Class A, B, C, D, E, F, G, and H) encoded by distinct and unrelated genes [3]. Scavenger receptor classes are grouped by the presence of shared structural domains; however there is great structural diversity between the different classes. Despite this lack of sequence similarity or identity, all scavenger receptors retain the capacity to bind modified lipid particles in addition to a diverse range of polyanionic ligands of host-derived or exogenous origins, for example, pathogens [4, 5].

2. Genetics of Scavenger Receptors

Class A scavenger receptors comprise at least four related genes: scavenger receptor A (SR-A), macrophage receptor with collagenous structure (MARCO), scavenger receptor with C-type lectin (SRCL), and scavenger receptor A-5 (SCARA5) [6–10]. The human and murine *SR-A* genes are located on chromosome 8 and can be transcribed to produce three (SR-AI/II/III) or two SR-A splice variants, respectively [11]. SR-AI/II is largely found on macrophages

but are also present on endothelial cells and vascular smooth muscle cells (VSMCs). Oxidative stress, OxLDL, macrophage colony-stimulating factor (M-CSF), and phorbol esters can elevate SR-A levels [12–15]. SR-A is postulated to be proatherogenic due to its ability to mediate uptake of OxLDL in macrophages [16, 17]. Deficiency of SR-AI and SR-AII not only led to the formation of smaller atherosclerotic lesions, but also to a reduction of macrophage adhesion and increased susceptibility to bacteria and viruses [18, 19]. *MARCO* is located on human chromosome 2 or mouse chromosome 1 [20], and the gene product is expressed largely on macrophages and on splenic dendritic cells to a lower extent [9, 21]. MARCO is implicated in host defense and pathogen clearance since binding to dead or apoptotic cells, bacteria, and lipopolysaccharides elevates MARCO levels [22, 23]. When challenged with *Streptococcus pneumoniae*, wild type mice could clear the infection whereas the ability was impaired in MARCO$^{-/-}$ mice, demonstrating the role of MARCO in the innate immune response against pathogens [24]. MARCO expression in human alveolar macrophages also plays a crucial role in the innate immunity against bacteria [25]. Human and murine *SRCL* genes are both located on chromosome 18 and can generate at least 2 splice variants in humans. In contrast to the other Class A gene products, SRCL is detected on endothelial cells but not macrophages [26] and may be involved in the innate immune response against fungal infections [27]. *SCARA5* is located on mouse chromosome 14: the resulting gene product is detected on epithelial cells but not macrophages [7] and may play unique role(s) in the innate immune system and atherosclerosis [28].

Class B contains at least four members: CD36, SR-B (also known as CLA-1 in humans), LIMPII-related genes, and CD163. *CD36* is located on human chromosome 7 or murine chromosome 5 [29] and its expression is mostly limited to cells of lymphoid and hematopoietic lineages including leukocytes, platelets, endothelial cells, adipocytes, VSMCs, and some epithelial cells; its levels are highest in macrophages [30, 31]. Double knockout SR-A$^{-/-}$/CD36$^{-/-}$ mice show increased foam cell formation and atherosclerotic lesion size, suggesting that CD36 acts as a major cellular receptor for OxLDL [16, 32, 33]. However, a different study using a triple knockout SR-A$^{-/-}$/CD36$^{-/-}$ApoE$^{-/-}$ mouse demonstrated no change in atherosclerotic lesion size but decreased levels of various inflammatory gene products; ∼30% decrease in macrophage apoptosis and ∼50% decrease in plaque necrosis suggested delayed progression towards advanced, unstable atherosclerotic lesions [34]. In the nematode *C. elegans,* the CD36 orthologue (C03F11.3) mediates host defense against fungal pathogens [35]. Higher levels of a soluble form of CD36 are biomarkers of insulin resistance and plaque instability in patients with diabetes and internal carotid stenoses, respectively [36, 37]. CD36-deficient mice when challenged with pathogens were significantly more susceptible to the infections [38, 39]. Humans expressing CD36 allelic variants were also more susceptible to malaria [40] demonstrating its important role in the immune system. *SR-BI* (*SCARB1*) is located on human chromosome 12 or mouse chromosome 5 [41] and encodes two protein isoforms (SR-BI/II) [42] in monocytes, macrophages, hepatocytes, and adipose and steroidogenic tissues [43]. SR-BI expression is elevated by either PPARα, PPARγ, testosterone, PUFA, or TSA [44–47] and downregulated by either OxLDL, TNF-α, IL-1, or lipopolysaccharides [48, 49]. SR-B1 is a receptor for hepatitis C virus, *Plasmodium,* and mycobacteria pathogens [50–52]. In contrast to other scavenger receptors, SR-BI could provide protective function(s) against atherosclerosis by increasing the macrophage-based cholesterol efflux into HDL particles followed by liver HDL clearance and excretion [47, 53–56]. LIMPII is located on human chromosome 4 or mouse chromosome 5 and has a similar expression profile to SR-BI [31]. *CD163 (M130)* [57] is located in human chromosome 12 [58] and expressed in monocytes and macrophages in both membrane-bound and soluble forms [59] where it plays an important role in the regulation of anti-inflammatory responses, pathogen recognition, and atheroprotection probably through elevation in expression of heme oxygenase and in removing free hemoglobin [60–63]. Patients with hematological, inflammatory, and lysosomal storage diseases have also a high level of soluble CD163, and it may thus serve as a biomarker for such conditions [64, 65].

Class C comprises of just one scavenger receptor, dSR-C1 which has only been so far identified in the fruit fly *Drosophila melanogaster*. dSR-C1 is a pattern recognition receptor for bacteria expressed in hemocytes and macrophages during fly embryonic development [66]. It can recognize bacteria and may play a role in the innate immune system of the insect [67].

Class D comprises the CD68 and lysosomal membrane glycoprotein (Lamp) gene products. *CD68* is located on human chromosome 17 and the murine orthologue (also called macrosialin) is located on murine chromosome 11 [68]. Macrophages, Langerhans cells, dendritic cells, and osteoclasts express CD68 in pattern similar to the class B gene products [69]. The expression levels can be elevated by OxLDL, GM-CSF, and phorbol ester but inhibited by TNF-α and lipopolysaccharides [70–72]. Macrosialin levels are also observed to be increased by a proatherogenic diet: OxLDL and macrosialin were both found in macrophages within atherosclerotic plaques from ApoE-deficient mice [73]. Macrosialin has been identified as a receptor for OxLDL [74–76] although this view has been challenged [77]. The three *Lamp* genes (1, 2, and 3) are located in human chromosome 13, X, and 3 or murine chromosomes 8, X, and 16, respectively [78]. Lamp-1 and -2 are constitutively and widely expressed whereas Lamp-3 is elevated during dendritic cell maturation implying a functional link to the immune system [79].

Class E comprises of just one member: the lectin-like oxidized low density lipoprotein receptor 1 (LOX-1). *LOX-1* (*OLR1*) is located on human chromosome 12 [80] or mouse chromosome 6 and is expressed on endothelial cells, macrophages, smooth muscle cells, and platelets [81, 82]. The resting levels are relatively low but elevated by proinflammatory stimuli including OxLDL, inflammatory cytokines, for example, TNF-α, shear stress, oxidative stress, phorbol ester, endothelin-1, and angiotensin II [83–87].

A splice variant (LOXIN) conferred protection against the proatherogenic LOX-1 effects by forming inactive heterodimers with LOX-1 and blocking OxLDL-induced apoptosis in macrophages [88, 89]. A human LOX-1 allelic polymorphism (K167N) is postulated to increase the risk of CVD in a patient cohort [90]. However, further investigations into the associations between the LOX-1-K167N polymorphism, myocardial infarction (MI) and cardiovascular disease (CVD) have produced conflicting data [91, 92] suggesting that this polymorphism has no effects on CVD incidence [93]. The expression profile of a soluble LOX-1 species was elevated in obese postmenopausal women [94], and it is a biomarker for type 2 diabetes mellitus and atherogenesis [95–97]. In dendritic cells, LOX-1 can act as a receptor that mediates the uptake of antigens [98]. Overexpression of LOX-1 in CHO cells led to bacterial binding and uptake [99]. Macrophage LOX-1 depletion inhibits foam cell formation suggesting a role in atherosclerotic plaque initiation and progression [100]. Importantly, the incidence of atherosclerotic plaques is significantly lowered in LOX-1-deficient mice [101].

Class F consists of the SREC gene products (scavenger receptors expressed by endothelial cells) which are expressed on mammalian endothelial cells and macrophages [102] and also in nematodes [103]. The *SREC-I* gene is related to the EGF precursor gene [104] and is located on human chromosome 17 but the murine orthologue called *SCARF-1* is located on mouse chromosome 11. In humans, alternative splicing gives rise to at least five different membrane-bound and soluble protein isoforms [105]. SREC-I levels are elevated by lipopolysaccharides [102] and repressed by cytokines such as IL-1α, IL-1β, and TNF-α [105]. In humans, another gene called *SREC-II* that displays ~35% similarity to *SREC-I* is located on chromosome 22 [104, 106]. Murine *SREC-II* is located on chromosome 16 [107]. In *C. elegans*, a SREC-like gene product called CED-1 is implicated in the engulfment of apoptotic cells during animal development and immune defense against pathogens [35, 108, 109]. SREC-I is a receptor for Ac-LDL [102].

The chemokine ligand CXCL16 is a class G scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX). *SR-PSOX* is located on human chromosome 17 and mouse chromosome 11. SR-PSOX is highly expressed on macrophages, smooth muscle cells, dendritic cells, kidney and B cells with lower levels detected on the endothelium, and T cells [110–119]. Monocyte SR-PSOX expression is increased by TNF-α, IFN-γ, LPS, or OxLDL stimulation [113, 115, 120]. In addition to SR-PSOX links to atherosclerosis [120, 121] where the molecule was induced *in vitro* and *in vivo* by atherosclerosis-promoting inflammatory signals [122], it is also involved in acute and adaptive experimental autoimmune encephalomyelitis [123], CD8+ T cell recruitment during inflammatory valvular heart disease [124], and bacterial phagocytosis [116]. A soluble form of SR-PSOX functions as an activated T cell and NK cell-recruiting chemokine [112, 125] and is a biomarker for acute coronary syndrome [126].

Class H scavenger receptors consist of Fasciclin, EGF-like, lamin type EGF-like and link domain-containing scavenger receptor-1 (FEEL-1), also known as stabilin-1 or CLEVER-1 [127] and FEEL-2 (stabilin-2) a paralogous protein with 39% sequence identity to FEEL-1 [128–130]. The *FEEL-1* gene (*STAB1*) is located on human chromosome 3 and mouse chromosome 14, and the *FEEL-2* gene (*STAB2*) is located on human chromosome 12 and murine chromosome 10. Expression levels of both FEEL-1 and -2 are high in the liver and lymph nodes. FEEL-1 is expressed on monocytes, macrophages, and endothelial cells whereas FEEL-2 expression was not detected on these cell types in humans [128]. FEEL-2 was found to be expressed in sinusoidal endothelial cells in the liver, lymph node, spleen, and bone marrow in mice as well as heart valve mesenchyme, brain, eyes, and kidneys [131], with expression levels being increased during development in the zebrafish *Danio rerio* [132]. FEEL-1 is known to undergo alternate splicing to yield an isoform lacking exon 27 [130]. Sorting nexin 17 (SNX17) is required for maximum cell surface expression of FEEL-1 [133]. Knockdown of SNX17 leads to a dramatic reduction in cell surface expression, due to increased degradation of the receptor.

3. Scavenger Receptor Structure and Function

Scavenger receptors are present on different tissues ranging between macrophages, monocytes, platelets, endothelial, smooth muscle, and epithelial cells. In addition to vascular tissues, they are also detected in adipose and steroidogenic tissues (Table 1) [9, 13, 82, 110, 134–137]. A general mechanism underlying scavenger receptor levels is the elevation of gene expression in response to ligand binding to cell surface receptors, thus generating a positive feedback loop that mediates enhanced ligand clearance and/or accumulation [138, 139]. This is in contrast to other membrane-bound receptors such as the low-density lipoprotein receptor (LDL-R) that is downregulated in response to binding LDL ligand, thus exhibiting a negative feedback mechanism [140].

Scavenger receptors are generally classified as membrane-bound proteins that bind modified LDL particles and other polyanionic ligands. These include AcLDL, OxLDL, Gram-positive and Gram-negative bacteria, apoptotic cells, β-amyloid fibrils, and advanced glycation end products (AGE) (Table 1) [4, 5]. Following ligand binding, scavenger receptors can mediate intracellular signaling and/or ligand internalization. A generic model for such regulation is outlined in Figure 1. Although there is little structural homology between the ligand-binding domains of scavenger receptors from different classes, mutagenesis studies have revealed some conserved characteristics. These include positively charged arginine or lysine clusters in the ligand-binding domain of either the LOX-1 scavenger receptor [141] or CD36 [142], respectively. Such amino acid clusters appear to be required to mediate electrostatic interactions with the predominantly negatively charged modified lipid particle or polyanionic ligand although other noncharged hydrophilic residues may also be involved [141]. The avidity of ligand binding is also enhanced through the formation of scavenger receptor dimers [143], trimers [144], and higher-order oligomers [145].

Table 1: The major scavenger receptor ligands and expression profiles.

Class	Scavenger receptor	Ligands	Expression profile	Involvement in CVD?
A	SR-A	AcLDL, OxLDL, β-amyloid, molecular chaperones, ECM, AGE, apoptotic cells, activated B-cell, bacteria	Macrophages, mast, dendritic, endothelial and smooth muscle cells	Yes—involved in OxLDL uptake by macrophages leading to foam cell formation
A	MARCO	AcLDL, OxLDL, apoptotic cells, B cells, bacteria	Macrophages, dendritic cells	No
B	SR-B	HDL, LDL, OxLDL, apoptotic cells	Monocytes/macrophages, hepatocytes and adipocytes	Reduces atherosclerosis through reverse cholesterol transport of HDL
B	CD36	AcLDL, OxLDL, HDL, LDL, VLDL, β-amyloid, AGE, apoptotic cells	Macrophages, platelets, adipocytes, epithelial and endothelial cells	Yes—OxLDL uptake into macrophages leading to foam cell formation
E	LOX-1	OxLDL, molecular chaperones, ECM, AGE, apoptotic cells, activated platelets, bacteria	Endothelial and smooth muscle cells, macrophages, and platelets	Yes—OxLDL uptake in endothelial cells, leads to endothelial dysfunction
F	SRECI/II	AcLDL, OxLDL, molecular chaperones, apoptotic cells	Endothelial cells and macrophages	Low levels of AcLDL uptake
G	SR-PSOX	OxLDL and bacteria	Macrophages, smooth muscle, dendritic, endothelial cells, and B- and T cells.	Yes—involved in OxLDL uptake in macrophages
H	FEEL-I/II	AcLDL, molecular chaperones, ECM, AGE, bacteria	Monocytes/macrophages, endothelial cell	No known link

SR-A: scavenger receptor class A, AcLDL: acetylated low density lipoprotein, OxLDL: oxidised low density lipoprotein, ECM: extracellular matrix, AGE: advanced glycation end products, MARCO: macrophage receptor with collagenous structure, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low density lipoprotein, LOX-1: lectin-like oxidized low density lipoprotein receptor-1, FEEL-I/II: fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1.

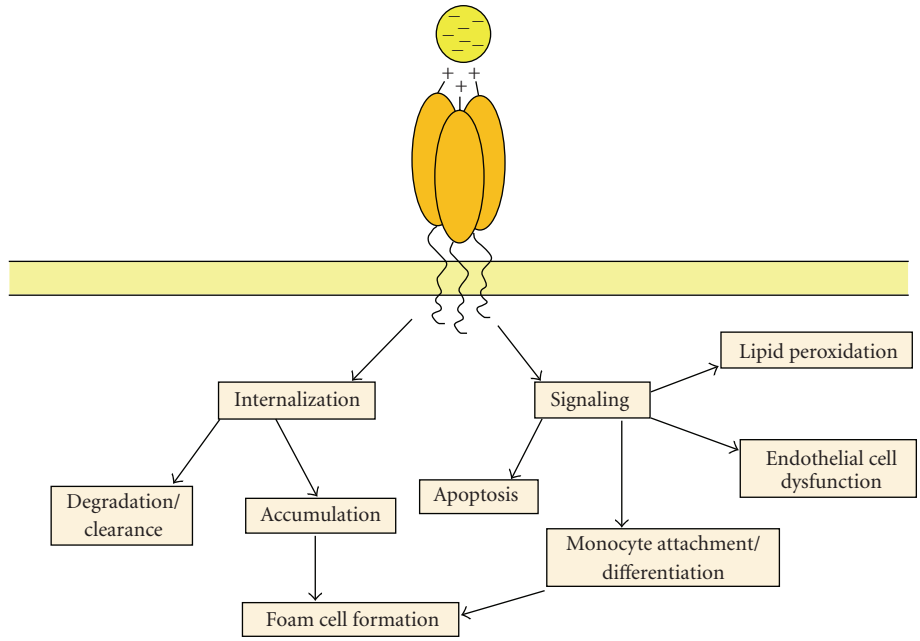

Figure 1: *A generic model for scavenger receptor-mediated ligand binding, internalization, and signal cascade activation.* Scavenger receptors bind negatively charged ligands through clusters of conserved positively charged residues. Ligands are internalized by scavenger receptors using a range of different clathrin-dependent and independent pathways. Ligands can be degraded or accumulate. Ligand binding can activate signaling cascades leading to diverse cellular functions including lipid peroxidation, apoptosis, endothelial cell dysfunction, and monocyte attachment and differentiation leading to foam cell formation.

Scavenger receptor-ligand complexes can undergo receptor-mediated endocytosis, trafficking through the endosome-lysosome system leading to degradation or accumulation of ligand. Different mechanisms of endocytosis have been postulated for the individual classes of scavenger receptors including clathrin-dependent [146], clathrin-independent [147], and lipid raft-mediated [148] events. This diversity in scavenger receptor endocytosis is not surprising considering the sequence diversity and different endocytic motifs within the cytoplasmic domains of the different scavenger receptors [146, 147]. Following endocytosis and delivery to endosomes, it is likely that many scavenger receptors are recycled back to the plasma membrane where they can mediate further ligand binding, clearance, or accumulation.

Ligand binding to scavenger receptors activates intracellular signaling cascades leading to diverse physiological outputs including apoptosis, endothelial cell dysfunction, and lipid peroxidation. One aspect of scavenger receptor activation is monocyte infiltration and differentiation leading to foam cell formation, a key event in atherosclerotic plaque initiation and progression. For example, activation of the Class B CD36 scavenger receptor is linked to phosphorylation and activation of c-Src and MAP kinase pathway thus triggering macrophage differentiation into foam cells [149]. Another model is the LOX-1 scavenger receptor where ligand binding stimulates reactive oxygen species (ROS) production, both MAPK and NF-κB activation leading to increased expression of different adhesion gene products. Such elevated expression in endothelium can enable monocyte infiltration, ultimately leading to monocyte differentiation and foam cell formation.

4. Current Atherosclerosis Therapies

Atherosclerosis is a leading cause of mortality in Europe and Western countries [150]. The subversion of human vascular function by atherosclerosis can lead to cardiovascular morbidity and mortality, including ischemic stroke, ischemic heart disease, myocardial infarction, and peripheral arterial disease. The causes of atherosclerosis are multifactorial, meaning that single intervention therapy has as yet not succeeded in major reductions in disease incidence. Ongoing large investments by many countries worldwide are directed towards the prevention of cardiovascular disease by modifying environmental risk factors. Within the United Kingdom alone, a vascular risk and assessment program is currently in its initial roll out phase, aiming to tackle modifiable risk factors in a healthy 40–74-year age group (http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_083822). Despite economic modeling predicting a relatively large annual cost (US$60 million), this approach is predicted to prevent approximately 9500 cases of myocardial infraction and strokes annually. This would thus also be cost effective in the long term. (http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_085869).

Pharmacological agents have only been partially successful in attenuating the clinical manifestations of atherosclerosis with the most dramatic effects achieved by statins, which inhibit 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase. Statin therapy has reduced the 5-year incidence of major cardiovascular events by ~20% with each millimole per liter reduction in levels of cholesterol in LDL particles [151]. It is thought that statins exert their effect through inhibiting the rate-limiting step in cholesterol biosynthesis, which in turn leads to elevation of LDL receptor expression. However there is much debate over the importance of additional lipid-independent modes of action including anti-inflammatory effects [152]. Clinical treatment of established atherosclerotic plaques is becoming more technologically advanced, with routine intra-arterial catheterization and angioplasty of damaged arterial blood vessels. Stents are commonly used, and new technologies, such as drug-eluting stents, raise exciting possibilities for potential gene therapy as well. Evaluation of a patient's genetic background in atherosclerotic plaque initiation and progression could be essential to provide major disease alleviation by combining intervention, medication, and lifestyle modification in tailored therapies. Targeting the scavenger receptor gene products that mediate the response to and/or uptake of modified LDL holds great promise in the prevention of cardiovascular disease.

5. Gene Therapy

Gene therapy is the process of ameliorating or curing a genetic disease by introducing a fragment of genetic material into diseased or dysfunctional cells. Viral or nonviral vectors are the vehicles used to transfer and express specific genes within a target cell and thus used to correct genetic disorders. The idea of using genetic material to treat human diseases gained prominence in 1960s [153], and in 1973, the first attempt used a wild-type human papilloma virus in attempting to correct hyperargininaemia [154] but this was unsuccessful. Even though subsequent gene therapy attempts were controversial [155], this area of biomedicine obtained the first signs of success by tackling adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID) [156]. The first ADA-SCID patient treated successfully using this technology has subsequently led a relatively normal lifestyle with amelioration of the disease symptoms [157]. By 2007, >1300 gene therapy clinical trials have taken place of which the majority of trials (67%) aimed at cancer treatment; gene therapy of cardiovascular diseases formed the second largest cohort of clinical trials (9%). The number of clinical gene therapy trials currently exceeds 1500 (http://www.wiley.co.uk/genmed/clinical/) and may well live up to the expectations of becoming the "twenty-first century medicine" to deliver personalized healthcare [158].

Nevertheless, successful clinical gene therapy has encountered numerous problems. The two major problems that usually hamper gene therapy efficacy are (1) immune response(s) against the protein products of the transgene or the vector and (2) insertional mutagenesis by the viral vector. Since viral vectors are based on pathogenic viruses, they can induce immune responses [159–161], and much of the human population may have preexisting immunity

against human viruses. Depending on the conditions used, this immunogenicity can lead to adverse effects. During one study on adenoviral-mediated treatment of ornithine transcarbamylase (OTC) deficiency, one of the eighteen subjects died as a result of an exacerbated immune response to the injected adenoviral vector carrying an *E1-E14* deletion [162, 163]. Leukemia induction was also noticed in a mouse model following gene transfer using retroviral vectors caused by vector integration into the ecotropic viral integration *Site-1* (*Evi1*) [164]. Two different gene therapy studies on X-linked SCID (SCID-X1) using retroviral vector integration resulted in leukemia induction in four out of nine subjects with one death [165, 166] and leukemia in one out of ten subjects [167, 168]. Retroviruses and lentiviruses undergo obligatory integration of the provirus into the host genome as a part of the life cycle, and this may lead to activation or increased expression of nearby host genes [169, 170]. In the case of retro- and lentiviral vectors, the promoter enhancer elements located in the viral termini appear responsible for this altered host gene expression profile [171, 172].

In contrast, although the viral termini located within adeno-associated viruses [173, 174] and adenoviruses [175–177] have enhancer and promoter activities, insertional mutagenesis caused by vectors based on these viruses have not as yet been reported. However, even in the case of adenoviral vectors which have been perceived as nonintegrating vectors, there are instances of viral integration into the chromosomal DNA close to or within host genes and instances of genetic mutations and rearrangements [178, 179]. A slightly higher percentage of adeno-associated viral vector DNA [180, 181] integrated into genes causing chromosomal deletions and translocation [182–184]. Despite the lack of adverse affects in cystic fibrosis gene therapy trials, the effects caused by administration of different viral and nonviral gene therapy vectors were not sufficient to cause regression of clinical symptoms; natural lung adaptation to alien particle administration may have also hindered patient gene transfer [185]. However, successful gene therapy in the treatment of ADA-SCID and lack of adverse effects [186] holds much promise for further work.

6. Gene Therapy Vectors

Gene therapy vectors are genetic vehicles used to transfer DNA sequences or specific genes from the laboratory bench into the diseased cells or tissues and are viral or nonviral in origin. Viruses are well adapted to infect cells or tissues, and these adaptations have been utilized to generate viral vectors for gene therapy. Such viral vectors constitute 66% of clinical gene therapy trials worldwide (http://www.wiley.co.uk/genmed/clinical/). Both viral and nonviral vectors have their advantages and disadvantages in gene therapy (summarized in Table 2).

6.1. Nonviral Gene Therapy Vectors. Nonviral gene delivery systems utilize physical force or chemical methods to deliver the genetic material to the cell. Major nonviral vectors used in gene therapy include circular plasmid or linear DNA complexed with nanoparticles [187, 188] or liposomes (cationic lipid-DNA complex) [189, 190]. Plasmid DNA can be transferred into the cells using a gene gun [191] where the DNA is bound to high density particles like gold and transferred at high velocities into the cell [192] or by electroporation [193], using transposable elements [194] or DNA:RNA oligonucleotide hybrids [195]. Even though nonviral gene therapy methods are used in clinical trials [196] they usually exhibit lower gene transfer efficiency and transient gene expression [197–200]. This is especially true when both viral and nonviral systems were compared simultaneously [201]; the immune response [202–205] may also limit the therapeutic capability of nonviral gene therapy.

6.2. Viral Gene Therapy Vectors. Since viruses are well adapted to evade the host immune responses and to deliver the genetic material into the host cells, gene therapy vectors based on viruses have been more effective so far and currently account for two-thirds of all gene therapy clinical trials worldwide (http://www.wiley.co.uk/genmed/clinical/). Barring the use of vectors in suicide gene therapy [206], viral gene delivery systems utilize viral vectors with defective replication capabilities. The coding region of the viral genome is replaced by foreign genetic material, leaving only the *cis*-acting elements essential for viral packaging and/or integrating into the host genome on the vector. Producer cell lines can provide the essential viral gene products either totally by themselves or by the assistance of other systems, which are used to generate nonreplicating viral vectors [207]. Currently, vectors based on adeno-associated viruses, retroviruses, and adenoviruses form the majority of the viral vectors used as gene delivery systems.

6.2.1. Adeno-Associated Viral (AAV) Vectors. Adeno-associated viruses belong to the *Parvoviridae* family which have a nonenveloped icosahedral capsid containing a single-stranded DNA genome. This viral DNA has *cis*-acting palindromic inverted terminal repeats at each end which form hairpins that are essential for DNA replication and packaging [208]. Most of the current AAV-based gene therapy vectors are derived from AAV-2 subtype. This virus is dependent on coexpression of an adenovirus or herpes helper virus for gene products essential for lytic productive infection where the genome is replicated, and virions are produced. In the absence of the helper virus, AAV-2 undergoes site-specific integration to establish a latent state. The provision in *trans* of the AAV Rep (regulatory) and Cap (structural capsid) genes together with the adenoviral early viral genes (provided by a helper virus) is needed to generate AAV vectors for gene therapy [158, 207, 209]. Despite the smaller AAV transgene capacity [210] and the preexisting immunity against AAV [211], these vectors have been used successfully in animal models of retinal disorders [212, 213], cystic fibrosis [214, 215], hemophilia B [216, 217], muscular dystrophy, and DNA vaccination [218]. They are currently used in human clinical gene therapy trials [219].

6.2.2. Adenoviral Vectors. Adenoviruses belong to the *Adenoviridae* family and contain nonenveloped icosahedral capsid with a double-stranded DNA genome. A *cis*-acting inverted

terminal repeat is present at each end of the DNA and a packaging signal at the 5′ terminus [220]. Some of the early adenoviral genes (e.g., *E1*) have transforming and transactivating functions and were thus replaced by inserted DNA sequences or the gene of interest in the first generation adenoviral vectors. However, this did not prevent low level expression of other adenoviral gene products, including those with immunogenic and toxic properties causing rapid clearance from host *in vivo* [221, 222]. To both avoid this adaptive immune response and to increase viral transgene capacity, high capacity adenoviral vectors (HC-AdV) were developed where the only viral elements present are the *cis*-acting ITRs and packaging signal with viral gene products needed for replication provided in *trans* by a packaging-deficient helper virus [223]. Even though the innate immunity against adenoviral capsid would still elicit an immune response [161], this may be circumnavigated either by using adenoviral vectors of different serotypes [224] or by modifications with synthetic polymers [225]. The large cloning capacity of 36 kb and the longevity of transgene expression [226–228] in tissues with low cellular turnover hint at potentially successful gene therapy. Adenoviral vectors have been used successfully in rodent, canine, and primate models of cardiovascular diseases [229, 230], muscular dystrophy [231, 232], glycogen storage diseases [233], hemophilia [234], cancer [235–237], retinal disorders [238], and DNA vaccination studies [239]. Adenoviral vectors comprise the largest group of the largest group of vectors (24%) used in clinical gene therapy trials (http://www.wiley.co.uk/genmed/clinical/) [240, 241].

6.2.3. Retro- and Lentiviral Vectors. Retroviruses are enveloped single-stranded RNA viruses where the RNA genome is reverse transcribed into a DNA provirus which then integrates into the host chromosomal DNA during its life cycle. The viral genome is flanked by long terminal repeats (LTRs) which along with the packaging signal and a truncated *gag* gene comprising the *cis*-acting elements essential for functionality. Retroviral genes encoding the capsid proteins, the viral protease, the reverse transcriptase, and the integrase are supplied in *trans* by transient transfection of plasmids to generate an assembled virus with gene delivery capability [242–244]. Lentiviruses are more complex, regulatory and accessory genes, and have the capability to infect dividing and nondividing cells in contrast to retroviruses which can only infect dividing cells. Functional lentiviral vectors also need expression of the Rev (cytoplasmic transport of the RNA) and the Tat (viral promoter transactivator) viral gene products.

One biosaftey issue when using viral gene therapy is that extensive genetic recombination of the viral and host genomes could generate replication-competent viruses. Whilst the generation of replication-competent adenoviruses may cause only relatively mild health problems, this is likely to be dangerous if replication-competent lentiviruses are generated. To reduce this risk during the packaging process *in vitro* using cultured cell lines, *gag* and *pol* (with additional *tat* and *rev* for lentiviral vectors) and *env* are present on different plasmids. A further biosafety improvement is removal of the U3 region (enhancer-promoter) of the viral LTR to generate self-inactivating (SIN) vectors [245]. Since reverse transcription means that both the 5′ and 3′ U3 regions of the provirus DNA are transcribed, this deletion would abrogate synthesis of a complete RNA viral genome packaged into virions. One feature of the retrovirus life cycle is integration of the provirus into the host genome causing persistent gene expression [246]. Gene therapy using retro- or lentiviral vectors has been successfully used in the gene therapy experiments for treating Duchenne muscular dystrophy [247], hemophilia [248], Fanconi anemia [249], diseases of the central nervous system [250], DNA vaccination [251], X-linked SCID, adenosine deaminase SCID, and chronic granulomatous disease [168, 252]. These successes of these studies in a combination of rodent, canine, primate models have now led to ∼21% of current human clinical trials for gene therapy.

7. Scavenger Receptor Gene Therapy

Even in the late 1980s, cardiovascular dysfunction was a major focus of gene therapy trials. In a rabbit model of homozygous familial hypercholesterolemia, a retroviral LTR promoter was used to overexpress human LDL-R in fibroblasts [253]. Elsewhere, retroviral vectors containing *LacZ* encoding β-galactosidase were used for *ex vivo* transduction of canine and porcine endothelial cells: β-galactosidase expression could be detected after surgical implantation into canine and porcine models [254, 255]. Currently, the proportion of clinical gene therapy trials for cancer (65%) is followed by the second largest trial group on cardiovascular disease (9%) (http://www.wiley.co.uk/genmed/clinical/).

As mentioned earlier, scavenger receptor function is associated with both healthy and pathophysiological processes ranging between homeostasis, apoptotic cell clearance, diabetic necropathy, age-induced cardiomyopathy, and antigen cross-presentation in Alzheimer's disease [256–260]. Importantly scavenger receptor function is heavily implicated in atherosclerotic plaque initiation and progression [261], making this diverse protein supergroup [1] an attractive target for gene therapy (Figure 3). Currently, the majority of scavenger receptor gene therapy studies have utilized adenoviral vectors (Table 3).

7.1. LOX-1 Gene Therapy. A mouse knockout model lacking LOX-1 suggested that this was a key contributory factor in driving lipid accumulation in vascular tissues [101], raising the question as to whether these properties could be manipulated using gene therapy. A first-generation adenoviral vector expressing human LOX-1 was used to successfully express LOX-1 transiently in vascular smooth muscle cells (VSMCs) and other cells [262]. A first-generation first-generation adenoviral vector was used to provide ectopic expression of LOX-1 in hepatic tissues of the ApoE-deficient mice, leading to increased OxLDL excretion, reduction in plasma OxLDL, and complete loss of atherosclerotic plaque initiation and progression [263]. In addition, oxidative stress and inflammatory responses were reduced in the mice infected with adenovirus LOX-1. Nevertheless, OxLDL levels returned to

Table 2: The advantages and disadvantages of the major gene therapy vectors currently used.

Gene therapy vector	Genetic material	Advantages	Disadvantages
Nonviral vectors	Mainly DNA	Large transgene capacity, biosafety	Low efficiency, immune response (cationic lipids and polymers), toxicity
Retro-/lentiviral vectors	RNA	Stable integration, lack of immune response, up to 10 kb cloning capacity	Insertional mutagenesis following integration is higher
Adeno-associated viral vectors	DNA	Long-term expression, site-specific integration	Immune response, small transgene capacity
First-generation adenoviral vectors	DNA	High titer, up to 8 kb of cloning capacity	Immune response and toxicity leading to shortened duration of transgene expression *in vivo*
High capacity adenoviral vectors	DNA	High titer, longevity of transgene expression, up to 36 kb cloning capacity	Immune response directed against the viral capsid

control baseline levels 3 weeks after hepatic LOX-1 overexpression. This profile of gene expression correlates with the expected duration of transgene expression using first generation adenoviral vectors [221]. This raises the question whether more sustained long-term LOX-1 expression using stable ectopic expression systems or integrating viruses would be a better strategy to inhibit atherosclerosis.

7.2. SR-A Gene Therapy. A retroviral vector with a bovine SR-AII cDNA transgene was used to show increased lipid accumulation, foam cell formation, and predisposition to apoptosis in fibroblasts and smooth muscle cell lines [264]. This suggests that manipulation of SR-A levels might be advantageous in hindering proatherogenic responses in vascular tissues [264]. A hybrid gene containing the human CD68 promoter upstream of truncated human *SR-AI* encoding the extracellular domain alone and expressed using an adenoviral vector inhibited degradation of AcLDL and OxLDL particles and subsequent foam cell formation [265]. One conclusion is that soluble SR-AI binds to modified LDL particles and sequesters such ligands away from the wild-type membrane-bound scavenger receptors.

Using the LDL-R knockout mouse that develops atherosclerotic lesions, overexpression of soluble human SR-AI using this adenoviral system completely blocked plaque initiation and progression [266]. However, similar to gene expression profiles for first- and second-generation adenoviral vectors, the plasma soluble SR-AI returned to control baseline levels after 4 weeks [266]. When adeno-associated viral vectors were used to express soluble SR-AI in the same mouse model, the atherosclerotic lesion area was reduced and persistence of soluble SR-AI plasma levels was observed for 6 months [267]. Expression of murine MARCO using a lentiviral vector in cultured cells suggests that quality control along the secretory pathway is essential for scavenger receptor assembly and presentation at the plasma membrane [274]. SR-A may also be a receptor for adenovirus binding and host cell entry [275], and this could be further exploited to block macrophage lipid accumulation leading to foam cell formation during atherosclerosis.

7.3. SR-BI Gene Therapy. High-density lipoprotein (HDL) particles can mediate reverse cholesterol transport, have antiatherogenic properties, and are recognized by the SR-BI glycoprotein [276, 277]. Current gene therapy used first-generation adenoviral vectors to express murine SR-BI. Transient hepatic expression of murine SR-BI in mice increased HDL clearance, reduction in plasma HDL levels, and increased biliary cholesterol levels [54]. These effects are either due to increased hepatic uptake of HDL and/or the increased cholesterol secretion into the bile. Similar results were obtained with the same adenoviral vector delivered into LDL-R knockout mice with reduction of both early and advanced atherosclerotic lesions [55]. One explanation is that SR-BI overexpression resulted in a reduction of all three of HDL, LDL, and VLDL levels [278]. However, in human ApoB transgenic mice, SR-BI expression from the same vector resulted in a much lesser LDL-metabolism compared to HDL metabolism [279]. A xenogenic model comprising *ApoAI* knockout mice and SR-BI overexpression was used to examine transplanted human HDL processing. Here, small and dense HDL particles are not cleared from the circulation but remodel in the plasma to form larger HDL particles [280]. Similar SR-BI expression in a rabbit model caused reduction in HDL levels and increased LDL levels [268]. Again, SR-BI overexpression increased biliary excretion of cholesterol [269].

HDL binding to SR-BI can activate intracellular signaling leading to increased endothelial nitric oxide synthase (eNOS) activity [281]. Coexpression of SR-BI and Apobec 1 (essential in ApoB mRNA editing, resulting in the truncated ApoB48 product) in immortalized hepatic cells using a HC-Ad vector caused reduction in ApoB levels. Using the same system on immortalized endothelial cells caused increased eNOS phosphorylation and elevated nitric oxide levels [270]. However, the amount of HDL normally absorbed by the liver from the bloodstream is relatively low [282]. *SR-BI* allelic polymorphisms also do not correlate with variations in plasma HDL levels [283]. More clinical gene therapy studies are desirable to fully test whether this molecule is a good candidate for alleviating atherosclerosis.

Table 3: List of the main viral gene therapy experiments examining the therapeutic potential of scavenger receptors.

SR used/targeted	Vector used	Outcome	References
LOX-1	FG AdV	Inhibition of the progression of atherosclerosis	Ishigaki et al. [263]
Soluble SR-A1	FG AdV	Foam cell formation inhibited	Laukkanen et al. [265]
Soluble SR-A1	FG AdV	Abrogation of the atherosclerotic lesion area	Jalkanen et al. [266]
Soluble SR-A1	AAV	Abrogation of the atherosclerotic lesion area	Jalkanen et al. [267]
SR-B1	FG AdV	Reduction of plasma HDL	Kozarsky et al. [54]
SR-B1	FG AdV	Reduction of plasma HDL	Kozarsky et al. [55]
SR-B1	FG AdV	Reduction of plasma HDL, increase of LDL	Tancevski et al. [268]
SR-B1	FG AdV	Increased biliary secretion of cholesterol	Wiersma et al. [269]
SR-B1 and Apobec 1	HC-AdV	Reduction of Apo B levels, elevation of NO	Zhong et al. [270]
SR-B1/CD36	FG AdV	SR-B1 mediated uptake of cholesterol esters higher than that by CD36. However, CD36 resulted in higher levels of Ox-LDL degradation	de Villiers et al. [271] and Sun et al. [272]
CD36	FG AdV	Increased hepatic fatty acid uptake	Koonen et al. [273]
SR-PSOX	LV	Decreased foam cell formation	Zhang et al. [120]

SR: scavenger receptor, FG AdV: first-generation adenoviral vector, AAV: adeno-associated viral vector, HC-AdV: high capacity adenoviral vector, LV: lentiviral vector, SR-A: scavenger receptor class A, LOX-1: lectin-like oxidised low density lipoprotein receptor-1, SR-B1: scavenger receptor class B 1, SR-PSOX: scavenger receptor that binds to phosphatidylserine and oxidized lipoprotein, Apobec 1: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 1, CD36: cluster of differentiation 36, LDL: low density lipoprotein, HDL: high density lipoprotein, and NO: nitric oxide.

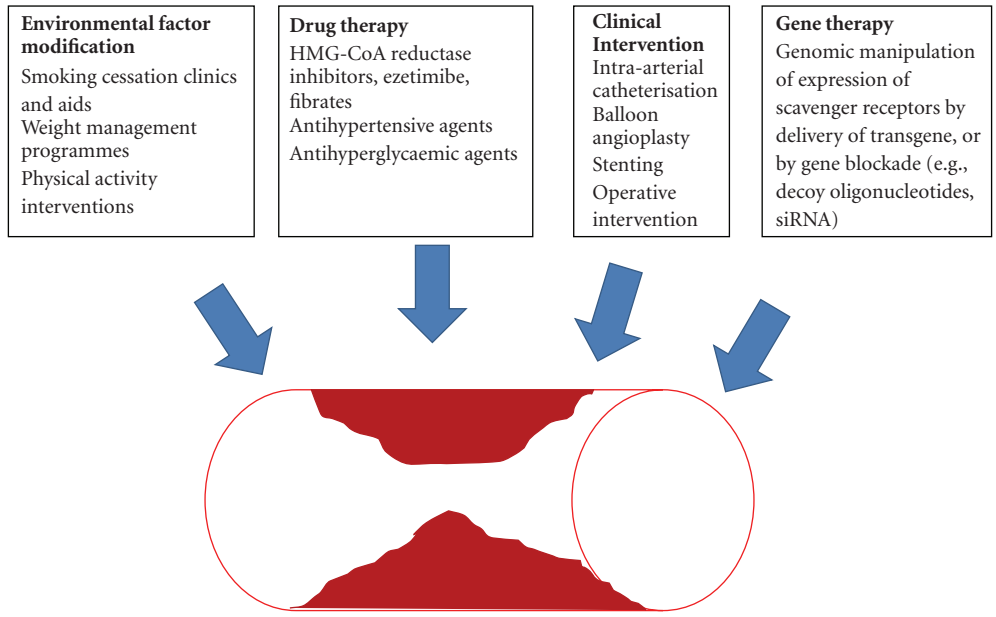

Figure 2: *Treatment of atherosclerosis.* A schematic to display the potential synergistic role of gene therapy in the treatment of atherosclerosis.

7.4. CD36 Gene Therapy. CD36 is another Class B scavenger receptor that can mediate oxidized LDL binding and internalization [284]. Liver overexpression of CD36 using a first-generation adenoviral vector significantly increased cellular fatty acid uptake including hepatic fatty acid, plasma, and hepatic triglycerides [273]. Constitutive expression of murine SR-BI or the murine CD36 using a first-generation adenoviral vector in cultured cells *in vitro* showed that SR-BI-mediated uptake of cholesterol esters was higher than CD36 [271]. Hepatic SR-BI overexpression significantly reduced HDL levels whereas CD36 overexpression had little effect [271]. However, in another study using the same *in vitro* model, CD36-mediated OxLDL internalization resulted in significantly higher lipid particle degradation, compared to SR-BI [272]. These findings suggest significant differences between the two proteins within the same class and that

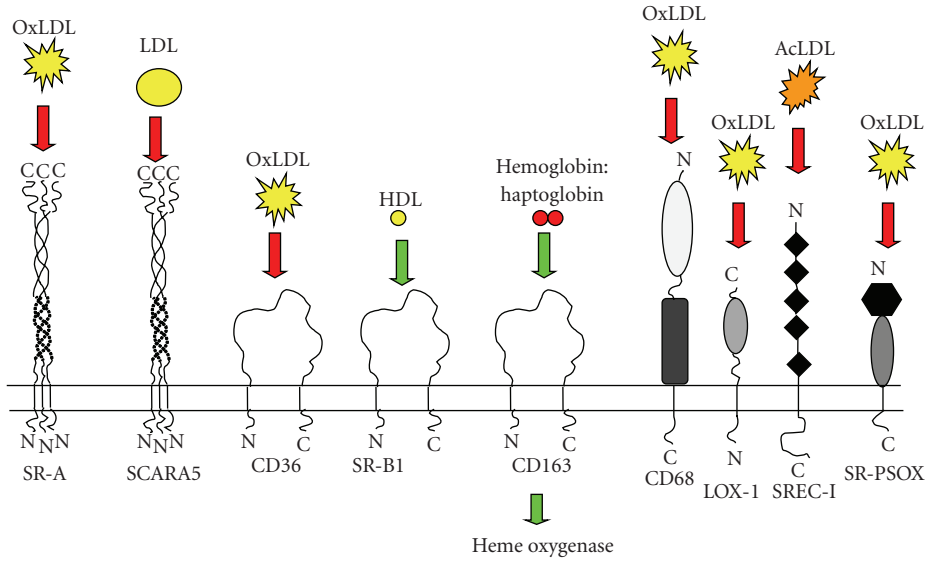

Figure 3: *Major scavenger receptors and their effects on atherosclerosis*. A schematic showing the role of major scavenger receptors in atherosclerosis. Red arrows indicate proatherogenic effects; green arrows indicate antiatherogenic or protective effects. SR-A (scavenger receptor class A) mediates uptake of OxLDL (oxidised low density lipoprotein) in macrophages; SCARA5 (scavenger receptor A5) down-regulation results in reduction of aortic LDL (low density lipoprotein) deposition; CD36 is probably a receptor of OxLDL; SR-B (scavenger receptor B) increases cholesterol efflux; CD163 exerts its protective actions through elevation of IL-10 and heme oxygenase; CD68 is a possible receptor for OxLDL; LOX-1 is a receptor for OxLDL; SREC-1 (scavenger receptors expressed by endothelial cells) is a receptor for AcLDL (acetylated low density lipoprotein); SR-PSOX (scavenger receptor that binds to phosphatidylserine and oxidized lipoprotein) binds to OxLDL (figure adapted from [1]).

CD36 overexpression may be more beneficial by promoting the clearance of modified lipid particles.

7.5. SR-PSOX Gene Therapy. The scavenger receptor that binds to phosphatidylserine and oxidized lipoprotein (SR-PSOX) is highly expressed within atherosclerotic lesions [111], and elevated expression in macrophages stimulates OxLDL uptake [285]. Reduction of human SR-PSOX levels in immortalized monocytes by RNAi using a lentiviral vector decreased lipid accumulation and foam cell development [120]. However, in double knockout mice lacking both LDL-R and SR-PSOX, there was accelerated atherosclerosis with increased macrophage recruitment to the aortic arch [121]. The potential role of SR-PSOX in the innate immune system to mediate pathogen clearance [125, 286] suggests that more studies may be needed to ascertain potential benefits of the SR-PSOX manipulation *in vivo*.

8. Conclusions

Treatment of atherosclerosis using pharmacological agents has only been partially successful, and therefore newer therapies, either stand-alone ones or in combination with the pharmacological agents, are desirable (Figure 2). Gene therapy has been successful in human and animal models with more than 1500 clinical trials worldwide (http://www.wiley.co.uk/genetherapy/clinical/). Viral vectors appear better than nonviral vectors to deliver transgenes to target cells and tissues. Despite the setbacks to viral gene therapy due to insertional mutagenesis caused by the viral vectors or anti-viral host immune responses, better vectors with significant safety biosafety improvements have been developed Self-inactivating lentiviral vectors [245] are safer, and modifications of the viral long terminal repeats further reduce the potential of insertional mutagenesis by retro- and lentiviral vectors [287]. Even though viral integrations have been observed near transcription start sites and genes, foamy viruses have reduced preferential integration into host genes [288] in comparison to other viral systems [170, 179], and thus this class of viruses may be significantly safer than retro- or lentiviruses when used as vectors. Most of the adenoviral vectors currently being used are based on Ad5 serotype; however, since neutralizing host antibodies to the Ad5 serotype exceeds that to the Ad36 serotype in the human population [289], Ad36-derived vectors may be better for human clinical trials. Intrahepatic injection of adenoviral vectors also help in reducing the immune response and increasing transgene expression [290]. The problems arising from the preexisting host immune responses against Ad5 serotype can also be circumnavigated using vectors based on a canine adenovirus [224]. Viral vectors can also be made to specifically target the desired cell type [291–294], and this strategy should help in improving the overall efficacy of the therapy.

Even though the first successful clinical gene therapy trial took place almost twenty years ago, work on its utility for modulating scavenger receptor function is still in its infancy (summarized in Table 3). Hepatic overexpression of scavenger receptors using viral vectors resulted in the inhibition of atherosclerosis initiation and progression

[263, 265, 266] even though therapy efficacy was hampered by lack of stable long-term gene expression. Better results were obtained when a different viral vector was used [267]. A study of the long-term effect of the transgene expression *in vivo* using the other available viral vector types would be very desirable to ascertain the potential of using viral gene therapy using scavenger receptors. The splice variant isoforms of some scavenger receptors can confer protection against myocardial infarction [88], and it might be interesting to examine expression of these molecules in an *in vivo* model. The long-term effects of the expression of soluble scavenger receptors on atherosclerosis would be interesting [265–267]. Since scavenger receptor knockout models can be antiatherogenic [120], suppression of gene expression appears to be a promising strategy although effects on the host immune responses have yet to be fully understood. A dual approach where genetic manipulation of a candidate scavenger receptor is supplemented by the action of another transgene [270] combined with longer duration of transgene expression might increase therapeutic benefits in disease models. Adeno-associated viral vectors may not be very useful in the simultaneous expression of a number of transgenes from a single vector due to the limitations in their packaging capacity, but multiple transgenes can be expressed in the model system with large vectors such as HC-AdVs. Transgene expression from viral vectors such as HC-Ad and lentiviruses is relatively stable and long-lived but these vectors have been used sparingly for such studies with scavenger receptors. With the advent of gene therapy vectors with higher biosafety such as SIN-LV vectors and HC-AdV vectors, development of less immunogenic viral vectors based on nonhuman viruses [224], and suppression of transgene-specific immune responses [295] gene therapy with scavenger receptors along with other therapies might be useful in providing sustained long-term amelioration of the clinical manifestations of atherosclerosis.

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Review Article

Cardiovascular Disease, Single Nucleotide Polymorphisms; and the Renin Angiotensin System: Is There a MicroRNA Connection?

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Essential hypertension is a complex disorder, caused by the interplay between many genetic variants, gene-gene interactions, and environmental factors. Given that the renin-angiotensin system (RAS) plays an important role in blood pressure (BP) control, cardiovascular regulation, and cardiovascular remodeling, special attention has been devoted to the investigation of single-nucleotide polymorphisms (SNP) harbored in RAS genes that may be associated with hypertension and cardiovascular disease. MicroRNAs (miRNAs) are a family of small, ~21-nucleotide long, and nonprotein-coding RNAs that recognize target mRNAs through partial complementary elements in the 3′-untranslated region (3′-UTR) of mRNAs and inhibit gene expression by targeting mRNAs for translational repression or destabilization. Since miRNA SNPs (miRSNPs) can create, destroy, or modify miRNA binding sites, this review focuses on the hypothesis that transcribed target SNPs harbored in RAS mRNAs, that alter miRNA gene regulation and consequently protein expression, may contribute to cardiovascular disease susceptibility.

1. Introduction

Identifying the genes and mutations that contribute to disease is a central aim in human genetics. Single nucleotide polymorphisms (SNPs) are mutations that occur at genome positions at which there are two distinct nucleotide residues (alleles) that each appear in a significant portion (i.e., a minor allele frequency greater than 1%) of the human population [1]. There are some estimated 14 million SNPs [2] in the human genome that occur at a frequency of approximately one in 1,200–1,500 bp [3]. SNPs can affect protein function by changing the amino acid sequences (nonsynonymous SNP) or by perturbing their regulation (e.g., affecting promoter activity [4], splicing process [5], and DNA and pre-mRNA conformation). When SNPs occur in 3′-UTRs, they may interfere with mRNA stability and translation by altering polyadenylation and protein/mRNA regulatory interactions. Recently, a new layer of posttranscriptional miRNA-mediated gene regulation has been discovered and shown to control the expression levels of a large proportion of genes (reviewed in [6]). Importantly, SNPs in microRNA (miRNA) target sites (miRSNPs) represent a specific class of regulatory polymorphisms in the 3′-UTR that may lead to the dysregulation of posttranscriptional gene expression. Thus, for miRNAs acting by this mechanism, the miRSNPs may lead to heritable variations in gene expression. Given that the renin angiotensin system (RAS) is intricately involved in the pathogenesis of cardiovascular disease [7–12], we review and discuss the presently available evidence for miRSNPs-mediated RAS gene regulation and its importance for phenotypic variation and disease.

2. Current View of the Renin Angiotensin System

The RAS plays a critical role in regulating the physiological processes of the cardiovascular system [reviewed in [7–14]]. The primary effector molecule of this system, angiotensin II (Ang II), has emerged as a critical hormone that affects the function of virtually all organs, including heart, kidney, vasculature, and brain, and it has both beneficial and

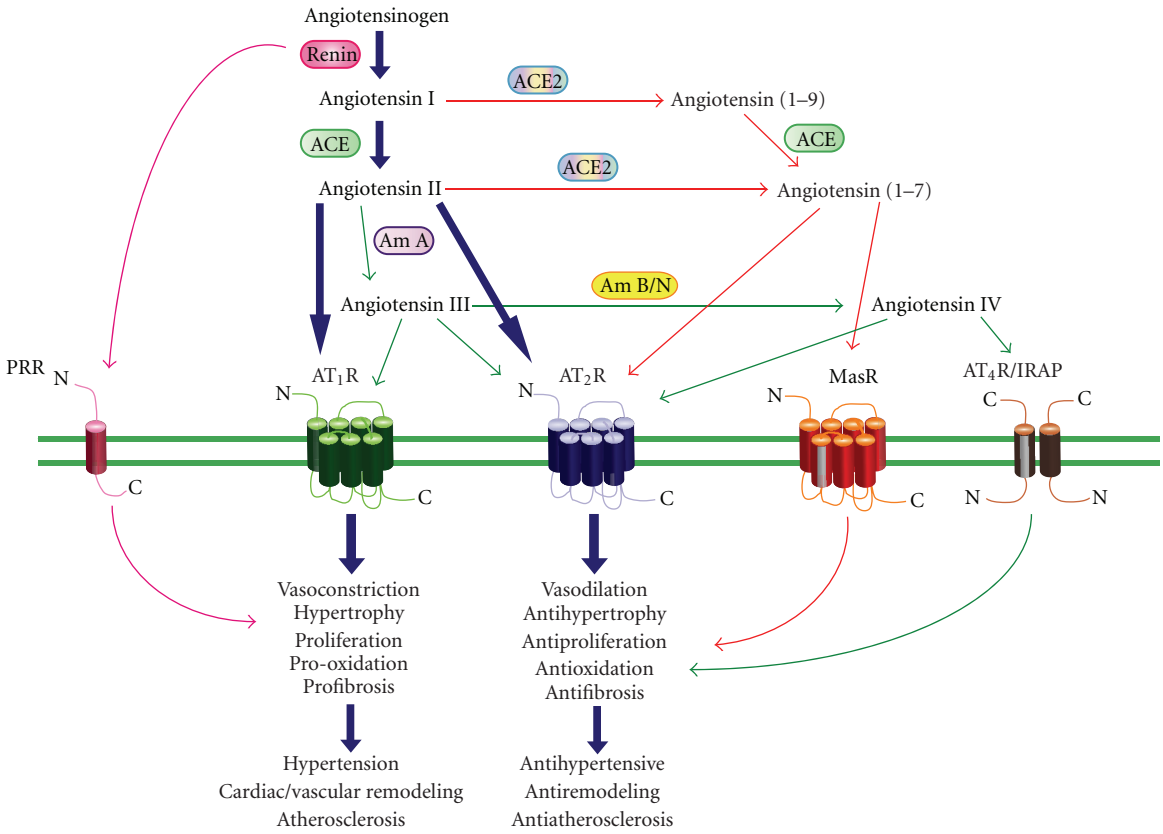

Figure 1: Summary of the RAS incorporating the Ang peptide family and physiological effects mediated via ATR subtypes. Under the classical RAS schema, Ang II is produced, via renin and ACE, to act with equal affinity on two ATR subtypes, AT_1R and AT_2R (large arrows). However, it is now appreciated that a number of breakdown products of Ang II, namely, Ang (1–7), Ang III, and Ang IV exert their own unique effects that are distinct (and often opposite) to those of Ang II. Such effects are often mediated via newly recognized receptors such as MasR for Ang (1–7) and AT_4R (also known as IRAP) for Ang IV, or additionally via AT_2R stimulation. ACE2 is also a new pathway for the formation of Ang (1–7). Newly identified Ang receptor binding proteins associated with different ATR subtypes may also modify ATR activation. Thus, overstimulation of AT_1R (and PRR) by Ang II, which can contribute to a plethora of cardiovascular disease processes, may be counter-regulated by a number of non-AT_1R mechanisms. Most notably, AT_2R stimulation usually causes opposing effects to AT_1R, as indicated. It is also likely that the MasR exerts a similar counter-regulatory role whereas the evidence is more preliminary and speculative for AT_4R/IRAP. In terms of mediators, Ang II itself stimulates AT_2R whereas the shorter Ang peptides stimulate their cognate receptors and possibly also AT_2R.

pathological effects [7–14]. Acute stimulation with Ang II regulates salt/water homeostasis and vasoconstriction, modulating blood pressure, while chronic stimulation promotes hyperplasia and hypertrophy of vascular smooth muscle cells (VSMCs). In addition, long-term exposure to Ang II also plays a pathophysiological role in cardiac hypertrophy and remodeling, myocardial infarction, hypertension, atherosclerosis, in-stent restenosis, reduced fibrinolysis, and renal fibrosis [7–14].

Ang II, an octapeptide hormone, is produced systemically via the classical RAS and locally via the tissue RAS [7–14]. In the classical RAS, circulating renal-derived renin cleaves hepatic-derived angiotensinogen to form the decapeptide angiotensin I (Ang I), which is converted by angiotensin-converting enzyme (ACE) in the lungs to the biologically active Ang II (Figure 1). Alternatively, a recently identified carboxypeptidase, ACE2, cleaves one amino acid from either Ang I or Ang II [15–18], decreasing Ang II levels and increasing the metabolite Ang 1–7, which has vasodilator properties. Thus, the balance between ACE and ACE2 is an important factor controlling Ang II levels [15–18]. Ang II is also further degraded by aminopeptidases to Ang III (Ang 2–8) and Ang IV (Ang 3–8) (Figure 1) [7]. Although the RAS was originally regarded as a circulating system, many of its components are localized in tissues, including the heart, brain, blood vessels, adrenal, kidney, liver and reproductive organs, indicating the existence of local tissue RASs [19]. In addition to ACE-dependent pathways of Ang II formation, non-ACE pathways have also been described. Chymotrypsin-like serine protease (chymase) may represent an important mechanism for conversion of Ang I to Ang II in the human heart, kidney, and vasculature and may be particularly important in pathological conditions such as coronary heart disease [20].

The biological responses to Ang II are mediated by its interaction with two distinct high-affinity G protein-coupled receptors (GPCRs) designated AT_1R and AT_2R (Figure 1) [7]. Both AT_1R and AT_2R possess similar affinity for Ang II [21]; however, pharmacologically, these receptors can be distinguished according to inhibition by specific antagonists. For example, AT_1R are selectively antagonized by biphenylimidazoles such as losartan (angiotensin receptor blockers, ARB) [21] whereas tetrahydroimidazopyridines such as PD123319 specifically inhibit AT_2R [21, 22]. Interestingly, all of the classical actions of Ang II, including vasoconstriction, effects on fluid and electrolyte homeostasis, and influences on cellular growth and differentiation, have been shown to be due to stimulation of AT_1R located on the plasma membrane of cells [7–12]. Additionally, the majority of the pathophysiological effects (i.e., cardiac hypertrophy and remodeling, myocardial infarction, hypertension, etc.) of Ang II are also mediated via the AT_1R [7–12]. In contrast, it is thought that the AT_2R counter-regulates AT_1R function (reviewed in [13, 14]). It is also speculated that during cardiovascular disease, AT_2R upregulation and activation by Ang II, or angiotensin peptide fragments (i.e., Ang III, Ang IV, and/or Ang 1–7) may limit AT_1R-mediated overactivity and cardiovascular pathologies [13, 14].

Although the AT_1R and AT_2R have been intensively investigated it is now clear that angiotensin fragments can bind to and activate other receptor subtypes. For example, Ang 1–7 acts on the Mas GPCR (MasR) and has vasodilatory and antiproliferative effects. This arm of the RAS is also thought to counterbalance the effects of Ang II acting on the AT_1R (Figure 1) (reviewed in [18]). Additionally, Ang IV can bind to the angiotensin II type 4 receptor (AT_4R) or the membrane-bound, insulin-regulated aminopeptidase (IRAP) (Figure 1) and mediate the enhancement of cognitive function, modulate blood flow, increase natriuresis, inhibit cardiomyocyte hypertrophy, and improve endothelial function in animal models of atherosclerosis [23, 24].

Finally, recent studies now suggest that renin, the aspartyl protease that cleaves angiotensinogen into Ang I, and prorenin, its proenzyme inactive form, can bind to what is now designated as the (pro)renin receptor (PRR) (reviewed in [25–27]). Interestingly, the binding of renin/prorenin to PRR has been shown to have two major consequences. First, the binding of renin to its receptor increases angiotensinogen conversion to Ang I by five-fold, and prorenin, which is virtually inactive in solution, also displays enzymatic activity following receptor binding [25–27]. Second, receptor-bound renin/prorenin activates the MAP kinases ERK1/2 and p38 pathways, which in turn, leads to the upregulation of profibrotic and cyclooxygenase-2 genes independent of Ang II generation [25–27]. Therefore, the activation and potentiation of renin/prorenin enzymatic activity, together with specific PRR-mediated signaling, could have striking effects on cardiovascular regulation. Taken together, these studies suggest that RAS is unexpectedly complex and multilayered. New components and functions of the RAS are still being unraveled and the physiological significance, and ultimately the clinical relevance, of these factors remain largely undefined.

3. Overview of miRNA Biology

MicroRNAs (miRNAs) are endogenous, short (20–23 nucleotide), and single-stranded nonprotein-coding RNA molecules that regulate gene expression (reviewed in [28]). These molecules act by binding to their target mRNAs, preferentially to the 3′-UTR, using a partial base-pairing mechanism. In order for a miRNA to give rise to functional consequences, the 7-8 nucleotides (nt) at the most 5′ end must have exact complementarity to the target mRNA, generally referred to as the "seed" region [29]. The current model for inhibition of expression by a miRNA suggests that a miRNA either inhibits translation or induces degradation of its target mRNA, depending upon the overall degree of complementarity of the binding site, number of binding sites, and the accessibility of those binding sites [30–32].

In mammals, computational predictions indicate that miRNAs may regulate 60% of all human protein coding genes [33], and have been increasingly implicated in the control of various biological processes, including cell differentiation, cell proliferation, development and apoptosis, and many pathological processes such as cancer, Alzheimer's disease, and cardiovascular disease [34–36]. There are estimated to be >1,000 miRNAs encoded by the human genome [37, 38], each of which can act on multiple target mRNAs. Conversely, individual mRNAs are commonly targeted by multiple miRNAs, which results in a combinatorial repression of gene expression more robust than the suppression that results from a single miRNA [39, 40]. Although miRNAs are known to mediate posttranscriptional gene silencing in the cytoplasm, recent evidence indicates that at least some fraction of mammalian miRNAs may also activate or inhibit gene expression at the transcriptional level [41, 42]. Taken together, these miRNA phenomena allow for enormous combinatorial complexity and regulatory potential.

4. miRNA Biogenesis

Mature miRNAs are processed from primary miRNA transcripts (pri-miRNAs), which are either transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase II transcripts (Figure 2) [43–45]. miRNAs tend to cluster throughout the genome and many of these clusters are likely transcribed as polycistrons [46–48]. Although little is known regarding the regulation of miRNA transcription, it is recognized that miRNA expression is usually regulated by established transcriptional mechanisms. Interestingly, however, it has been shown that each miRNA located within the same genomic cluster may be transcribed and regulated independently [49].

During the transcriptional process, pri-miRNAs fold into hairpin structures containing imperfectly base-paired stems and are endonucleolytically cleaved by the nuclear microprocessor complex formed by the RNase III type endonuclease Drosha and the DiGeorge critical region 8 (DGCR8) protein [50]. The Drosha/DGCR8 complex processes pri-miRNAs into ~70-nucleotide hairpins known as pre-miRNAs (Figure 2) [28, 51]. In animals, pre-miRNAs

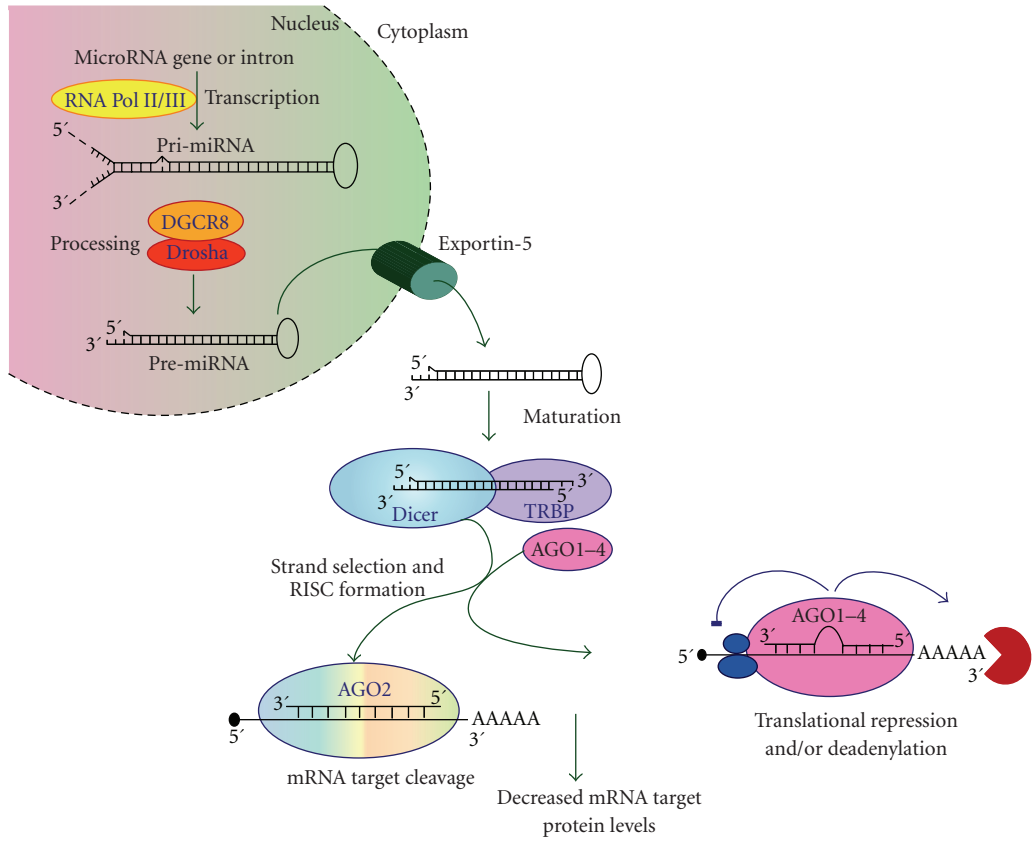

Figure 2: Schematic representation outlining miRNA biogenesis including transcription, maturation, and miRNA/mRNA targeting. This diagram also outlines two potential mechanisms for miRNA/mRNA silencing. The specific details discussing these processes are included in the text.

are exported from the nucleus to the cytoplasm via Exportin-5, where they are cleaved by Dicer complexed with the TAR RNA binding protein (TRBP), to yield ~20-bp miRNA duplexes. In principle, the miRNA duplex could give rise to two different mature miRNAs. However, in a manner similar to siRNA duplexes, only one strand is usually incorporated into miRNA-induced silencing complexes (miRISCs) and guides the complex to target mRNAs; the other strand is degraded (the complementary miRNA* strand) [52]. This functional asymmetry depends on the thermodynamic stability of the base pairs at the two ends of the duplex, with the miRNA strand, which has the least stable base pair at its 5′ end in the duplex, being loaded into the miRISC [53]. Still, recent data suggest that both arms of the pre-miRNA hairpin can give rise to mature miRNAs [28, 45, 51].

5. miRNA/mRNA Silencing

Although the details are not well understood, pre-miRNA processing by Dicer is coupled with the assembly of miRNAs into ribonucleoprotein complexes called micro-RNPs (miRNPs) or miRISCs [28, 51, 54]. One of the key components of miRNPs is the Argonaute (AGO) protein family, AGO1 to AGO4, and while all four AGO proteins function in miRNA repression only AGO2 functions in mRNA target cleavage (Figure 2) [54, 55]. Once the miRNA is processed, the mature miRNA acts as an adaptor for miRISC to specifically recognize and regulate particular mRNAs. It is currently thought that the miRNA-loaded RISC is targeted to a given mRNA by a mechanism where the miRISC binds many sites nonspecifically until the correct target site is found [56].

With few exceptions, miRNA binding sites in animal mRNAs are present in the 3′-UTR and mature miRNAs base pair with their target mRNAs imperfectly, following a set of rules that have been identified by experimental and bioinformatics analyses [29, 57–60]. First, miRNA/mRNA target recognition involves Watson-Crick base pairing that must be perfect and contiguous at the 5′-end of the miRNA from nucleotides 2 to 8. This section represents the "seed" region and nucleates the miRNA-mRNA association. Second, G:U wobble pairing in the seed sequence is highly detrimental to miRNA function despite its favorable contribution to RNA:RNA duplexes. Third, an A residue across position 1 of the miRNA, and an A or U across position 9, improve the site efficiency, although they do not need to base pair with miRNA nucleotides. Fourth, it has been established that miRNAs that have suboptimal 5′ Watson-Crick base pairing need substantial complementarity to the miRNA 3′ half to stabilize the interaction and to be functional. Finally, the context of the miRNA's binding sites harbored in the 3′-UTR

of target mRNAs, also influence the functional importance of these sites [61]. For example, miRNA site efficacy can be improved if the site is positioned at least 15 nt downstream from the stop codon, away from the center of long 3′-UTRs, and near AU-rich nucleotide regions. These factors can make the 3′-UTR regions less structured and hence more accessible to miRNP recognition.

When an endogenous miRISC programmed with miRNA binds to a recognition site that is perfectly complementary, this target mRNA will be cleaved by the miRISC (Figure 2) [56, 62–65]. In contrast, miRISCs that are imperfectly matched with target mRNAs can repress translation initiation at either the cap-recognition stage [66–70] or the 60S subunit joining stage [71]. Alternatively, binding of miRISCs can induce deadenylation and decay of target mRNAs [62].

6. Computational Algorithms to Predict miRNA/mRNA Targets

Computational miRNA/mRNA target programs remain the only source for rapid prediction of miRNA recognition sites harbored within the 3′-UTR of target mRNAs. Therefore, the development of reliable computational target prediction programs is critical in advancing our understanding of miRNA function. Given that miRNA functionality usually requires seed sequence complementarity [28, 61] the main prediction feature used in most of these programs is the sequence alignment of the miRNA seed to the 3′-UTR of candidate target genes. Additionally, many current algorithms also utilize conservation of miRNA/mRNA target sites across species as an important parameter for the identification of bona fide targets; notably however, the conservation of a miRNA binding site harbored in a given mRNA target is not a requirement for a functional miRNA.

A recent review article [72] comparing eight of the most commonly used algorithms for miRNA target prediction for the human and mouse genome programs demonstrated that the four top algorithms, DIANA-microT 3.0 (http://microrna.gr/microT) [73], TargetScan 5.0 (http://www.targetscan.org/) [74], Pictar (http://pictar.org/) [75], and ElMMo (from http://www.mirz.unibas.ch) [76] all have a precision of ∼50% with a sensitivity that ranges from 6% to 12%. Of the top four performing programs, it is important to note that TargetScan is the most up-to-date regarding the number of miRNAs and genes used and Pictar is least updated [72]. Most investigators assume that mRNA targets predicted by more than one algorithm are more accurate than other targets thus leading to higher prediction precision. However, Alexiou et al. [72] demonstrated that many of the algorithm combinations performed worse than the prediction of a single algorithm. These investigators reason that the better specificity of a combination is achieved by a higher price for the sensitivity. Taking this into account, our laboratory has analyzed all of the classical and nonclassical RAS components for putative miRNA binding sites by the TargetScan algorithm (Table 1). Importantly, this analysis suggests that miRNAs may play a major role in regulating the expression of RAS proteins.

Although many programs are available online for the prediction of individual mRNA targets of miRNAs (see above), the identification of authentic mRNA targets remains problematic. Mammalian miRNAs bind to the mRNA with imperfect complementarity thus how binding sites are recognized is only partially understood. Therefore, some bioinformatically predicted targets turn out to be false and others are entirely overlooked. Experimental validation of targets is therefore an important step in defining the functions of individual miRNAs (for review, see [77]).

7. Experimentally Validated miRNA/RAS Targets

Although classical and nonclassical RAS components harbor putative miRNA binding sites, very few of these sites have been experimentally validated. Our laboratory has demonstrated that miR-155 specifically interacted with the algorithm-predicted binding site harbored in the 3′-UTR of the human AT_1R (hAT_1R) mRNA (Table 1) [78, 79]. Additionally, miR-155 gain-of-function experiments (i.e., cells were transfected with partially double-stranded RNAs that mimic the Dicer cleavage product and are subsequently processed into their respective mature miRNAs) inhibited the expression of the hAT_1R and also attenuated Ang II-induced signaling via the hAT_1R in fibroblasts and vascular smooth muscle cells (VSMCs) [78, 79]. These results also demonstrated that transfection with miR-155 did not significantly decrease hAT_1R steady state mRNA levels, suggesting that miR-155 can decrease hAT_1R expression by inhibiting translation of the mRNA, rather than targeting it for degradation. In contrast, loss-of-function experiments (i.e., cells were transfected with miRNA inhibitors; antisense single-stranded chemically-enhanced oligonucleotides, ASO) demonstrated that transfection of anti-miR-155 not only increased hAT_1R expression but also enhanced Ang II-induced signaling via the hAT_1R, indicating that miR-155 plays a physiological role in regulating the expression of hAT_1Rs in human fibroblasts and VSMCs [78, 79]. Recently, our laboratory also demonstrated that hAT_1R expression can be regulated by miR-802 [80].

In support of the miR-155/hAT_1R studies described above, trisomy 21 (Ts21) mediated overexpression of miR-155 (the bic/miR-155 gene is located on human chromosome 21 and is triplicated in Down syndrome [DS] individuals) [81, 82] resulted in the attenuation of hAT_1R protein levels in fibroblasts isolated from one monozygotic twin with DS when compared to fibroblasts isolated from the unaffected euploid twin [83]. Interestingly, individuals with DS have significantly lower systolic and diastolic blood pressures [84–87], a reduced risk of vascular anomalies [88], and a low prevalence of coronary artery disease [89–93] when compared with the general population. Given that the overexpression of miR-155 in Ts21 results in attenuated hAT_1R protein levels, we speculate that this may be one mechanism which contributes to the lack of cardiovascular disease observed in individuals with DS [84–87].

Table 1: TargetScan* algorithm-predicted RAS putative miRNA/mRNA target sites.

RAS Component	Total Conserved miRNA Targets	Total Poorly Conserved miRNA Targets	Overall Total miRNA Targets
AGTR1 (AT_1R)	0	56	56
AGTR2 (AT_2R)	2	96	98
ACE	0	26	26
ACE2	1	57	58
AGT	1	31	32
REN	0	15	15
ATP6AP2 (PRR)	4	49	53
MAS1 (MasR)	0	12	12
LNPEP (AT_4R)	5	42	47

* Source: TargetScan (April, 2009). The number of potential targets is dramatically dependent upon the algorithm utilized.

Finally, Boettger et al. [94] demonstrated by genomic, proteomic, and transcriptional analyses that mouse ACE mRNA is a miR-145 target. The miR-143/-145 gene cluster includes miR-143 and miR-145, which lie within a 1.7-kb highly conserved region of mouse chromosome 18 [94]. miRNA microarray hybridization experiments revealed that miR-143 and miR-145 are enriched in murine vascular smooth muscle cells (VSMCs) [94, 95]. In the mouse embryo, miR-143/-145 expression is restricted to heart, vascular, and visceral SMCs [94, 95]. During late fetal and postnatal development, miR-143/-145 expression is downregulated in the heart but persists in vascular and visceral SMCs. Homozygous miR-143/-145 knockout mice were viable but exhibited thinning of the arterial tunica media (muscular layer), with a reduction in the number of contractile VSMCs and a concomitant increase in the number of proliferative VSMCs [94]. Physiological characterization of miR-143/-145 deficient mice and arterial segments from these animals revealed defects in Ang II-induced VSMC contractility and homeostatic control of blood pressure. Consistent with this observation, pharmacological inhibition of ACE or the AT_1R partially reversed vascular dysfunction and normalized gene expression in the mutant mice [94]. Since miR-145 regulates the expression of ACE, when the miR-143/-145 gene cluster is mutated, the levels of membrane-bound ACE in VSMCs increase, causing the chronic stimulation of VSMCs by Ang II, which in turn results in desensitization and "angiotensin resistance" of VSMCs. Given that miR-143/145 mutant mice developed neointimal lesions in the absence of hyperlidemia, lipid depositions, and foam cells also highlights the potential role of VSMCs in the pathogenetic process leading to atherosclerosis. The enhancement of Ang II signaling due to the increased expression of ACE would certainly contribute to this process, since increased levels of Ang II have been shown to promote atherosclerotic lesions in apoE-deficient mice [96].

Although the hAT_1R and mouse ACE are experimentally validated targets of miRNAs, it is important to note that none of these recognition sites are conserved across species (Table 1). Therefore, although miR-155 and -802 repress hAT_1R expression in humans, these miRNAs will not lead to the repression of AT_1R levels in mice or rats. Likewise, miR-145 will repress ACE expression in mice but will not regulate ACE levels in humans.

8. miRSNPs

Since a large number of miRNA binding sites are harbored in the 3′-UTRs of RAS component mRNAs (Table 1), there is a high probability that SNPs will occur within miRNA target sites. By definition, miRSNPs have the potential to create, destroy, or modify the efficiency of miRNA binding, if the SNP occurs in the seed region (i.e., the region of base-pairing between nucleotides 2 and 8 of the miRNA and complementary nucleotides in the target mRNA) [97–99]. Based on this definition, there are two mechanisms by which miRSNPs can be functionally important: as a gain- or as a loss-of-function variation. A gain-of-function effect would result if the SNP enhances the targeting of the miRNA or creates a new target site in the 3′-UTR of the mRNA. In this scenario, protein expression of the target mRNA would be attenuated. In contrast, a loss-of-function effect would result when the SNP decreases or abolishes the interaction of the miRNA with its mRNA target, thus resulting in an augmentation of protein expression. In support of this conclusion, miRSNPs have been implicated in Tourette syndrome [100], papillary thyroid cancer [101], muscularity in sheep [102], hereditary spastic paraplegia type 31 [103], methotrexate resistance [104], breast cancer [105], and tumor susceptibility [106]. These examples include both gain- and loss-of-function miRSNPs.

9. The Human AT_1R Gene and miRSNPs

The hAT_1R gene has been found to be highly polymorphic [107]. In particular, a SNP has been described in which there is an A/C transversion at position +1166 (i.e., 1166 base-pairs downstream from the start codon, dsSNP# rs5186) located in the 3′-UTR of the hAT_1R gene. The increased frequency of the +1166 C-allele has been associated with hypertension [108–115], cardiac hypertrophy [116–118], aortic stiffness [119–121], myocardial infarction [122], heart failure [123–125], abdominal aortic aneurysms [126, 127],

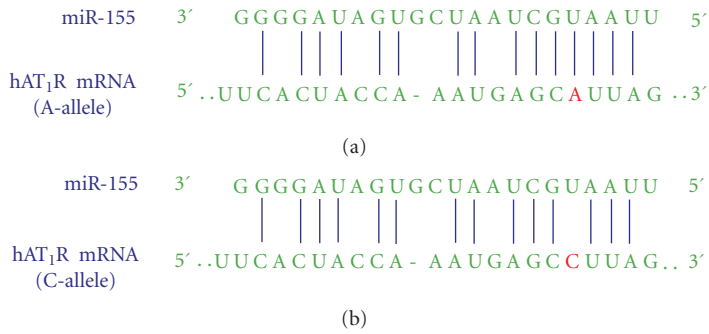

Figure 3: The human AT_1R +1166 A/C SNP occurs in the miR-155-binding site. (a) Complementarity between miR-155 and the hAT_1R 3′-UTR site targeted (70–90 bp downstream from the human AT_1R stop codon). The +1166 A/C SNP corresponds to the nucleotide 86 bp downstream from the human AT_1R stop codon (shown in red print). The binding of miR-155 to the hAT_1R 3′-UTR target site fulfills the requirement of a 7-bp seed sequence of complementarity at the miRNA 5′ end when the +1166 A-allele is expressed. (b) Complementarity between miR-155 and the human AT_1R 3′-UTR harboring the +1166 C-allele. If the +1166 C-allele is expressed, the seed sequence requirement would not be met and, as a consequence, it would be expected that human AT_1R expression would be elevated [79].

and increased oxidative stress levels in human heart failure [128]. However, the physiological relevance of this polymorphism is uncertain because of its location within the noncoding region of the hAT_1R gene. Since our laboratory has previously demonstrated that miR-155 interacted with a specific *cis*-response element localized in the hAT_1R 3′-UTR [78], we investigated whether or not there was a correlation between the +1166 A/C SNP and the miR-155-binding site [79, 83]. Importantly, computer alignment revealed that the +1166 A/C SNP occurs within the *cis*-response element at the site where miR-155 was shown to interact (Figure 3). The interaction between miR-155 and the hAT_1R 3′-UTR harboring the A-allele fulfills the seed sequence rules [29] since there is a 7-bp region of complementarity between the 5′ end of miR-155 and the hAT_1R mRNA target site (Figure 3(a)). In contrast, if a hAT_1R mRNA that harbors the +1166 C-allele is expressed, the complementary seed site is interrupted (Figure 3(b)), and the thermodynamics of the miRNA:mRNA duplex would be significantly altered (i.e., a decrease in free energy) [79]. Therefore, the presence of the +1166 C-allele miRSNP would decrease the ability of miR-155 to interact with the *cis*-regulatory site located in the hAT_1R 3′-UTR. As a consequence, it would be expected that aberrantly high levels of the hAT_1R would be synthesized. In support of this hypothesis we demonstrated that when the hAT_1R *cis*-response element harboring the C-allele was present in luciferase mRNAs, the ability of miR-155 to inhibit luciferase activity was significantly attenuated [79]. When identical experiments were performed utilizing mutant miR-155, which restored perfect Watson-Crick complementarity, luciferase activity was decreased to levels that were comparable with experiments utilizing the hAT_1R *cis*-response element harboring the A-allele and miR-155 [79]. To further demonstrate that the presence of the +1166 C-allele can influence hAT_1R density, expression constructs that produced hAT_1R mRNAs containing either the A- or C-allele were cotransfected with miR-155 or mut-miR-155. These experiments again demonstrated that when seed sequence complementarity was not fulfilled, regardless of whether miR-155 or mutant miR-155 was utilized, hAT_1R levels were always higher than the levels obtained when perfect complementarity was present between the miRNA and the hAT_1R *cis*-response element [79]. Taken together, these studies provide the first feasible biochemical mechanism by which the +1166 A/C polymorphism (i.e., miRSNP) can lead to increased hAT_1R densities and possibly cardiovascular disease.

10. miRSNPS and RAS Component Genes

To begin to investigate whether other RAS components harbored SNPs in their 3′-UTRs which created miRSNPs, the SNP Geneview Report (http://www.ncbi.nlm.nih.gov/nucleotide/) for each gene was surveyed and all of the identified SNPs were analyzed utilizing the Patrocles algorithm (http://www.patrocles.org/) [99]. Specifically the "Patrocles Finder" was utilized since it allows one to compare two sequences and subsequently determines the miRNA binding sites that are different between the two sequences. Thus, a region of the transcribed 3′-UTR which harbored the common allele was compared with the same region containing the mutated allele (select motif length: human 7 mers) and the generation of putative miRSNPs was determined (Table 2). Importantly, this analysis demonstrated that the 3′-UTRs of the RAS components harbor a number of miRSNPs some of which alter or destroy legitimate miRNA binding sites and others that create novel, illegitimate target sites. Hypothetically, if a loss-of-function miRSNP occurred in the AGTR1, ACE, AGT, REN, or ATP6AP2 gene, an increased incidence of hypertension, cardiac/vascular remodeling, and atherosclerosis would be observed (Table 3). In contrast, if a loss-of-function miRSNP occurred in the AGTR2, ACE2, MAS1, or LNPEP gene, a decreased incidence of hypertension, cardiac/vascular remodeling, and atherosclerosis would be expected (Table 3).

Table 2: Patrocles* algorithm-predicted RAS component miRSNPs.

RAS Component	dbSNP#	Hetero	dbSNP	bp from Stop Codon	Loss of Function miRSNP	Gain of Function miRSNP
AGTR1 (AT_1R)	rs5184	0.069	A>G	2	miR-668	
	rs56343250	N.D.		40	mR-1237, -1248	
	rs5185	0.130	T>G	70	miR-302c, -573	miR-143*, -301a
	rs12721277	0.028	G>A	82	miR-579	
	rs5186	**0.500**	**A>C**	**86**	**miR-155**	
	rs5187	N.D.	A>G	93	miR-562, -548n	miR-646
	rs1799870	0.055	C>T	135		
	rs5188	0.109	G>A	317		miR-1197
	rs55707609	N.D.	A>T	335	miR-299-3p	
	rs5189	0.500	G>T	437		miR-570
	rs12721276	0.061	C>A	461		miR-128, -27a
	rs12721275	0.055	C>T	484	miR-143*	
	rs12721274	0.028	T>C	556	miR-641	
	rs440881	N.D.	A>C	565	miR-30b	
	rs1051649	N.D.	C>T	680	miR-1197	miR-7
	rs35533650	N.D.	A>G	704		
	rs380400	0.500	A>G	798		
	rs35393661	N.D.	->AT	803		
AGTR2 (AT_2R)	rs34589510	N.D.	->T	57		
	rs5193	0.241	G>T	199		
	rs5194	0.499	A>G	205		miR-1229
	rs11091046	0.496	A>C	501		
	rs17231436	0.131	G>C	581	miR-384	
	rs41312570	N.D.	C>T	645	miR-301a, -130a	
	rs17231443	0.025	C>G	816		miR-548f, -570
	rs17237806	0.050	C>T	837		
	rs12858432	N.D.	C>T	906		
	rs12845035	0.030	C>G	1103	miR-361-3p	
	rs17237820	0.073	A>T	1111	miR-548a-3p	
	rs17231478	0.038	G>T	1185		
	rs17237827	0.025	A>G	1274		
	rs17231450	0.050	C>A	1318	miR-150*	
	rs17231457	0.050	A>C	1536		miR-571
ACE	None					
ACE2	None					
AGT	rs61762526	0.007	G>T	33		
	rs5042	0.008	C>T	40	miR-486-3p	
	rs4753	0.021	G>C	158		miR-337-5p
	rs61751079	0.002	G>A	159		miR-639, -539
	rs5043	0.021	T>C	167		
	rs61751080	0.002	T>A	168		
	rs11684	N.D.	C>T	184	miR-103, -107	
	rs1803103	N.D.	G>T	187	miR-483-3p	
	rs15022	N.D.	G>T	188	miR-483-3p	
	rs1803104	N.D.	A>G	316		
	rs1803106	N.D.	A>C	338	miR-129-5p	miR-335*
	rs61751081	0.002	C>T	350		
	rs5044	0.029	T>G	423		miR-1283, -606
	rs61762525	0.004	C>T	473		miR-548l, -559
	rs7079	0.312	C>A	556	miR-218-1*, -584	
	rs55720804	0.035	C>-	573		

Table 2: Continued.

RAS Component	dbSNP#	Hetero	dbSNP	bp from Stop Codon	Loss of Function miRSNP	Gain of Function miRSNP
	rs61751082	0.023	C>-	575		
REN	rs11799601	0.500	C>A	48		miR-326, -330-5p
	r11571124	0.041	C>T	145	miR-138	miR-150*
ATP6AP2 (PRR)	rs5963816	0.296	A>T	266	miR-664	
	rs6609080	0.186	A>G	358	miR-1179	
	rs9062	N.D.	G>T	654	miR-508-5p	miR-410
	rs10536	0.334	A>G	761	miR-802	miR-140-3p, -497*
	rs1060063	0.340	T>C	809		
MAS1 (MasR)	None					
	rs17087239	0.004	C>T	61		miR-992, -15b
	rs39602	0.488	C>G	217		
	rs75912980	0.180	T>A	364		miR-1225-5p, -9
	rs1057808	0.014	T>C	408		miR-922, -15b
LNPEP (AT_4R)	rs3756618	0.028	A>T	501	miR-22*, -26b*	miR-223
	rs35838718	N.D.	A>-	561		
	rs79818663	0.105	G>T	594		
	rs77639920	N.D.	C>T	614	miR-664*	
	rs62377081	N.D.	G>T	695	miR-302a*, -1264	miR-548l

* Source: http://www.patrocles.org/.

Table 3: Physiological ramifications of RAS miRSNPs.

RAS Component	Loss of Function miRSNP	Physiological Result	Gain of Function miRSNP	Physiological Result
AGTR1 (AT_1R)	AT_1R↑	Hypertension Cardiac/vascular remodeling Atherosclerosis	AT_1R↓	Antihypertensive Antiremodeling Anti-atherosclerosis
ACE	ACE↑		ACE↓	
AGT	AGT↑		AGT↓	
REN	REN↑		REN↓	
ATP6AP2 (PRR)	PRR↑		PRR↓	
AGTR2 (AT_2R)	AT_2R↑	Antihypertensive Antiremodeling Antiatherosclerosis	AT_2R↓	Hypertension Cardiac/vascular remodeling Atherosclerosis
ACE2	ACE2↑		ACE2↓	
MAS1 (MasR)	MasR↑		MasR↓	
LNPEP (AT_4R)	AT_4R↑		AT_4R↓	

11. Conclusion

miRNAs have been increasingly implicated in the control of various biological processes, including cell differentiation, cell proliferation, development and apoptosis, and many pathological processes such as cancer, Alzheimer's disease, and cardiovascular disease [34–36]. Importantly, several studies which have now demonstrated that polymorphisms at the miRNA target site in the 3′-UTRs of transcribed mRNAs can have detrimental effects given that miRSNPs can lead to the modulation of gene expression [79, 83, 100–106]. In support of this hypothesis, a recent study suggested that differences in SNP allele frequency among ethnic groups account for differences in gene expression [129]. Therefore, we speculate that miRSNPs can modulate RAS phenotypic gene expression diversities, at least in part, through alteration of miRNA target binding capability, ultimately leading to differences in the susceptibility to complex genetic disorders, such as cardiovascular disease.

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Review Article

Roles of Beta2- and Beta3-Adrenoceptor Polymorphisms in Hypertension and Metabolic Syndrome

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Hypertension, diabetes mellitus (especially type 2 diabetes mellitus), metabolic syndrome and obesity are rapidly growing public health problems. Sympathetic nerve activation is observed in obesity, hypertension and diabetes mellitus, which have strong genetic as well as environmental determinants. Reduced energy expenditure and resting metabolic rate are predictive of weight gain, and the sympathetic nervous system participates in regulating energy balance through thermogenesis. The thermogenic effects of catecholamines in obesity have been mainly mediated via the β2- and β3-adrenergic receptors in humans. Further, β2-adrenoceptors importantly influence vascular reactivity and may regulate blood pressure. Genetic polymorphistns of the β-adrenoceptor gene have been shown to alter the function of several adrenoceptor subtypes and thus to modify the response to catecholamine. β2-adrenoceptor polymorphisms (Arg16Gly, Gln27Glu, and Thr164Ile) have been studied in relation to hypertension. Genetic variations in the β3-adrenoceptor (i.e. Try64Arg variant) are also associated with both obesity and hypertension. However, the precise relationships of the polymorphisms of β2- and β3-adrenoceptor genes with sympathetic nervous system activity, hypertension, and metabolic syndrome have not been fully clarified. This paper will discuss the current topics involving the influence of the sympathetic nervous system and β2- and β3- adrenoceptor polymorphisms in hypertension and metabolic syndrome.

1. Introduction

Obesity, hypertension, and metabolic syndrome (type 2 diabetes mellitus) are major and growing health problems and are known as high-risk factors for subsequent cardiovascular and renal complications [1–3]. Obesity, hypertension, diabetes, and metabolic syndrome are intimately associated [4–6], and sympathetic nervous activation is frequently observed in those conditions. Thus, sympathetic nerve activation may play a major role in the onset and development of hypertension, obesity, and metabolic syndrome (diabetes mellitus) as well as cardiovascular complications in patients with hypertension, diabetes and obesity [2, 7].

The sympathetic nervous system plays an important role in the regulation of energy expenditure. Reduced energy expenditure and resting metabolic rate are predictive of weight gain (obesity). The sympathetic nervous system participates in regulating energy balance through thermogenesis [8]. A large part of the sympathetic nervous system-mediated energy expenditure takes place in skeletal muscle, via the coupling of catecholamines with β2-adrenoceptors. Catecholamines are also powerful regulators of lipolysis and act via β1-, β2-, β3- (stimulatory), and α2- (inhibitory) adrenoceptor subtypes in adipose tissue, where their role becomes especially important during both exercise and energy restriction, when increased need for fat as a fuel exists. Thus, β-adrenoceptors play important roles in energy expenditure and control body weight [9–13].

Recently, there is evidence that human hypertension and obesity have strong genetic backgrounds [14–16]. Harrap et al. reported that about 46% of the phenotype of systolic blood pressure are determined genetically for hypertension [17, 18]. Masuo et al. [18–22] have reported close relationships between β2- and β3-adrenoceptor polymorphisms

accompanying elevated sympathetic nervous activity, blood pressure elevation (hypertension), weight gain (obesity), and insulin resistance in a series of longitudinal study. Many epidemiological studies on the relationships between β-adrenoceptor polymorphisms, hypertension, obesity, and diabetes (metabolic syndrome) have still been discordant.

This paper will discuss the current topics involving the contribution of the sympathetic nervous system and β2- and β3-adrenoceptor polymorphisms in the onset and the development of hypertension and metabolic syndrome (type 2 diabetes mellitus).

2. Subtypes of Adrenoceptors (Table 1)

The adrenoceptors (or adrenergic receptors) are a class of G protein-coupled receptors which specifically bind their endogenous ligands, the catecholamines (epinephrine and norepinephrine). Many tissues possess these adrenoceptors, and the binding of an agonist generally elicits a "typical" sympathetic response (i.e., the fight-or-flight response). Table 1 shows the effects of catecholamines bound to adrenoceptors (Table 1) and these effects on sympathetic nervous activity are through α- and β-adrenergic receptors.

There are several types of adrenergic receptors, but there are two main groups: α-adrenoceptors (α1- and α2-adrenoceptors) and β-adrenoceptors (β1-, β2-, and β3-adrenoceptors). Table 1 also summaries the distributions and functions of the α1-, α2-, β1-, β2-, and β3-adrenoceptors [24, 25]. The α-receptors bind norepinephrine and epinephrine, though norepinephrine has higher affinity. Phenylephrine is a selective agonist of the α-adrenoceptors (both α1- and α2-receptors), thus phenylephrine is usually used to investigate the α-adrenoceptors function. β-adrenoceptors are linked to G proteins, which are linked to adenyl cyclase. β-adrenoceptor agonists cause the intracellular elevation of the second messenger cyclic AMP. Downstream effects of cyclic AMP include cyclic AMP dependent protein kinase, which mediates the intracellular events following hormone binding.

3. Sympathetic Nervous Activity and Insulin Resistance in Hypertension (Figure 1)

Insulin resistance in hypertension has been well documented in many epidemiological and clinical studies [8, 26, 27]. Several investigators have reported that chronic insulin administration elevates blood pressure in rats and in humans [28], although insulin also has effects on vasodilation. In addition, many clinical and epidemiological studies have demonstrated the close relationships between sympathetic nerve activity, insulin resistance and hypertension [19, 29–32].

Landsberg and other investigators examined the effect of feeding and starvation on sympathetic nerve activity in the cardiac tissue of animals, noting that feeding raised sympathetic nerve activity, and starvation had the opposite effect [33–35]. Energy intake stimulates hyperinsulinemia and sympathetic nerve activity resulting in blood pressure elevations in a cycle to inhibit thermogenesis. Insulin-mediated sympathetic nerve stimulation in obese subjects is a compensatory mechanism aimed at restoring the energy balance by increasing the metabolic rate [33]. Therefore, hyperinsulinemia and insulin resistance in obese subjects are all part of a response to limit further weight gain via stimulating sympathetic nerve activity and thermogenesis [28].

On the other hand, Julius et al. [36] have hypothesized that increased sympathetic nerve activity in skeletal muscle causes neurogenic vasoconstriction, thereby reducing blood flow to muscle and consequently inducing a state of insulin resistance by lowering glucose delivery and uptake in hypertension and obesity. Both blood pressure elevation and weight gain may reflect a primary increase in sympathetic nervous tone. Masuo et al. [30, 37] supported Julius's hypothesis. They described that high plasma norepinephrine might predict future blood pressure elevations and weight gain accompanying deterioration in insulin resistance observed in HOMA-IR (homeostasis model assessments of insulin resistance) [30, 37]. Rocchini et al. [38] reported that clonidine prevented insulin resistance in obese dogs over a 6-week period. Their results suggest that sympathetic nerve activity might play a major role in the development of insulin resistance accompanying blood pressure elevations. Valentini et al. [39] reported attenuation of hemodynamic and energy expenditure responses to isoproterenol infusion in hypertensive patients, suggesting that sympathetic nerve activity-induced hypertension may subsequently lead to the development of obesity.

Many epidemiological studies showed close linkages of beta2- and beta3-adrenoceptor polymorphisms with obesity, hypertension, and the metabolic syndrome shown in Tables 2, 3, and 4. Sympathetic nervous activity is related to body weight or blood pressure through β-adrenoceptors. Thus, close linkages between sympathetic nerve activity and insulin resistance might depend on the β-adrenoceptor polymorphisms. Thus, one could speculate that the strong associations between β-adrenoceptor polymorphisms and insulin resistance might provide evidence that heightened sympathetic nerve activity followed by insulin resistance might play a major role in hypertension and obesity, because β-adrenoceptor polymorphisms might relate to insulin resistance through heightened sympathetic nerve activity (Figure 1).

4. Role of β-Adrenoceptor Polymorphisms in Hypertension, Obesity, and Diabetes

The sympathetic nervous system plays an important role in the regulation of energy expenditure and blood pressure regulation. A large part of the sympathetic nervous system-mediated energy expenditure takes place in skeletal muscle, via the coupling of catecholamines with β2-adrenoceptors. Catecholamines are also powerful regulators of lipolysis and act via β1-, β2-, β3- (stimulatory), and α2- (inhibitory) adrenoceptor subtypes in adipose tissue, where their role becomes especially important during both exercise and

Table 1: Comparisons of adrenergic receptor subtypes.

Receptor type	Agonist potency order	Action sites	Functions
α1-adrenoceptor	norepinephrine≥	*blood vessels* of skim, gastrointestinal, kidney	*vasoconstriction*
	epinephrine⋙	ureter, uterus, urethral sphincter, bronchioles	smooth muscle contraction,
	isoprenaline	urinary bladder,	contraction,
		iris, blood vessels of erectile tissue,	smooth muscle relaxation,
		heart muscle,	*positive ionotropic effect*
		salivary gland,	increase in secretion,
		adiposetissue, liver	*glycogenolysis and gluconeogenesis,*
		sweat glands,	increase in secretion,
		kidneys	*Na reabsorption*
α2-adrenoceptor	epinephrine>	pancreas and	*inhibition of insulin secretion,*
	norepinephrine⋙		*induction of glucagon release, and*
	isoprenaline	gastrointestinal tract	contraction of sphincters
β1-adrenoceptor	isoprenaline>	*heart,*	*increase cardiac output,*
	Norepinephrine>	*kidneys* (juxtaglomerular cells),	*increase renin release, and*
	Epinephrine	*adipose tissue*	*lipolysis*
β2-adrenoceptor	isoprenaline>	Bronchi,	smooth muscle relaxation,
	epinephrine≫	urinary sphincter, bladder wall,	smooth muscle relaxation,
	norepinephrine	*skeletal muscle,*	*dilate arteries*
		adipose tissue, liver	*glycogenolysis and gluconeogenesis,*
		gastrointestinal tract,	contract sphincters,
		salivary glands,	thickened secretions,
		mast cells, and	inhibit histamine release, and
		kidneys (juxtaglomerular cells)	*increase renin release*
β3-adrenoceptor	isoprenaline> norepinephrine = epinephrine	*adipose tissue*	*enhancement of lipolysis*

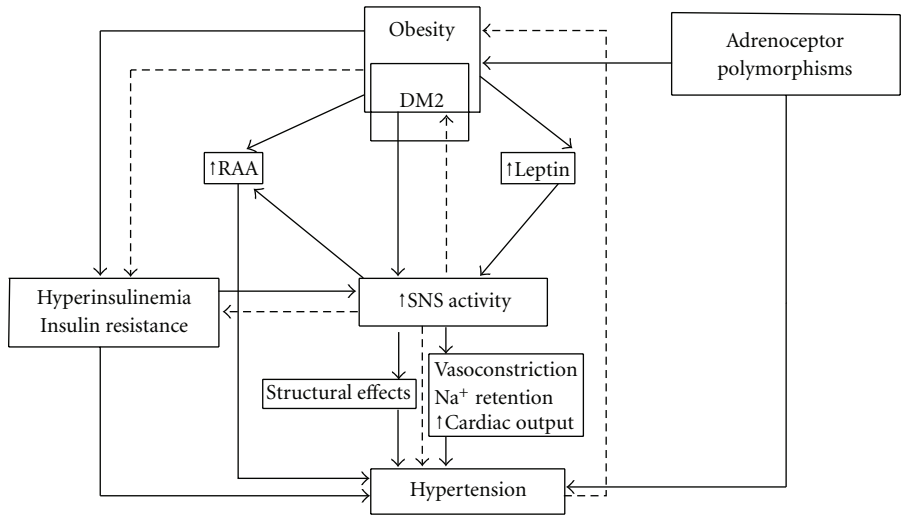

Figure 1: Potential pathophysiological mechanisms by which obesity may contribute to hypertension (modified figure from [23]). RAAS: renin-angiotensin-aldosterone system; SNS: sympathetic nervous system; OSA: obstructive sleep apnea; BRS, baroreflex sensitivity.

Table 2: Arg16Gly, β2-adrenoceptor polymorphisms: association with hypertension, metabolic syndrome (type2 diabetes: (DM)), and obesity.

Authors	Year	Populations	Subjects	Associations with the polymorphism
Large et al. [40]	1997	Swedish	140 Caucasian women with a wide range of obesity	Obesity
The Quebec Family Study [41]	2000	Canada	Caucasian men and women	Obesity, hyperlipidemia
Hayakawa et al. [42]	2000	Japanese	210 Japanese men from a population	No association with obesity
Jia et al. [43]	2000	USA	Caucasians (298 hypertensive versus 298 normotensive subjects)	No association with hypertension
Xie et al. [44]	2000	USA	Black and white Americans (including normotensive and hypertensive subjects)	No associations with hypertension
Candy et al. [45]	2000	English	England Black African men (including 192 hypertensive and 123 normotensive men)	No association with hypertension
Cockcroft et al. [46]	2000	Caucasian	127 young normotensive men	Forearm vascular responses (hypertension)
Meirhaeghe et al. [47]	2000	French	1195 middle-aged Caucasian from the urban population	Obesity, if subjects carry Gln27Gln
Kato et al. [48]	2001	Japanese	842 hypertensive and 633 normotensive subjects	BP levels (hypertension) in normotensives
Bengtsson et al. [49]	2001	Swedish	Hypertensive patients with and without type 2 DM	Hypertension in subjects with DM
The Bogalusa Heart Study [50]	2002	USA	1151 Caucasian and Black Africans children (including boys and girls)	Weight gain in males
Kim et al. [51]	2002	Korean	type 2 DM patients	Obesity, DM, hyperlipidemia
Chang et al. [52]	2002	Taiwanese	type 2 DM patients	Type 2 DM
Van Rossum et al. [53]	2002	Dutch	286 subjects with a significant weight gain over 7 years including men and women	Weight gain in men, but not in women
The HERITAGE family study [54]	2003	Canada	Sedentary black and white women	Lower fat in obese white women
Pereira et al. [20]	2003	Brazilian	1576 ethnically mixed population (including men and women)	Systolic BP, BMI
The Olivetti heart study [55]	2004	Italian	993 middle-aged men regardless of BP levels or BMI	No association with obesity or hypertension
Ikarashi et al. [56]	2004	Japanese	type 2 diabetic patients	Association with IR
Tafel et al. [57]	2004	Germany	extremely obese children	No association with obesity
Ellsworth et al. [58]	2005	USA	Black and white American men and women	BMI (obesity) in only men
Trombetta et al. [59]	2005	Brazilian	Brazilian healthy women	Hypertension (blunted forearm vasodilation response)
Masuo et al. [21]	2005	Japanese	Nonobese, normotensive men	Weight gain, BP elevation, obesity-HT
Masuo et al. [60]	2005	Japanese	Nonobese, normotensive men	Insulin resistance
Masuo et al. [61, 62]	2006	Japanese	Normotensive men (including nonobese and obese men)	Weight gain, blunted leptin-sympathetic axis
Kurabayashi et al. [63]	2006	Japanese	PCOS patients	Association with high prevalence of PCOS Accompanying IR
Gjesing et al. [64]	2007	Dutch	7808 white subjects	No association with hypertension or obesity
Masuo et al. [65]	2007	Japanese	219 nonobese, normotensive men	Association with high SNA followed by IR

BP: blood pressure; BMI: body mass index; HT: hypertension; DM: diabetes mellitus; IR: insulin resistance; PCOS: polycystic ovary syndrome; SNA: sympathetic nervous activity.

Table 3: Gln27Glu, β2-adrenoceptor polymorphisms: association with hypertension, metabolic syndrome (type2 diabetes (DM)), and obesity.

Authors [reference number]	Year	Populations	Subjects	Associations with the polymorphism
Large et al. [40]	1997	Swedish	Caucasian women with a wide range of obesity	Association with obesity
Echwald et al. [66]	1998	Danish	Caucasian juvenile-onset obese men	No association with obesity
Hellström et al. [67]	1999	Swedish	Caucasian men and women	Association with obesity only in women
Kortner et al. [68]	1999	German	Caucasian with morbid obesity	No association with obesity
Xie et al. [44]	2000	USA	Black and white Americans	No associations with hypertension
The Quebec Family Study [41]	2000	Canada	Caucasian men and women	Association with obesity and hyperlipidemia
Hayakawa et al. [42]	2000	Japanese	210 Japanese men from a population	No association with obesity
Candy et al. [45]	2000	England	Black African men (including 192 hypertensive and 123 normotensive men)	No association with hypertension
Meirhaeghe et al. [47]	2000	French	1195 middle-aged Caucasian in the urban population	Association with obesity in men
Kato et al. [48]	2001	Japanese	842 hypertensive and 633 normotensive subjects	Association with BP levels (hypertension) in NT
Kawamura et al. [69]	2001	Japanese	Japanese-Americans	No association with obesity or DM
Ukkola et al. [70]	2002	USA	12 pairs of twins, Caucasians	Association with weight gain (obesity)
Kim et al. [51]	2002	Korean	Patients with type 2 DM	Association with obesity, DM, and hyperlipidemia
Gonzalez-Sanchez et al. [71]	2003	Spanish	666 Caucasian-based study (including men and women)	Association with obesity only in men
The HERITAGE family study [49]	2003	Canada	Sedentary black and white men	Association with lower fat in obese white men
Pereira et al. [20]	2003	Brazilian	1576 ethnically mixed population (including men and women)	No association with systolic BP or BMI
The Olivetti heart study [55]	2004	Italian	993 middle-aged men (regardless of BP levels or BMI)	No association with obesity or hypertension
Tafel et al. [57]	2004	Germany	Extremely obese children	No association with obesity
Masuo et al. [21]	2005	Japanese	Nonobese, normotensive men	Association with BP elevation, but no association with IR
Trombetta et al. [59]	2005	Brazilian	Brazilian healthy women	Association with hypertension (blunted forearm vasodilation response)
Kurabayashi et al. [63]	2006	Japanese	PCOS women	Association with high prevalence of PCOS accompanying IR
Gjesing et al. [64]	2007	Dutch	7808 white subjects	No association with hypertension or obesity
Masuo et al. [65]	2007	Japanese	219 nonobese, normotensive men	No association with IR

BP: blood pressure; BMI: body mass index; DM: diabetes mellitus; NIDDM: noninsulin-dependent diabetes mellitus; IR: insulin resistance; PCOS: polycystic ovary syndrome; NT: normotensive subjects.

energy restriction, when increased need for fat as a fuel exists. Stimulation of β-adrenergic receptors by the sympathetic nervous system is a significant physiological modulator of pre- and postprandial energy expenditure [11–13] and total daily energy expenditure [9, 10].

Recent studies show that β-adrenoceptors are polymorphic. Single nucleotide polymorphisms might have functional consequences in terms of receptor activity and regulation and hence may contribute to the pathophysiology of hypertension and obesity. On the other hand, there are few studies on the relationships between α-adrenoceptor polymorphisms, hypertension, obesity, and metabolic syndrome.

4.1. β1-Adrenoceptor Polymorphisms. The β1-adrenoceptor is predominantly expressed in cardiac myocytes and adipose

Table 4: Trp64Arg, β3-adrenoceptor polymorphisms: association with hypertension, metabolic syndrome (type2 diabetes (DM)), and obesity.

Authors [reference number]	Year	Populations	Subjects	Associations with the polymorphism
Clement et al. [76]	1995	French	185 subjects with morbid obesity and 94 subjects with normal weight	Increased capacity of weight gain
Widen et al. [77]	1995	Finns	335 subjects including 207 non-DM and 128 patients with NIDDM	Insulin resistance
Walston et al. [78]	1995	Pima Indians	390 with NIDDM and 252 without NIDDM	Association with the early onset of DM2
Fujisawa et al. [79]	1996	Japanese	Patients with NIDDM	Type 2 DM, weight gain (obesity)
Silver et al. [80]	1996	Nauruans	65 obese subjects with NIDDM	No association with DM2 or IR
Fujisawa et al. [81]	1997	Japanese	Essential hypertension patients	No association with IR during hyperinsulinemia euglycemic glucose clamp
Sakane et al. [82]	1997	Japanese	131 obese women versus 218 controls	Association with IR and obesity
Rissanen et al. [83]	1997	Finns	110 with NIDDM, 183 with IR, and 82 controls	No association with NIDDM or IR
McFarlane-Anderson et al. [84]	1998	Jamaican	Population study	Association with hyperglycemia only in women, but not in men
Gracía-Rubi et al. [85]	1998	American	Postmenopausal women	Association with IR
Janssen et al. [86]	1998	Dutch	Postmenopausal women	Association with IR
Shiwaku et al. [87]	1998	Japanese	Moderate overweight men	No association with obesity
Ongphiphadhanakul et al. [88]	1999	Thais	76 men and 135 women	No association with IR assessed by fasting insulin/glucose ratio
Pulkkinen et al. [89]	1999	Finns	185 untreated non-DM and 119 untreated NIDDM	No association with IR or CHD in both non-DM and NIDDM
Christiansen et al. [90]	1999	Danish	196 dizygotic twins	Association with lower insulin secreting capacity
Kawamura et al. [69]	1999	Japanese-American	Japanese living in USA versus living in Japan	Similar distribution between Japanese-America and Japanese-Japanese. Association with IR in subjects with impaired oral glucose tolerance test.
Stangl et al. [91]	2001	German	1000 with CHD and 1000 controls	No association with prevalence of CHD or IR
Strazzullo et al. [92] (The Olivetti Prospective Heart Study)	2001	Italian	979 population study	No association with IR observed in HOMA-IR
Ishii et al. [93]	2001	Japanese	196 young normoglycemic men, 186 old normoglycemic men, and 122 old hyperglycaemic men	No association with IR or NIDDM
Kurokawa et al. [94]	2001	Japanese	meta-analysis in 6582 subjects	BMI (obesity)

Table 4: Continued.

Authors [reference number]	Year	Populations	Subjects	Associations with the polymorphism
Ochoa et al. [95]	2004	Spanish	185 obese and 185 nonobese children	BMI (obesity)
Porto et al. [96]	2004	Argentina	121 NT and 54 HT from 934 high school students	Association with central obesity, but no association with IR
Tsai et al. [97]	2004	Taiwanese	299 pregnant women	No association with gestational IR
Ellsworth et al. [58]	2005	USA	1179 African-Americans and white-Americans	BMI (obesity)
Masuo K, et al. [21]	2005	Japanese	Nonobese, normotensive men	BP elevation
Masuo et al. [62]	2006	Japanese	55 obese normotensive men	Weight gain (obesity), BP elevation (hypertension)
Højlund et al. [98]	2006	Danish	10 male twins	No association between heterozygous for Trp64Arg and IR or NIDDM
Tamaki et al. [99]	2006	Japanese	1416 population study without HT, DM, or hyperlipidemia	No association with metabolic syndrome
Morcillo et al. [100]	2008	Spanish	1020 population study	Join association of alleles of -75A and Arg64 with the risk of DM
Gjesing et al. [101]	2008	Danish	7605 population study	Association with NIDDM and IR, but no association with obesity
Dunajska et al. [102]	2008	Polish	284 postmenopausal women	No association with metabolic syndrome

BP: blood pressure; BMI: body mass index; DM: diabetes mellitus; NIDDM: noninsulin-dependent diabetes mellitus; DM2: type 2 diabetes mellitus; IR: insulin resistance.

Table 5: Confounding variables considered to cause the discrepancy of the relationships between β-adrenoceptor polymorphisms and phenotypes of hypertension and metabolic syndrome in obesity.

Variables [reference number]	Findings in the studies
Severity of obesity [16, 57, 62, 76, 95]	In lean subjects, β2-AR polymorphisms linked to obesity and obesity-related hypertension, but in obese subjects, β2- and β3-AR polymorphisms relate to obesity and obesity-related hypertension. Morbid obesity is linked with β3-AR polymorphisms, but overweight or mild obesity is not associated with those.
Gender differences [71]	Interaction between β1- and β2-AR polymorphisms with changes in BMI was observed in men only, while in women an interaction between β1- and β3-AR polymorphisms was observed in a longitudinal over a 24-year period large cohort study.
Ethnic difference [103, 104]	Distributions of β-AR polymorphisms are different in 8 different ethnic populations.
Haplotype [20, 58, 59, 105–107]	Functions expressed of β-AR polymorphisms are different due to the other β-AR polymorphisms.

AR: adrenoceptor; BMI: body mass index.

tissue, where its activation leads to increased heart rate and contractility and stimulation of lipolysis, respectively. The two most common β1-adrenoceptor polymorphisms are Ser49Gly and Arg389Gly, with relative allele frequencies of 0.85/0.15 and 0.70/0.30 in the Caucasian population, respectively. The β1-adrenoceptor is a candidate gene for obesity because of its role in catecholamine-mediated energy homeostasis [72, 73]. For example, in obese individuals, the degree of weight loss during a very low calorie diet has been shown to correlate with changes in β1-adrenoceptor protein concentration in adipose tissue [72]. A population cohort of 761 women showed that women carrying the Gly49 genotype had greater increases in BMI over15 years compared to those with the Ser49 genotype [73]. Conversely, the distribution of the Arg389Gly polymorphism is similar in lean and obese subjects [74] and in a large cohort study including 3981 normotensive and 2518 hypertensive subjects [75]. The factors which might explain the discrepancy of published data are shown in the later section.

4.2. β2-Adrenoceptor Polymorphisms. The β2-adrenoceptor is the dominant lipolytic receptor in white human adipose tissue [13] and in skeletal muscle [12]. It also plays an important regulatory role in the peripheral vasculature. Genetic polymorphisms of the β2-adrenoceptor have been associated with hypertension, obesity, and metabolic syndrome (diabetes mellitus). The most common polymorphisms are Arg16Gly, with an allele frequency of 0.40/0.60, and Gln27Glu, with an allele frequency of 0.55/0.45, in the Caucasian population. The Thr164Ile polymorphism is rare, occurring in only 3 to 5% of the general Caucasians population.

Studies of agonist stimulation in cultured cells demonstrate that Gly16 receptors have a greater reduction in numbers or enhanced downregulation when compared with Arg16 whereas the Glu27 receptor is resistant to down regulation when compared with the Gln27 variant [108]. A number of clinical studies have investigated the impact of these polymorphisms on vascular responsiveness [40, 109]. Gratze et al. [110] found that young normotensive white men homozygous for the Gly16 allele had higher blood pressure and lower peripheral vasodilation after infusion of the β2-agonist salbutamol. Similar results were obtained by Hoit et al. [111] using the agonist terbutaline. On the other hand, three studies investigating isoprenaline induced increase in the limb blood flow Thus, volunteers homozygous for Gly16 exhibited larger vasodilatory responses than did volunteers homozygous for Arg16 [23]. Conflicting results have also been published with regard to the effects of genetic variants on the sympathetic nervous system modulation of energy expenditure. Bell et al. [112] reported that the response of resting energy expenditure to nonspecific β-adrenoceptor stimulation (with isoproterenol infusion) was not different between the 3 genotypes of Arg16Gly. Stob et al. [41] showed that individuals carrying the Arg16Arg variant of the β2-adrenoceptor gene have a reduced thermogenic response to selective β2-adrenoceptor activation.

Associations of β2-adrenoceptor polymorphisms with hypertension and metabolic syndrome have been reported in many epidemiological studies but results are also discordant (summarised in Tables 2 and 3).

4.3. β3-Adrenoceptor Polymorphisms. The β3-adrenoceptor, which is mainly expressed in adipose tissue, differs from the β2-adrenoceptor in two ways: it has a lower affinity for catecholamines, and it resists desensitisation (i.e., downregulation). These characteristic differences might lead to the different effects of catecholamine on β2-adrenoceptors and β3-adrenoceptors. β3-adrenoceptors stimulate the mobilization of lipids from the white fat cell and increase thermogenesis in brown fat cell. Decreased function of β3-adrenoceptor in white adipose tissue could slow lipolysis and thereby cause the retention of lipids in fat cells. Slow lipolysis may contribute strongly to visceral obesity in human, and treatment of obese animal models with selective β3-adrenergic agonists reduces fat stores most effectively [94, 113, 114]. Many epidemiological studies have shown the strong relationships between β3-adrenoceptor polymorphisms (mainly Trp54Arg), hypertension, metabolic syndrome, and obesity [78, 94, 113–117] (Table 4).

4.4. Confounding Variables Affecting the Relationships of β-Adrenoceptor Polymorphisms with Obesity, Hypertension, and Diabetes (Table 5). Tables 2, 3, 4, and 5 show the discordant contributions of β-adrenoceptor polymorphisms to hypertension, metabolic syndrome (type 2 diabetes), and obesity. Table 5 summarizes factors which might explain the discrepancy of published data. Further, haplotypes of polymorphisms have strong influence on β-adrenoceptor function in each polymorphism [20, 58, 59, 105–107].

5. Conclusions

The role of the sympathetic nervous system β2- and β3-adrenoceptor polymorphisms in hypertension, metabolic syndrome (diabetes mellitus), and obesity is discussed through a literature review. Sympathetic nervous system activity and β-adrenoceptor polymorphisms (mainly β2- and β3-adrenoceptor polymorphisms) might contribute to the onset and maintenance of hypertension, metabolic syndrome, and obesity; however, the findings have been discordant. Further, few studies have been performed to evaluate the relationship between β2- and β3-adrenoceptor polymorphisms and sympathetic nervous system activity in the same study. A better understanding for the relationships of genetic background (polymorphisms) with sympathetic nervous system activity as the cause for hypertension (blood pressure elevation), metabolic syndrome (insulin resistance), and obesity (weight gain) might help for clinical treatment for obesity-related hypertension and metabolic syndrome. In fact, a number of studies have investigated genetic polymorphisms as determinants of cardiovascular response to antihypertensive drug therapy [103, 104]. But further research on gene-drug interactions is necessary. In addition, to clarify the pathogenesis and mechanisms may lead to the prevention of hypertension and metabolic syndrome in obesity.

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Research Article

Polymorphisms in α- and β-Adrenergic Receptor Genes, Hypertension, and Obstructive Sleep Apnea: The Skaraborg Sleep Study

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The sympathetic nervous system and the adrenergic receptors play an important role in regulation of blood pressure. This study explored the associations between functional polymorphisms of the α_{2B}-, β_1-, and β_2-adrenergic receptor genes and obstructive sleep apnea (OSA) in hypertensive patients and hypertension in patients with OSA in a populationbased sample of 157 hypertensive patients and 181 healthy control subjects. Only the Arg389Gly polymorphism of the β_1-adrenergic receptor gene was associated with increased risk for mild OSA in hypertensive patients (Arg/Arg versus Gly/Arg/Gly/Gly, 2.1, 95% CI, 1.02–4.7). Hypertensive men carrying the Arg389Arg genotype had higher crude and age-adjusted AHI than carriers of the Arg389Gly/Gly389Gly genotypes. When adjusted also for BMI this difference became borderline significant. This difference was not observed in women. The risk of hypertension in mild OSA was associated with increasing number of Arg-alleles (Arg/Arg OR 5.4, 95% CI 1.4–21.2).

1. Introduction

Obstructive sleep apnea (OSA) is a disorder characterized by upper airway collapse and is common in middle-aged adults [1]. OSA often leads to hypoxia and activation of the sympathetic nervous system [2, 3] which might lead to adverse metabolic reactions and high blood pressure [4, 5]. OSA has in humans been associated with hypertension [6, 7] and cardiovascular complications [8, 9]. In an earlier study of a population-based sample of hypertensive patients and normotensive controls from primary care in Sweden, OSA was found to be more prevalent in hypertensive patients, specifically in male patients compared to controls, 83% versus 64% (58% versus 49% in females) when OSA was defined as apnea/hypopnea index (AHI) $\geq$ 10 events per hour and 47% versus 25% (26% versus 24% in females) when OSA was defined as AHI $\geq$ 30 events per hour [7].

The sympathetic nervous system plays an important role in regulation of blood pressure and the adrenergic receptors are important components of this system. The aim of this study was to explore the associations between functional polymorphisms of the α_{2B}-, β_1-, and β_2-adrenergic receptor genes and occurrence of OSA in hypertensive patients and occurrence of hypertension in patients with OSA in a population-based sample.

The α_{2A}-adrenergic receptor and the α_{2B}-adrenergic receptor are expressed in the central nervous system. Blood pressure is regulated by the opposing action of these

receptors upon agonist binding. The α_{2A}-adrenergic receptor has an inhibiting effect on the sympathetic outflow while the α_{2B}-adrenergic receptor has an excitatory effect [10]. The α_{2B}-adrenergic receptor gene is located on chromosome 2 in a region where several genome scans, including one from the population in the current study [11], have found linkage to blood pressure variation and hypertension [12–14]. In the third intracellular loop of the receptor, in an area of importance in downregulation, there is a polymorphism consisting of either an insertion (I) or a deletion (D) of three amino acids at positions 301–303. Earlier studies have shown that the deletion variant confers an increased activity in the sympathetic nervous system [15] and that it is associated with hypertension in the current and other populations [16, 17].

The β_1-adrenergic receptor is expressed in cardiac myocytes [18] and upon agonist binding it confers excitatory reactions in the myocyte leading to higher cardiac output through increased cardiac inotropy and chronotropy.

The β_1-adrenergic receptor gene on chromosome 10 harbors two polymorphisms with functional properties. The Ser49Gly, located in the extra cellular portion of the receptor, has been associated with variations in resting heart rate in a dose dependent way [19]. The other polymorphism, Arg389Gly, is located in the intracellular cytoplasmic tail of the receptor near the seventh transmembrane region, where the stimulatory G-protein probably binds. In vitro studies have shown that the Arg389 variant mediates a higher isoproterenol stimulated adenylate cyclase activity than the Gly389 variant [20]. Homozygous carriers of Arg389 have been shown to have a higher risk for hypertension and higher diastolic blood pressure levels and heart frequency than carriers of the other genotypes in the current population [21] and a minor influence on both systolic and diastolic blood pressure in a larger population [22]. The homozygous Arg389 carriers have also been shown to have a greater blood pressure lowering response to atenolol [23] and metoprolol [24] compared with the homozygous Gly389 carriers strengthening its functional importance in cardiovascular regulation. Whether the polymorphism is associated with higher risk for cardiovascular complications is not clear [25, 26]. Recently, it has been shown that the Arg389Gly polymorphism can modify the beneficial effect of continuous airway pressure [27].

The β_2-adrenergic receptor is expressed in most arteries in the body and in the heart and also in the respiratory tract. Upon agonist binding it elicits dilation of the arteries and bronchi. The β_2-adrenergic receptor gene is located on chromosome 5 in an area where already in 1996 genetic variation was found to be associated with hypertension [28]. Linkage to blood pressure levels has been found in genome wide scans [29] and subsequent fine mapping has revealed association between hypertension and the β_2-adrenergic receptor gene variants [30] in the cytoplasmic tail of the receptor. Two polymorphisms, the Arg16Gly and Gln27Glu, have been found in vitro to confer different degree of downregulation of the receptor [31]. Studies on humans have given conflicting results possibly because the two polymorphisms have different properties and are in linkage disequilibrium with each other [32]. In the Ectim and PEGASE studies there was no association between the polymorphisms of the receptor and myocardial infarction [33]. In studies of Scandinavian subjects, the Arg16 and Gln27 variants were found to be associated with an increased risk of hypertension [34] and with hypertension associated with type 2 diabetes [35].

2. Subjects and Methods

2.1. Study Population. The Skaraborg Hypertension and Diabetes Project cohort have previously been described in detail [36]. This sample comprises the majority of patients with hypertension and/or type 2 diabetes ($n = 1149$) in the local community of Skara investigated in 1992-1993 and an age-stratified, random sample from 40 years of age from Skara population ($n = 1109$) that was investigated in 1993-1994 [37]. In 2000, a random sample of hypertensive and normotensive men and women from 40 to 64 years at the baseline investigations was drawn from the previous cohorts. The diagnosis of hypertension was based upon at least three consecutive diastolic blood pressure measurements ≥90 mmHg diastolic blood pressure (DBP) according to the current National Guidelines [38, 39] or ongoing antihypertensive treatment at baseline. Most hypertensive subjects in the present study (at least 85%) were already diagnosed according to the criteria used before 1989 (≥160/95 mmHg, WHO, 1989 [40]) and had ongoing antihypertensive treatment. Normotension was defined as a systolic blood pressure (SPB) < 160 mmHg and a DBP < 90 mmHg and no antihypertensive medication. Type 2 diabetes was diagnosed according to the current WHO recommendations [41] in 21 hypertensive patients. A full-night polysomnography (PSG) at home was performed in 344 subjects, 161 patients with hypertension, and 181 individuals without hypertension. The local ethics committee approved the study and written informed consent was obtained from all the participants.

2.2. Study Procedure. At baseline 1992–1994 all subjects were surveyed for cardiovascular disease (CVD) risk factors using a previously published protocol [42]. Sleep studies were conducted during 2000–2002. A specially trained study nurse performed the basic investigations. The blood pressure was measured with Tricuff [43] in the supine position with a sphygmomanometer after 5 minutes of rest. A standardised interview on medical history and medications was performed and a questionnaire on sleep quality, daytime sleepiness, and symptoms of depression was administered [44]. Height was measured to the nearest centimetre and weight to the nearest 0.1 kg and body mass index (BMI) was calculated. A full night PSG recording was performed in the subjects' home using a portable Embla-A10 polysomngraphy system (Flaga, Reykjavik, Iceland). Each recording included three-channel electroencephalography (EEG, C_4/A_1, C_3/A_2), two-channel electrooculography, and one-channel submental electromyography (EMG). Limb movements were monitored by tibial surface electrodes. Ventilatory monitoring included oxygen saturation (SaO_2) by an oximeter probe,

respiratory movements by chest and abdominal belts, nasal and oral airflow by thermistor and nasal pressure by pressure sensors. Two cotrained staff members manually scored the recordings according to a validated scoring manual [45]. A successful PSG required a minimum of 4 hours sleeping time including at least one REM-sleep period. The number of apnea/hypopnea events per hour of sleep (AHI) was used to dichotomise subjects into two groups, one with a cutoff level of 10 events per hour (mild OSA, OSA10, AHI $\geq$ 10 events per hour) and one with a cutoff level of 30 events per hour (severe OSA, OSA30, AHI $\geq$ 30 events per hour). Separate statistical analyses were undertaken to compare the two groups with the control subjects (those with AHI <10 and <30 events per hour, resp.).

2.3. Genotyping. High molecular weight DNA was extracted from blood leukocytes according to standard techniques [46] and used as template in all polymerase chain reactions (PCRs). The genotyping procedures have been described elsewhere [16, 35, 47]. The PCR conditions and digestion conditions in the restriction fragment length polymorphism (RFLP) reactions are shown in Table 1.

2.4. Statistical Methods. Hypertensive and normotensive subjects were analysed separately. Continuous variables are presented as means $\pm$ standard deviation (SD) and discrete variables as frequencies (%). Differences between means were tested by ANCOVA. Differences in proportions were tested by χ^2-test and by logistic regression. Confounding from age and sex was accounted for by multivariate analyses or by stratification. Multiple logistic regressions were performed with mild OSA (OSA10) and severe OSA (OSA30) as the dependent variable and with age, sex, BMI and the α_{2B}-adrenergic receptor, β_1- and β_2-adrenergic receptor polymorphisms as independent variables. Associations were expressed as odds ratio (OR) with 95 per cent confidence interval (95% CI). For the α_{2B}-adrenergic receptor gene the II genotype was used as reference in the comparison with the ID and DD genotypes. For the β_1-adrenergic receptor gene the combination of Ser49Gly and Gly49Gly was used as a reference and compared with the Ser49Ser genotype. The combination of Gly389Gly and Arg389Gly genotypes was used as reference and compared with the Arg389Arg genotype. Finally, for the β_2-adrenergic receptor gene the Gly16Gly and Glu27Glu genotypes were used as references compared with the Arg16Gly and Arg16Arg and the Gln27Glu and Gln27Gln genotypes, respectively. Analyses were carried out using SPSS Base system for Macintosh 11.0 (Chicago, SPSS Inc.). All statistical tests were two sided. *P*-values less than .05 were considered statistically significant.

3. Results

The clinical characteristics of the subjects are shown in Table 2. As expected hypertensive patients had higher systolic and diastolic blood pressure but were also older and had higher BMI than normotensive subjects. AHI was higher in hypertensive men but did not differ between hypertensive and normotensive women. The observed genotype frequencies were all in Hardy Weinberg equilibrium.

3.1. The α_{2B}-Adrenergic Receptor. The genotype frequencies of the α_{2B}-adrenergic receptor gene were 15% for DD genotype, 51% for ID genotype, and 34% for the II genotype. There was no statistical difference in genotype or allele frequencies of the 301–303 I/D polymorphism in hypertensive patients with or without OSA (Table 3) or in normotensive controls (data not shown).

3.2. The β_1-Adrenergic Receptor. The Arg389Arg genotype of the β_1-adrenergic receptor gene was more common in male hypertensive patients with mild OSA than in those without mild OSA (Table 3). There was a similar trend in hypertensive females and in hypertensive males with severe OSA. Among the 62 analyzed men there was no one with OSA carrying the Gly389Gly genotype (Table 3). In a recessive model comparing Arg389Arg to Arg389Gly and Gly389Gly there was a significant association with mild OSA in hypertensive subjects, OR 2.3 (95% CI 1.10–4.87), P = .027, crude and when adjusted for age and sex OR 2.3 (95% CI 1.09–4.96), P = .029 and still significant when adjusted also for BMI OR 2.1 (95% CI 1.02–4.71), P = .045. In sex specific analysis the patterns were very similar in men and women; however, neither crude nor adjusted models turned out significant. In the full model we found OR 3.4 (95% CI 0.85–13.40), P = .085 in men, and 2.26 (95% CI 0.85–5.99), P = .102 in women, respectively. Hypertensive men carrying the Arg389Arg genotype had higher crude and age-adjusted AHI than carriers of the Arg389Gly/Gly389Gly genotypes. When adjusted also for BMI this difference became borderline significant. This difference was not observed in women (Table 4). There was also a dose dependent increase in AHI according to genotype being the lowest in Gly389Gly carriers increasing in the Arg389Gly and the highest in Arg389Arg carriers, significant in all men (15.0 $\pm$ 13.7, 24.3 $\pm$ 18.5 and 32.3 $\pm$ 26.2 events per hour, P = .017). In the subgroups of men with and without hypertension AHI showed a similar increasing trend, 4.0 (one subject), 27.2 $\pm$ 20.1 and 39.5 $\pm$ 27.7 events per hour, P = .065 and 16.1 $\pm$ 13.9, 21.8 $\pm$ 16.9, and 25.9 $\pm$ 23.4 events per hour, P = .33, respectively. In women no such trend was observed (28.8 $\pm$ 27.5, 22.9 $\pm$ 25.3, and 23.7 $\pm$ 23.1 events per hour P = .72).

In analysis including both patients with hypertension and normotensive controls subjects the frequency of hypertension versus normotension differed significantly between the different genotype carriers in subjects with mild OSA (Table 5). When using the number of Arg389 alleles as an approximately linear variable the age and BMI adjusted OR for hypertension in patients with mild OSA were for one Arg389 allele 4.8 (95% CI 1.2–19.3), P = .028 and for two Arg389 alleles 5.4 (95% CI 1.4–21.2), P = .015. A test for trend adjusted for age, sex, and BMI also showed an increased OR for hypertension with increasing number of Arg389 alleles, OR 1.6, 95% CI 1.003–2.5, P = .048.

3.3. The β_2-Adrenergic Receptor. Neither of the investigated polymorphisms (Arg16Gly or Gln27Glu) was associated with

Table 1: PCR and digestion conditions for genotyping of the the α_{2B}-, β_1-, and the β_2-adrenergic receptor gene polymorphisms.

		PCR conditions					RFLP		Gel‡‡
Gene/polymorphism	Forward (sense) primer sequence								
N successfully genotyped	Reverse (antisense) primer sequence	Size (bp)	Annealing T (°C)	Extension time (s)	Mg^2+ mmol/L	Cycles	Enzyme	Time (h)	%
338 A2BAR									
I/D 301–303	5′-AGGGTGTTTGTGGGGCATCT	112/103	72	30	1.5	33	—		3
	5′-CAAGCTGAGGCCGGAGACACT								
337* B1AR									
Ser49Gly	5′-CCGGGCTTCTGGGGTGTTCC	562	64	30	1.5	35	*EcoO109I*	3	2
	5′-GGCGAGGTGATGGCGAGGTAGC								
Arg389Gly	5′-CGCTCTGCTGGCTGCCCTTCTTCC	530	64	30	1.5	33	*BcgI*	3	2
	5′-TGGGCTTCGAGTTCACCTGCTATC								
338 B2AR									
Arg16Gly	5′-CGCCTTCTTGCTGGCAC<u>G</u>CAAT **	203	60	30	1.5	30	*BsrDI*	2	4.5
	5′-CCAGTGAAGTGATGAAGTAGTT								
Gln27Glu	5′-CCGGACCACGACGTCAC<u>C</u>CAG ***	169	60	30	1.5	30	*BstNI*	2	4.5
	5′-CCAGTGAAGTGATGAAGTAGTT								

A2BAR: α_{2B}-adrenergic receptor gene; B1AR: β_1-adrenergic receptor; B2AR: β_2-adrenergic receptor; Ag: agarose; bp: base pair.
*The genotyping failed in one individual.
**The underlined nucleotide is a mismatch to create a *BsrDI* recognition site in case of the Gly16-allele.
***The underlined nucleotide is a mismatch to create a *BstNI* recognition site in case of the Glu27-allele.
‡‡The PCR products were separated on a multipurpose agarose gel (Ag) with ethidium bromideand visualised with UV-light.

Table 2: Clinical characteristics in normotensive and hypertensive men and women.

	Normotensive subjects		Patients with hypertension		
Variables	Mean	(SD)	Mean	(SD)	*P*
Men (n)	*96*		*74*		
Age (years)	60	(6.3)	62	(6.2)	.008
Systolic blood pressure (mmHg)	131	(13.2)	148	(15.7)	<.001
Diastolic blood pressure (mmHg)	77	(7.5)	84	(6.7)	<.001
Heart rate (beats per minute)	68	(8.3)	68	(8.2)	.653
AHI (events per hour)	23	(20.4)	34	(25.6)	.005
Body mass index (kg/m^2)	26.8	(3.0)	29.5	(4.6)	<.001
Women (n)	*85*		*83*		
Age (years)	60	(7.1)	64	(5.7)	<.001
Systolic blood pressure (mmHg)	132	(14.8)	150	(16.8)	<.001
Diastolic blood pressure (mmHg)	75	(7.5)	83	(8.0)	<.001
Heart rate (beats per minute)	70	(8.7)	69	(9.0)	.944
AHI (events per hour)	22	(26.0)	25	(22.2)	.655
Body mass index (kg/m^2)	28.1	(4.8)	30.3	(5.7)	.006

Data are mean (SD). Differences in means were analysed by general linear models with adjustment for age. AHI: apnea/hypopnea index.

increased risk of OSA in hypertensive subjects or with hypertension in patients with OSA (data not shown).

4. Discussion

We describe an association between the Arg389Arg genotype of the β_1-adrenergic receptor gene and OSA in hypertensive patients. Furthermore, occurrence of hypertension increased in subjects with mild OSA with increasing number of Arg389 alleles. The Arg389Arg genotype carriers had a borderline significantly higher AHI than the Arg389Gly/Gly389Gly carriers when adjusted for age and BMI. While the current study was designed to investigate the risk of OSA in patients with hypertension, the results showing that the risk of hypertension in subjects with mild OSA was associated with the Arg389 allele are in accordance with what have been previously published on association between hypertension and the Arg389Gly polymorphism [21].

The β_1-adrenergic receptor is important in the adrenergic activation of the heart by increasing both the inotropic and

Table 3: Genotype frequencies of adrenergic receptor genes: α_{2B}-adrenergic I/D, the β_1 Ser49Gly and Gly389Arg, and the β_2 Gly16Arg and Gln27Glu polymorphisms in hypertensive patients with and without mild (panel A) and severe (panel B) obstructive sleep apnoea (OSA).

A	Patients with mild OSA			Patients without mild OSA			*P*
	α_{2B}-adrenergic receptor gene I/D polymorphism ($n = 157$)						
Genotype	II	ID	DD	II	ID	DD	
Men OSA10 ($n = 74$)	18 (28.6)	32 (50.8)	13 (20.6)	5 (41.7)	6 (50.0)	1 (8.3)	.50
Women OSA10 ($n = 83$)	20 (35.7)	27 (48.2)	9 (16.1)	9 (34.6)	11 (42.3)	6 (23.1)	.73
	β_1 adrenergic receptor gene Ser49Gly and Gly389Arg polymorphisms ($n = 156$)						
Genotype (Ser49Gly)	S/S	S/G	G/G	S/S	S/G	G/G	*P*
Men OSA10 ($n = 74$)	37 (59.7)	24 (38.7)	1 (1.6)	9 (75.0)	3 (25.0)	0 (0.0)	.58
Women OSA10 ($n = 82$)	39 (69.6)	12 (21.4)	5 (8.9)	20 (76.9)	6 (23.1)	0 (0.0)	.29
Genotype(Gly389Arg)	G/G	G/A	A/A	G/G	G/A	A/A	*P*
Men OSA10 ($n = 74$)	0 (0.0)	23 (37.1)	39 (62.9)	1 (8.3)	6 (50.0)	5 (41.7)	.042
Women OSA10 ($n = 82$)	3 (5.4)	18 (32.1)	35 (62.5)	3 (11.5)	12 (46.2)	11 (42.3)	.20
	β_2 adrenergic receptor Gly16Arg and Gln27Glu ($n = 157$)						
Genotype (Gly16Arg)	A/A	A/G	G/G	A/A	A/G	G/G	*P*
Men OSA10 ($n = 74$)	8 (12.9)	35 (56.5)	19 (30.6)	1 (8.3)	5 (41.7)	6 (50.0)	.43
Women OSA10 ($n = 83$)	8 (14.0)	22 (38.6)	27 (47.4)	4 (15.4)	13 (50.0)	9 (34.6)	.54
Genotype (Gln27Glu)	Gn/Gn	Gn/Gu	Gu/Gu	Gn/Gn	Gn/Gu	Gu/Gu	*P*
Men OSA10 ($n = 74$)	24 (38.7)	28 (45.2)	10 (16.1)	1 (8.3)	8 (66.7)	3 (25.9)	.13
Women OSA10 ($n = 83$)	17 (29.8)	27 (47.4)	13 (22.8)	10 (38.5)	10 (38.5)	6 (23.1)	.70
B	Patients with severe OSA			Patients without severe OSA			
	α_{2B}-adrenergic receptor gene I/D polymorphism ($n = 157$)						
Genotype		II	ID	DD	II	ID	*P*
Men OSA30 ($n = 74$)	12 (34.3)	15 (42.9)	8 (22.9)	11 (27.5)	23 (57.5)	6 (15.0)	.43
Women OSA30 ($n = 83$)	8 (36.4)	12 (54.6)	2 (9.1)	21 (35.0)	26 (43.3)	13 (21.7)	.40
	β_1 adrenergic receptor gene Ser49Gly and Gly389Arg polymorphisms ($n = 156$)						
Genotype (Ser49Gly)	S/S	S/G	G/G	S/S	S/G	G/G	*P*
Men OSA30 ($n = 74$)	24 (68.6)	11 (31.4)	0 (0.0)	22 (56.4)	16 (41.0)	1 (2.6)	.41
Women OSA30 ($n = 82$)	15 (68.2)	5 (22.7)	2 (9.1)	44 (73.3)	13 (21.7)	3 (5.0)	.78
Genotype (Gly389Arg)	G/G	G/A	A/A	G/G	G/A	A/A	*P*
Men OSA30 ($n = 74$)	0 (0.0)	10 (28.6)	25 (71.4)	1 (2.6)	19 (48.7)	19 (48.7)	.11
Women OSA30 ($n = 82$)	2 (9.1)	7 (31.8)	13 (59.1)	4 (6.7)	23 (38.3)	33 (55.0)	.83
	β_2-adrenergic receptor Gly16Arg and Gln27Glu ($n = 157$)						
Genotype (Gly16Arg)	A/A	A/G	G/G	A/A	A/G	G/G	*P*
Men OSA30 ($n = 74$)	2 (5.7)	22 (62.9)	11 (31.4)	7 (18.0)	18 (46.2)	14 (35.9)	.19
Women OSA30 ($n = 83$)	4 (18.2)	8 (36.4)	10 (45.5)	8 (13.1)	27 (44.3)	26 (42.6)	.76
Genotype (Gln27Glu)	Gn/Gn	Gn/Gu	Gu/Gu	Gn/Gn	Gn/Gu	Gu/Gu	*P*
Men OSA30 ($n = 74$)	11 (31.4)	19 (54.3)	5 (14.3)	14 (35.9)	17 (43.6)	8 (20.5)	.62
Women OSA30 ($n = 83$)	6 (27.3)	10 (45.5)	6 (27.3)	21 (34.4)	27 (44.3)	13 (21.3)	.77

OSA10: subjects dichotomised by AHI < or ≥10 events/ hour; OSA30: subjects dichotomised by AHI < or ≥30 events/hour. *P*-values are estimated with chi-2 test. A: (Arg) arginine; D: deletion; I: insertion; G: (Gly) Glycine; Gn:(Gln) Glutamine; Gu: (Glu) glutamic acid; S:(Ser) serine; HT: hypertensive; OSA: obstructive sleep apnoea.

chronotropic responses upon agonist binding. The Arg389 variant of the β_1-adrenergic receptor has been found to increase both the basal and the isoprotenerol-stimulated adenylate cyclase activity compared with the Gly389 variant. Experimental hypoxia in rats is a powerful activator of the sympathetic nervous system leading to increased blood pressure [48]. This is consistent with our results that a variant of the β_1-adrenergic receptor that confers an increased cyclic AMP activity and has been shown to be associated with hypertension also could be associated with increased risk of OSA in hypertensive patients. The gender difference found in this study could be due to the lower impact of the genetic variants of the sympathetic nervous system in development of OSA in females compared with males; instead anatomic factors in the throat might play a greater role in females [49]. The analysis of AHI in different β_1-adrenergic receptor genotype carriers showed a significant difference only in males. In fact we found no difference in AHI between

Table 4: Differences in AHI between male and female carriers of the Arg389Gly genotypes of the Beta-1 adrenergic gene.

	Gly389Gly/Arg389Gly		Arg389Arg		
AHI (events per hour)	Mean	(SE)	Mean	(SE)	*P (adjusted)*
Males	$n = 96$		$n = 74$		
Normotensive subjects					
(crude)	20.5	(3.0)	25.9	(2.9)	.20
(adjusted for age)	20.1	(3.1)	26.3	(2.9)	.15
(adjusted for age, BMI)	20.5	(3.0)	25.9	(2.8)	.20
Hypertensive patients					
(crude)	26.5	(4.6)	39.5	(3.8)	.03
(adjusted for age)	26.3	(4.6)	39.6	(3.8)	.03
(adjusted for age, BMI)	27.4	(4.5)	38.6	(3.7)	.06
Females	$n = 85$		$n = 82$		
Normotensive subjects					
(crude)	23.5	(4.1)	21.3	(3.9)	.70
(adjusted for age)	23.4	(3.9)	21.3	(3.8)	.70
(adjusted for age, BMI)	22.8	(3.7)	21.9	(3.5)	.85
Hypertensive patients					
(crude)	24.4	(3.7)	26.0	(3.3)	.76
(adjusted for age)	23.9	(3.6)	26.3	(3.1)	.61
(adjusted for age, BMI)	24.3	(3.6)	26.1	(3.1)	.71

AHI: apnoea-hypopnoea index (events per hour). Data are mean (SE) and stepwise adjusted for age and age and BMI. One male hypertensive had missing BMI.

Table 5: Frequency of hypertension in patients with mild obstructive sleep apnea according to Beta-1 adrenergic receptor Arg389Gly polymorphism.

Beta-1 adrenergic Genotype	Hypertension	Normotension	*P*
All ($n = 229$)	$n = 118$	$n = 111$	
Gly389Gly%	2.5	9.9	
Gly389Arg%	34.8	37.8	
Arg389Arg%	62.7	52.3	.043
Males ($n = 124$)	$n = 62$	$n = 62$	
Gly389Gly%	0	9.7	
Gly389Arg%	37.1	37.1	
Arg389Arg%	62.9	53.2	.039

P-values are estimated with chi-2 test.

female hypertensive patients and controls. This suggests that hypertension plays a less important role in OSA in women than in men. One limitation of this study is the small number of subjects especially after dichotomization into hypertension and normotension. In this particular polymorphism where the Gly389Gly genotype has a low frequency the data should be interpreted with care.

The polymorphisms in the other adrenergic receptor genes (the α_{2B}-adrenergic receptor and the β_2-adrenergic receptor) were not associated with OSA in this study population.

The α_{2B}-adrenergic receptor gene I/D 301–303 polymorphism studied here is proposed to influence the agonist-induced desensitization of the receptor. We found no association between this polymorphism and OSA in hypertensive or normotensive individuals. One explanation might be that the α_{2B}-adrenergic receptor acts on a level of sympathetic action that is only indirectly involved in the adrenergic response of the heart to hypoxia induced during OSA.

The lack of association between polymorphisms of the β_2-adrenergic receptor and OSA is in accordance with the results from a cohort with high prevalence of hypertension from Germany [50] although the risk of myocardial infarctions was associated with the polymorphisms. Furthermore, these polymorphisms have been shown to be associated with hypertension only in the presence of type 2 diabetes in the current population [35]. Compared to the β_1-adrenergic receptor the β_2-adrenergic receptor has a similar but less pronounced effect in the heart. This difference in action could explain why the β_2-polymorphisms investigated here do not seem to be of importance in development of OSA in hypertensive patients.

In conclusion, the Arg389Gly polymorphism of the β_1-adrenergic receptor was associated with increased risk of OSA in hypertensive men and with hypertension in subjects with mild OSA. Also, in men, there was a dose dependent increase in AHI being the lowest in Gly389Gly carriers and the highest in Arg389Arg carriers. These findings highlight the role of the sympathetic nervous system in OSA and hypertension.

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Research Article

An *NOS3* Haplotype Is Protective against Hypertension in a Caucasian Population

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The endothelial nitric oxide synthase gene (*NOS3*) has been implicated in the development of hypertension, although the specific role of variants and haplotypes has not been clarified. In this study, the association of three polymorphisms (promoter T786C, intronic 4a/b, and nonsynonymous G894T) was tested in a case-control sample of 230 patients with essential hypertension and 306 healthy controls. Haplotype analysis was also performed. The mutant allele a* of the 4a/b polymorphism showed a protective effect against hypertension under a dominant model (odds ratio = 0.64, 95% confidence interval (0.44–0.93)), although this effect was not significant after the adjustment for covariates ($P = 0.06$). The estimated frequency of the haplotype composed of the T786*, 4a*, and G894* alleles was significantly higher in controls (5.5%) compared to cases (2%). These results indicate that although individual *NOS3* polymorphisms are not associated with hypertension, a rare haplotype of the gene might be protective against the development of hypertension.

1. Introduction

Hypertension is a multifactorial disorder resulting from complex interactions between genetic and environmental contributions. An increase in vascular resistance and impairment of endothelial-dependent vasodilatation are involved in the pathogenesis of the disease. Nitric oxide (NO) synthesis by the vascular endothelium is important for the regulation of vasodilator tone and control of blood pressure in humans [1, 2]. Given the pivotal role of NO in vascular homeostasis, the endothelial nitric oxide synthase gene (*NOS3*) has emerged as a logical candidate gene in the investigation of hypertension genetics [3].

Variants of the *NOS3* gene located in the 7q35-q36 region have been investigated for association with hypertension and other cardiovascular disorders [3]. Among them, three polymorphisms have been widely examined for clinical relevance, based on their potential functional effects and their relatively high minor allele frequency in various ethnic groups [3, 4]: (i) a G894T substitution in exon 7 resulting in a Glu to Asp substitution at codon 298 (rs1799983), (ii) an insertion-deletion in intron 4 (4a/b) consisting of two alleles (the a*-deletion which has four tandem 27-bp repeats and the b*-insertion having five repeats), and (iii) a T786C substitution in the promoter region (rs2070744). In a survey of all published association studies on the relation between the *NOS3* gene polymorphisms and the risk of hypertension, a meta-analysis and subsequent sensitivity analyses supported an association only for the 4a/b polymorphism and hypertension [5]. Most of the studies in the field had investigated the effects of individual polymorphisms and reported marginal or even controversial associations. However, accumulating evidence shows that significant interactions between individual *NOS3* polymorphisms have a major influence on NO formation [6, 7], and consequently an analysis of haplotypes (and not individual *NOS3* polymorphisms) is expected to be a more powerful approach for detecting genetic susceptibility to hypertension [8, 9].

In order to replicate [10] the association described in the above meta-analysis [5] and explore the effects of *NOS3* haplotypes on the risk of developing hypertension, we performed a case-control study to test for association between *NOS3* polymorphisms (or haplotypes) and hypertension. We focused on the three previously studied polymorphisms (T786C, 4a/b, and G894T) because of their potential functional implications, their high minor allele frequency, and the extensive publication record on them that would allow for meaningful comparisons [3, 4]. The study presented here was conducted in a case-control sample from a homogeneous population of Caucasian origin (Greeks).

2. Materials and Methods

2.1. Study Population. All consecutive hypertensive patients presenting in our recruitment centers (primary care practices and outpatient clinics) were systematically evaluated for fulfillment of the following inclusion criteria: (1) patient age more than 20 years, (2) age-at-onset of hypertension before 60 years of age (in order to obtain a phenotype with enriched genetic component) [11], (3) established hypertension defined either as long-term treatment of the disease or in those previously untreated with systolic blood pressure (SBP) more than 140 mmHg or diastolic blood pressure (DBP) more than 90 mmHg, (4) documented absence of secondary forms of hypertension after clinical and laboratory work up (such as renal, renovascular or endocrine disease) and (5) absence of diabetes mellitus. A total of 230 nondiabetic patients with essential hypertension were finally recruited. Each patient's medical history was obtained using a standardized questionnaire regarding the lifestyle, smoking (current or ex-smokers), alcohol or drug intake. Medical history of hypertension, cardiovascular disease, hyperlipidemia, and current medications was recorded. A complete physical examination was performed including measurement of supine SBP and DBP (*using mercury* column sphygmomanometers), electrocardiogram recording, and measurement of somatometric parameters (height, weight, body mass index (BMI)). Spouses and friends of cases were interviewed to assess their appropriateness for inclusion as a healthy control group. A group of 306 normotensive, healthy control subjects was also recruited according to the following criteria: (1) subject age more than 20 years, (2) absence of hypertension or antihypertensive treatment in the medical history, (3) SBP/DBP less than 140/90 mmHg, respectively, (4) absence of chronic illness (renal, cardiovascular, mental, hepatic, endocrine disorders or cancer) or concomitant chronic medication, and (5) unrelated by blood to cases. A blood sample for biochemical measurements and DNA extraction was taken from each participant. All participants provided written informed consent.

2.2. Laboratory Assays. Genomic DNA was extracted from whole blood using the QIAamp DNA blood kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instruction. Genotyping of each polymorphism was performed by amplification from 50 to 100 ng of genomic DNA. The primer sequences used and the laboratory conditions for genotyping (polymerase chain reaction, restriction enzymes, agarose electrophoresis) for each *NOS3* polymorphism have been previously described [12, 13]. Genotyping was performed by laboratory personnel blinded to clinical status.

2.3. Statistical Analysis. All statistical analyses were performed using the SAS software (v9.1 SAS Institute Inc, NC, USA). The values for clinical parameters are expressed as mean ± standard deviation. The Student *t*-test and

Table 1: Clinical characteristics of cases and controls.

	Cases ($n = 230$)	Controls ($n = 306$)	*P*-value
Age (years)	63.9 (9.4)	40.8 (18.3)	<.05
Males, *n* (%)	125 (54.4)	68 (22.2)	<.05
BMI	28.7 (4.1)	27.5 (4.4)	<.05
Smokers, *n* (%)	84 (36.5)	80 (29.9)	.08
SBP (mmHg)	145.3 (15.7)	124.4 (12.3)	<.05
DBP (mmHg)	85.1 (10.2)	68.8 (9.7)	<.05
PP (mmHg)	59.3 (17.2)	57.1 (14.1)	.19
CAD, *n* (%)	65 (28.3)	—	N/A
Creatinine (mg/dL)	1.09 (0.8)	0.92 (0.9)	<.05
Urea (mg/dL)	40.8 (8.6)	36.5 (6.6)	<.05
Potassium (mmol/L)	4.3 (0.5)	4.4 (0.3)	.26
Sodium (mmol/L)	138.4 (3.1)	140.0 (4.5)	.38
Total Cholesterol (mg/dL)	190.88 (48.4)	—	N/A
LDL Cholesterol (mg/dL)	113.7 (39.5)	—	N/A
HDL Cholesterol (mg/dL)	46.9 (17.8)	—	N/A
Triglycerides (mg/dL)	132.4 (64.5)	—	N/A
Drug treatment (%)	89.6	—	N/A
ACE-inhibitors (%)	45.2	—	N/A
ARBs (%)	29.0	—	N/A
Beta-blockers (%)	50.3	—	N/A
CCBs (%)	36.8	—	N/A
Diuretics (%)	50.9	—	N/A
Nitrates (%)	13.5	—	N/A
Statins (%)	39.7	—	N/A

Values are mean (standard deviation), unless otherwise specified. Abbreviations: BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, PP = pulse pressure, CAD = coronary artery disease, LDL = low-density lipoprotein, HDL = high density lipoprotein, ACE = angiotensin converting enzyme, ARBs = angiotensin receptor blockers, CCBs = calcium channel blockers, N/A = nonapplicable.

the chi-square test were used for comparisons of continuous and categorical variables, respectively. Any result with $P < .05$ was considered statistically significant. The chi-square test with one degree of freedom was used in order to test whether the frequency distribution of genotypes in the control group was in Hardy-Weinberg equilibrium (HWE) ($P \geq .05$). Differences in genotype distribution and allele frequencies between hypertensive and control subjects were tested using the chi-square test. The association was expressed as an odds ratio (OR) with the corresponding 95% confidence interval (95% CI). Multivariate logistic regression analysis was also used to adjust for the effects of covariates (age, gender, BMI, and smoking status) on the genotype-phenotype association. Addition of a squared term of age was found to improve model fit, as tested with the likelihood ratio test, and was therefore kept in the final model. For the regression models, the genetic effect of the mutant allele (786C*, 4a*, and 894T*, resp.) was assumed to be dominant (mutant carriers versus wild type homozygotes), or recessive (mutant homozygotes versus wild type carriers), or additive (mutant homozygotes versus wild type homozygotes) or codominant (heterozygotes versus homozygotes), for each *NOS3* polymorphism [10]. Linkage disequilibrium (LD) and haplotype analysis were performed using the SHEsis and HAPSTAT software platforms [14, 15]. The order of variants in the inferred haplotypes was T786C, 4a/b, G894T, corresponding to the physical location of these variants in the *NOS3* gene. The threshold haplotype frequency value for inclusion in the analysis was set at 3%. Sample size calculations were performed by using the Power Calculator for Genetic Studies [16]. A modest genetic effect (OR = 1.3), and observed disease prevalence (30%), and minor allele frequencies (786C* = 40%, 4a* = 20% and 894T* = 35%) in Caucasian populations were imputed [17]. Sufficient power (>80%) to detect modest genetic effects was achieved with a sample of 230 cases and 306 controls for all three polymorphisms. A systematic review of the literature for studies investigating the association between *NOS3* haplotypes and hypertension was also conducted and the individual study results were summarized.

3. Results

The main clinical and demographic characteristics of the subjects are summarized in Table 1. Age, male gender, BMI, SBP, DBP, creatinine, and urea concentration were significantly higher in cases compared to controls. Among cases, 28% suffered from coronary artery disease and almost 90% were treated by antihypertensive medications or statins.

Genotyping was successful in 96%, 99%, and 99% of subjects for the T786C, 4a/b and G894T polymorphisms, respectively. The genotype distributions and allele frequencies are shown in Table 2. The genotype distributions in the control group were in HWE for all polymorphisms ($P = .39$, .63 and .16 for T786C, 4a/b, and G894T polymorphisms, resp.). The genotype distribution differed significantly between cases and controls only for the intronic 4a/b polymorphism (Table 2). In univariate analysis under a dominant model, carriers of the mutant allele a* were 36% less likely to develop hypertension (OR = 0.64, 95% CI (0.44–0.93)) compared to homozygotes of the wild type allele (b*). However, this protective effect of the a* allele was no longer significant after adjustment for possible confounding variables using multiple regression analysis (adjusted OR = 0.52, 95% CI (0.26–1.04)) (Table 3). No significant interaction between the a* allele carriership and smoking was observed ($P = .44$). The remaining analyses, both univariate and multivariate, did not show any significant association for the T786C and G894T polymorphisms and are shown in Table 3.

Table 2: Genotypic and allelic distributions of *NOS3* polymorphisms for cases and controls.

	Cases, *n* (%)	Controls, *n* (%)	*P*-value
T786C			
TT	69 (30.4)	101 (35.0)	
TC	118 (52.0)	148 (51.2)	
CC	40 (17.6)	40 (13.8)	.37[(a)]
T alleles	246 (55.4)	350 (60.6)	
C alleles	198 (44.6)	228 (39.4)	.12[(b)]
Intron 4a/b			
4b/b	165 (72.4)	190 (62.7)	
4a/b	59 (25.9)	101 (33.3)	
4a/a	4 (1.7)	12 (4.0)	.04[(a)]
b alleles	389 (85.3)	481 (79.4)	
a alleles	67 (14.7)	125 (20.6)	.03[(b)]
G894T			
GG	99 (43.4)	135 (44.7)	
GT	95 (41.7)	130 (43.1)	
TT	34 (14.9)	37 (12.2)	.67[(a)]
G alleles	283 (63.5)	400 (66.2)	
T alleles	163 (36.5)	204 (33.8)	.39[(b)]

[(a)] *P*-value for the comparison of genotypic distribution. [(b)] *P*-value for the comparison of the allelic distribution.

Pairwise LD among the three polymorphisms was measured by the Lewontin standardized disequilibrium coefficient D' and the squared correlation coefficient r^2 [18], in both groups separately. All pairwise comparisons showed statistically significant LD ($P < .05$), although strong LD ($D' > 0.8$) was evident only for the 4a/b and G894T polymorphisms in the control group (Table 4).

The distribution of the estimated haplotype frequencies for cases and controls is presented in Table 5. Six major haplotypes with frequencies > 3% were identified. The global chi-square test for association of haplotypes showed that there was an overall significant difference between cases and controls ($P = .03$). This difference was due to the T-a-G* haplotype, which was more frequent in controls (5.5%) compared to cases (2%) ($P = .02$), conferring a protective effect against the development of hypertension. Exploration of possible interactions of the T-a-G* haplotype with confounding variables (age, sex, BMI, smoking status) did not detect any significant results (*P*-values > .05).

Table 3: Unadjusted and adjusted odds ratios with the corresponding 95% confidence intervals for the association of *NOS3* genotypes and hypertension: comparisons for the dominant, recessive, additive, and codominant models of the mutant alleles (786C*, 4a*, 894T*).

Polymorphism	Unadjusted OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
T786C				
Dominant model	1.23 (0.85–1.79)	.28	1.30 (0.64–2.63)	.47
Recessive model	1.33 (0.83–2.15)	.24	0.77 (0.33–1.83)	.56
Additive model	1.46 (0.86–2.49)	.16	0.99 (0.38–2.62)	.98
Codominant model	1.03 (0.73–1.42)	.86	1.48 (0.75–2.93)	.26
Intron 4a/b				
Dominant model	0.64 (0.44–0.93)	.02	0.52 (0.26–1.04)	.06
Recessive model	0.43 (0.14–1.36)	.15	0.49 (0.08–3.07)	.45
Additive model	0.38 (0.12–1.21)	.10	0.40 (0.06–2.78)	.35
Codominant model	0.69 (0.48–1.02)	.06	0.56 (0.28–1.13)	.10
G894T				
Dominant model	1.05 (0.75–1.49)	.77	1.51 (0.78–2.92)	.22
Recessive model	1.25 (0.76–2.07)	.37	1.04 (0.38–2.77)	.94
Additive model	1.25 (0.74–2.14)	.41	1.28 (0.46–3.56)	.64
Codominant model	0.94 (0.67–1.34)	.75	1.51 (0.77–2.96)	.23

Table 4: Pairwise linkage disequilibrium metrics $[D', (r^2)]$ for *NOS3* polymorphisms in cases and controls.

Polymorphisms T786C	Intron 4a/b	G894T
Cases	0.46[a] (0.05)[a]	0.34[a] (0.09)[a]
Controls	0.44[a] (0.15)[a]	0.51[a] (0.11)[a]
Intron 4a/b		
Cases	—	0.44[a] (0.02)[a]
Controls	—	0.82[a] (0.09)[a]

[a] $P < .05$.

Table 5: *NOS3* haplotype distribution in cases and controls.

Haplotype[a]	Cases (%)	Controls (%)	*P*-value
T-b-G*	47.2	43.1	.26
C-b-T*	26.4	20.9	.07
C-a-G*	11.9	13.7	.43
T-b-T*	7.9	11.5	.09
C-b-G*	3.7	4.1	.77
T-a-G*	2	5.5	.02
other	0.9	1.2	—
Global test of association			.03

[a] The order of variants in the inferred haplotypes is [T786C-4a/b-G894T] in order to correspond to the physical location of these variants in the *NOS3* gene.

The literature search identified nine previously published studies on the association between *NOS3* haplotypes and hypertension [6, 7, 19–25] and their results are provided in Table 6.

4. Discussion

4.1. Novel Findings. This study evaluated relations between common genetic variants and haplotypes in the *NOS3* gene with essential hypertension. The single locus analysis among the three most commonly studied polymorphisms revealed an association only for the 4a/b polymorphism. Contrary to what was anticipated, carriership of the mutant allele a* was associated with a 36% reduction in the risk of developing hypertension. A meta-analysis conducted by our group has previously shown a detrimental effect for the a* allele; based on unadjusted estimates, the a* allele was associated with an 22% increased risk for hypertension [5]. Interestingly, this association was confined in Caucasians. In the current study, the unadjusted analysis in a Caucasian population revealed an association in the opposite direction (protective effect of the a* allele). However, this association was not significant when the effects of potential confounders were taken into account with a multivariate logistic regression model. Additionally, previous reports have described that the 4a/b polymorphism effects can be smoking-dependent [26, 27]. No significant genotype-smoking interactions were found in our analysis. The lack of association for the G894T and T786C polymorphisms was in concordance with the meta-analysis results [5].

In the haplotype-based association analysis, the distribution of a relatively infrequent haplotype (T-a-G*) was found to be different between cases and controls, resulting in a protective effect against hypertension. The mutant allele of the 4a/b polymorphism is incorporated in this haplotype, along with the two wild type alleles of G894T and T786C polymorphisms. Although not significant in individual analyses, the point estimates of the genetic effects of the alleles involved in this haplotype were also in the protective direction (Table 3). This study could not provide

Table 6: Summary description of previous studies on the association between *NOS3* haplotypes and essential hypertension.

Study first author, year of publication	Study population ethnicity, (Number of cases/Number of controls)	Evidence for association with *NOS3* haplotypes	Haplotypes with statistically significant results	Direction of haplotypic genetic effect
Sandrim, 2006 [21]	Caucasians (112/113)	Yes	T-b-T*	Protective
			C-b-G*	Protective
			C-b-T*	Susceptibility
	Blacks (91/87)	Yes	T-b-T*	Protective
			C-b-G*	Protective
Sandrim, 2006 [22]	Mixed (119/102)	Yes	C-b-G*	Protective
			C-b-T*	Susceptibility
Zhao, 2006 [23]	East-Asians (503/490)	No		
Sandrim, 2006 [24]	Mixed (216/111)	Yes	C-b-G*	Protective
			C-b-T*	Susceptibility
Sandrim, 2007 [7]	Mixed (154/98)	Yes	C-b-G*	Protective
			C-b-T*	Susceptibility
Nejatizadeh, 2008 [6]	Indian (455/345)	Yes	T-a-G*	Protective
			T-a-T*	Protective
			C-a-G*	Protective
			T-b-G*	Susceptibility
Conen, 2008 [25]	Caucasian (18436)	No		
Kumar, 2009 [19]	Indian (440/470)	Yes	T-a-G*	Susceptibility
			T-a-T*	Susceptibility
			C-a-G*	Susceptibility
			T-b-G*	Protective
Vasconcellos, 2010 [20]	Caucasians (173/101)	Yes	C-b-G*	Protective
			C-a-G*	Susceptibility

any further information regarding the underlying culprit functional variation captured by this haplotype; however, the LD analysis showed evidence of strong LD between the 4a/b and the G894T polymorphisms in controls, which could potentially pinpoint a locus of interest.

4.2. Haplotype Testing in Hypertension. Testing of haplotypes overcomes some of the problems encountered with using single polymorphisms in genetic association studies, because the interaction of multiple genetic markers within a haplotype could be a key determinant of disease susceptibility rather than the individual polymorphism [28, 29]. The haplotype analysis approach is expected to be more powerful than single-marker analysis, because of the ancestral structure incarcerated in the distribution of haplotypes [9, 10]. Particularly if the markers used define mutations within functional DNA then the haplotypes composed of these markers can have more of a biological role. In accordance with this hypothesis, no significant associations were detected for the single-marker analysis promoter (T786C) and the nonsynonymous (G894T) variants used in our study, despite the fact that functionality analyses have demonstrated a role for both variants [3].

Previous studies have yielded contradictory results regarding the role of the T-a-G* haplotype that was found to be protective in our study, as shown in Table 6. Although no correlation with NO production has been detected [7], the T-a-G* has been reported to confer susceptibility to hypertension [19] or to be protective against gestational hypertension and preeclampsia [30]. Since the haplotypic structure has not been previously examined in the Greek population, it is possible that ethnicity may account for the observed discrepancies. The T-a-G* haplotype had a lower frequency in our study (2% and 5.5% in cases and controls) compared to previous reports (ranging from 6% to 20%) [19, 30].

For complex disorders as hypertension, the rare haplotypes have been recently shown to play a significant

role in influencing disease susceptibility [31, 32]. Recent paradigms have also shown that hunting common variation will not probably suffice to track the "missing heritability" explained by variants detected in the genome-wide association era [33, 34]. Multiple rare pathogenic variants are also likely to be important determinants of complex disorders. Although such variants will not be detectable by current techniques based on the use of linked polymorphic markers, advances in genotyping technologies and novel genetic variation maps that capture rare variants (1,000 Genomes Project) will make whole-genome searches for rare variants feasible [35]. Findings from genome-wide agnostic approaches can nevertheless be complemented by prioritized results from additional venues of genomic research (genomic convergence) [36, 37] in order to select for replication studies the candidate genes with the stronger evidence support.

4.3. Study Limitations. Large sample sizes and independent replication are sine qua non principles for genetic epidemiology [10, 38]. Our findings are not supportive of a major contributory role of individual genetic polymorphisms but provide evidence for association for a rare haplotype. Given the logistic limitations of single centers, like ours, to recruit large numbers of participants and to replicate the association in multiple cohorts, it is important that the validity of the proposed association here is tested in other studies before scrutinizing the gene in search for causal variants. Additionally, complex disorders such as hypertension are considered to emerge from multiple epistatic and gene-environmental interactions [34]. Although the *NOS3* gene is though to be involved in critical pathway interplays [39], our sample size did not allow the testing for interactions with sufficient power. Finally, the case-control design may have allowed for some elusive misclassification of controls, since the development of the disease is age-related. Replicating our hypothesis in large prospective studies could overcome these unavoidable limitations [38].

In conclusion, our genetic association study detected a protective effect of a rare *NOS3* haplotype against hypertension. Although the underlying functional genetic variation in the *NOS3* gene remains to be defined, haplotype-based analyses are expected to be more informative regarding the role of the *NOS3* gene compared to single-marker analyses. Rare haplotypes and variants may not serve as markers of disease with clinical utility on a population-wide basis but they hold the potential to uncover critical pathways involved in the pathogenesis of hypertension and assist in defining novel molecular targets for intervention. Our results require replication in independent cohorts and additional studies in order to disentangle the molecular basis of the detected genetic effects.

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Research Article

Promoter Polymorphism of RGS2 Gene Is Associated with Change of Blood Pressure in Subjects with Antihypertensive Treatment: The Azelnidipine and Temocapril in Hypertensive Patients with Type 2 Diabetes Study

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We performed a prospective study to examine the genetic effect on the response to a calcium (Ca) channel blocker, azelnidipine and an ACE inhibitor, temocapril treatment in patients with hypertension, as a part of the prior clinical trial, the Azelnidipine and Temocapril in Hypertensive Patients with Type 2 Diabetes Study (ATTEST). *Methods and Results*. All subjects who gave informed consent for genetic research were divided into two groups: the subjects treated with azelnidipine or temocapril, for 52 weeks. We selected 18 susceptible genes for hypertension and determined their genotypes using TaqMan PCR method. RNA samples were extracted from peripheral blood, and quantitative real time PCR for all genes was performed using TaqMan method. One of the polymorphisms of the RGS2 gene was extracted as being able to influence the effect of these treatments to reduce BP. At eight weeks, BP change showed a significant interaction between the A-638G polymorphism of Regulator of G protein signaling-2 (RGS2) gene and treatment with azelnidipine or temocapril. There was no gene whose expression was associated with BP phenotypes or the polymorphisms of each gene. *Conclusions*. A-638G polymorphism of the RGS-2 gene could be a predictive factor for therapeutic performance of Ca channel blockers.

1. Introduction

Genetic approaches may provide a powerful tool for clarifying the pathogenesis of essential hypertension. Many reports have demonstrated that gene polymorphisms of the renin-angiotensin system (RAS) are associated with hypertension. There have been some reliable reports about susceptible genes for hypertension including the results from "The Millennium Genome Project for Hypertension in Japan (2000∼2005) [1, 2]; however, no convincing gene has yet been detected. Some of the genes regulating blood pressure might also be related to the response to antihypertensive medication [3, 4]. Indeed, we and other collaborators have investigated several susceptible genes related to hypertension [5–15], including genes of not only the renin-angiotensin system and sodium handling but also insulin resistance, oxidative stress, and sympathetic nervous system (described in the Section 2); however, the genes involved in the response to antihypertensive medication have not yet been identified. In addition, exhaustive gene expression analysis (transcriptome analysis) for lifestyle-related diseases has not been performed thus far. We performed a large collaboration with the study group led by Professor Katayama at Saitama Medical University to perform a randomized controlled trial

called "Azelnidipine (a calcium (Ca) channel blocker) and Temocapril (an ACE inhibitor) in Hypertensive Patients with Type 2 Diabetes Study (ATTEST) [16]", which included genetic analysis to evaluate the therapeutic effects of azelnidipine and temocapril.

The goals of this study were, first, to assess the association between polymorphisms of susceptible genes for hypertension (18 genes) and each treatment or phenotype and, second, to assess the association between expression in peripheral blood of susceptible genes for hypertension and each treatment or phenotype.

2. Methods

2.1. Study Subjects. ATTEST Study, a multicenter, randomized, open-label study, was originally performed to investigate the efficacy and safety of combination therapy using the calcium channel blocker (CCB) azelnidipine and angiotensin-converting enzyme (ACE) inhibitor temocapril in hypertensive diabetics, led by Professor Katayama of Saitama Medical University. All of the subjects fulfilled the following inclusion criteria: (1) age: 30~70 years, outpatients, (2) mean systolic BP (BP): 140~180 mmHg and/or mean diastolic BP: 90~110 mmHg in washout period (four weeks) and stable BP without fluctuation in systolic BP of more than 30 mmHg or diastolic BP of more than 15 mmHg, and (3) fasting blood glucose: more than 126 mg/dl or HbA1c: more than 6.5% at 6 months before entry. These mild or moderate hypertensive subjects with diabetes were treated with the following medication: CCB: azelnidipine and ACE inhibitor: temocapril in accordance with the protocol (Figure 1). Each medication was started at a dose of 8 mg azelnidipine or 2 mg temocapril and increased to 16 mg azelnidipine or 4 mg temocapril until BP of less than 130/80 mmHg was achieved. All subjects were measured for body height, body weight, systolic BP, diastolic BP, fasting blood glucose level, triglyceride level, low density lipoprotein (LDL) cholesterol level, and serum creatinine (Cr) level.

Informed consent for genetic analysis was obtained from all subjects, and finally a total of 44 subjects were recruited in this study.

2.2. Selection of Susceptible Genes for Hypertension and Genotyping and Quantitative Real Time PCR (RT-PCR). The 18 genes shown in Table 1 were selected for the current study. All of the genes were previously reported to be susceptible genes for hypertension. The genotypes of the 22 polymorphisms of the 18 susceptible genes (ACE Ins/del, ADD1 Gly460Trp (rs4961), ADIPOQ Ile164Thr, ADRB1 Ser49Gly (rs1801252) and Arg389Gly (rs1801253), ADRB2 Gly16Arg (rs1042713) and Glu27Gln (rs1042714), ADRB3 Trp64Arg (rs4994), AGT Met235Trp (rs699), AGTR1 A1166C (rs17231380), ALDH2 Glu487Lys (rs671), ARHGAP8 Arg338Gly (rs6007334), BDKRB2 T-58C, FASL G-670A, GRK4 Ala486Val (rs1801058) and Arg65Leu (rs2960306), hOGG1 Ser326Cys (rs1052133), MTHFR C677T (rs1801133), RGS2 A to G in promoter (rs3767489), RGS2 A-638G (rs2746071), SLC12A3 Arg904Gln (rs11643718), and TGFB1 Leu10Pro (rs1982073)) for which positive associations with hypertension were previously reported were successfully determined using TaqMan PCR method (Applied Biosystems Inc., Foster City, CA, USA).

Table 1: Susceptible genes for hypertension.

(1)	Angiotensin converting enzyme (ACE)
(2)	α-adducin (ADD1)
(3)	Adiponectin (ADIPOQ)
(4, 5, 6)	$\beta1/\beta2/\beta3$ adrenergic receptor (ADRB1/2/3)
(7)	Angiotensinogen (AGT)
(8)	Angiotensin II type1 receptor (AGTR1)
(9)	Aldehyde dehydrogenase 2 (ALDH2)
(10)	Rho-GTPase activating protein-8 (ARHGAP8)
(11)	Bradykinin receptor $\beta2$ (BDKRB2)
(12)	Fas ligand (FASL)
(13)	G protein-coupled receptor kinase 4 (GRK4)
(14)	Human 8-hydroxyguanine DNA-glycosylase (hOGG1)
(15)	Methylenetetrahydrofolate reductase (MTHFR)
(16)	Regulator of G protein signaling-2 (RGS2)
(17)	Solute carrier family 12 member 3 (SLC12A3)
(18)	Transforming growth factor-β (TGFB1)

According to quantitative RT-PCR analysis using cDNA extracted from peripheral blood, 11 genes were expressed in peripheral blood: ACE, ADD1, ADRB1, ADRB2, ARHGAP8, FASL, GRK4, MTHFR, RGS2, SLC12A3, and TGFB1. RNA samples were extracted from the peripheral blood of the subjects, and cDNA was made from RNA using reverse transcriptase. Quantitative real-time PCR (RT-PCR) for all the genes was performed using TaqMan PCR method to determine their expression levels.

2.3. Statistical Analysis. The associations between polymorphisms and clinical variables were analyzed using one-way analysis of variance (ANOVA). The difference in each genotype or allele distribution was examined by χ^2 analysis. Odd ratios were calculated as an index of the association of each genotype with the prevalence of hypertension. To assess the contribution of confounding factors, we performed multiple logistic regression analysis using the computer software application, JMP 7.0 (SAS Institute Inc., Cary, NC, U.S.A.). We focused on testing for SNP medication and gene-expression medication interactions (i.e., whether the effects of SNPs on systolic BP or diastolic BP differed between hypertensives on azelnidipine and temocapril). Multivariate analysis of variance (MANOVA) was performed to determine whether BP change showed a significant interaction of each antihypertensive medication with the genotypes of the susceptible genes.

3. Results

3.1. Baseline Characteristics of Subjects Treated with Azelnidipine and Temocapril. Baseline characteristics of the subjects treated with azelnidipine and temocapril are shown in

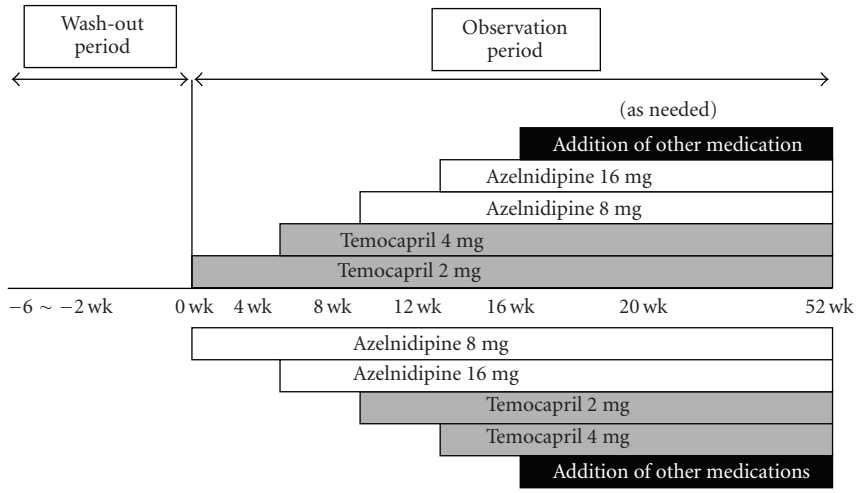

Figure 1: Study protocol; wk: week.

Table 2. There was no difference in sex, age, body mass index (BMI), affected period of hypertension, systolic BP, triglyceride level, LDL cholesterol level, and serum creatinine level between the azelnidipine and temocapril groups; however, fasting glucose level was higher in the azelnidipine group than in the temocapril group at the baseline.

3.2. BP Reduction with Azelnidipine and Temocapril. In the current study, the same level of BP reduction was observed in both the azelnidipine and temocapril groups, and both groups achieved a mean BP of less than 130/80 mmHg at 52 weeks. There was no difference in the dose of each treatment at 52 weeks (group started with azelnidipine: BP at 52 weeks 121.9 ± 11.4/74.7 ± 7.1 mmHg, final dose of azelnidipine 15.0 ± 2.7 mg, final dose of temocapril 3.8 ± 0.6 mg; group started with temocapril: BP at 52 weeks 125.6 ± 10.3/74.7 ± 6.1 mmHg, final dose of azelnidipine 14.9 ± 2.8 mg, final dose of temocapril 3.7 ± 0.7 mg, Figure 2).

3.3. Susceptible Gene Polymorphisms for Hypertension. The genotypes of 22 polymorphisms of the 18 genes were successfully determined at each time point (0, 8, 16, and 52 weeks). Only the Ile164Thr polymorphism of the adiponectin gene has no mutant genotype, and the other genotype frequencies were not significantly different from the values of Hardy-Weinberg's expectation (Figure 3).

According to the analysis of the association between BP, changes of BP, and all genotypes at each time point (0, 8, 16, and 52 weeks), only the A-638G polymorphism of the regulator of G protein signaling-2 gene (*RGS2*) showed a significant association with changes in BP (ΔBP: BP at 8 weeks—BP at 0 week). As shown in Figure 3, there was a significant relationship between the A-638G polymorphism of the RGS2 gene and the changes in BP between 0 and 8 weeks in subjects with azelnidipine (Δsystolic BP: $AA-28.0\pm 10.1$ mmHg, $AG-15.5\pm 12.6$ mmHg, $GG+7.0\pm 12.2$ mmHg, $P = .0013$; Δdiastolic BP: $AA - 17.2 \pm 9.8$ mmHg, $AG - 8.1 \pm 9.0$ mmHg, $GG - 4.0 \pm 11.0$ mmHg, $P = .067$), but not in subjects with temocapril (Δsystolic BP: $AA - 5.3 \pm 11.8$ mmHg, $AG - 8.4 \pm 13.0$ mmHg, $GG - 9.9 \pm 10.0$ mmHg, $P = .81$; Δdiastolic BP: $AA - 0.5 \pm 7.2$ mmHg, $AG - 4.4 \pm 3.5$ mmHg, $GG - 10.0 \pm 4.5$ mmHg, $P = .34$). At 8 weeks, changes in BP showed a significant interaction between *A-638G* and treatment with azelnidipine and temocapril (Δsystolic BP, Δdiastolic BP: $P = .0014$, $P = .036$, resp., by MANOVA, after adjustment for age, sex, and BMI) (Table 3).

There was no gene whose expression was associated with BP phenotypes or the polymorphisms of each gene through analysis of gene expression in peripheral blood. In terms of the RGS2 gene, the A-638G polymorphism was not related to the change of RGS2 expression between 0 and 8 weeks either in subjects with azelnidipine (ΔRGS2/18sRNA with azelnidipine: $AA - 1.06 \pm 2.1$, $AG + 0.31 \pm 0.85$, $GG + 0.57 \pm 0.44$, $P = .13$, Figure 4) or in subjects with temocapril (ΔRGS2/18sRNA with temocapril: $AA+0.75\pm 1.3$, $AG - 0.10 \pm 0.62$,$GG + 0.38 \pm 0.44$, $P = .24$, Figure 4).

4. Discussion

This study demonstrated the importance of pharmacogenetic research. Previously, Lynch et al. reported that the *NPPA* T2238C variant was associated with modification of antihypertensive medication effects on cardiovascular disease and BP, and TT allele carriers had more favorable outcomes when randomized to receive a CCB (amlodipine) [17]. Beitelshees et al. also demonstrated that the KCNMB1 genotype influenced responsiveness to verapamil SR and risk of adverse cardiovascular outcomes [18]. These reports could support the possibility of the existence of genes influencing drug efficacy.

Signaling by G-protein-coupled neurotransmitter receptors in the autonomic nervous system and vasoregulatory factor receptors in the periphery governs both blood pressure, by controlling the constriction and dilatation of resistance arterioles, and electrolyte and fluid balance by the kidney [19, 20]. The recently identified regulator of G-protein signaling (RGS) proteins is important in regulating

Table 2: Baseline characteristics of subjects treated with azelnidipine and temocapril.

	Azelnidipine	Temocapril	*P* value
n	23	21	
Male/Female (*n*), (%)	18/5 (78/22)	15/6 (71/29)	.60
Age (years)	60.8 ± 7.9	61.0 ± 8.5	.96
BMI (Kg/m^2)	25.6 ± 4.3	25.8 ± 3.3	.85
Period of HT (yr)	5.3 ± 5.7	9.0 ± 12.8	.21
Systolic BP (mmHg)	155.1 ± 11.1	157.2 ± 10.2	.52
Diastolic BP (mmHg)	97.8 ± 5.7	95.2 ± 5.4	.14
Fasting BG (mg/dL)	147.4 ± 27.5	132.3 ± 19.4	.04
TG (mg/dL)	127.3 ± 54.7	129.1 ± 61.3	.92
LDL-cholesterol (mg/dL)	136.1 ± 39.5	137.0 ± 39.0	.94
Serum Cr (mg/dL)	0.75 ± 0.12	0.77 ± 0.15	.58

HT: hypertension, BP: blood pressure, BG: blood glucose, TG: triglyceride, LDL: low density lipoprotein, and Cr: creatinine (mean±SD).

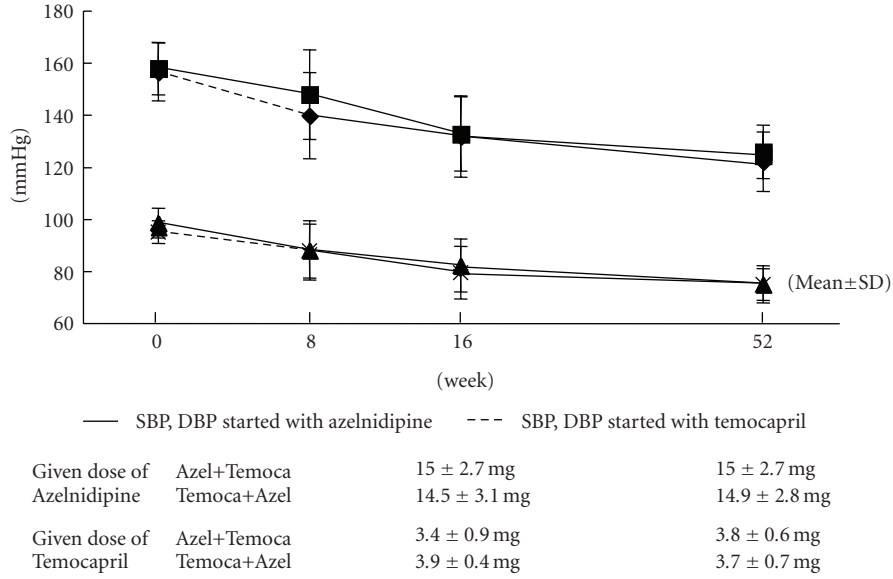

Figure 2: BP reduction with azelnidipine and temocapril; BP: blood pressure, SBP: systolic BP, and DBP: diastolic BP.

signaling cascades initiated by G-protein-coupled receptors (GPCRs) activation [21]. RGS proteins facilitate the intrinsic inactivating guanosine triphosphatase reaction of G-protein a-subunits , and thereby serve as effector channel blockers. RGS2 is unique among the RGS proteins in its apparent selectivity towards Gpa, which mediates the action of mouse physiological vasoconstrictors, including norepinephrine, angiotensin II, endothelin-1, and thrombin. RGS2 can also attenuate Gi- and Gs-mediated pathways [22, 23], which can also affect blood pressure via other physiologically important agonists such as serotonin, dopamine, and bradykinin. It was recently reported that mice lacking *RGS2* exhibit a strong hypertensive phenotype (increase in SBP of 50 mmHg) and resistance vasculature [24, 25]. Both heterozygous and homozygous *RGS2*-null mice exhibited a similar level of marked hypertension, suggesting that a naturally occurring mutation that affects the level of RGS2 protein may have a significant impact on blood pressure regulation. Recently there have been two reports that genetic changes in *RGS2* are associated with a hypertensive phenotype [26, 27]. A-638G, T1026A, and 1891-1892 del TC polymorphisms were extracted on the condition the allele frequencies of these polymorphisms were >0.1, and the T1026A and 1891-1892 del TC polymorphisms of this gene were associated with hypertension in women. These findings suggest that some functional variants of the RGS2 gene might be involved in regulating blood pressure in humans.

This ATTEST gene study revealed that one possible gene related to the effect of antihypertensive agents. In the current study, the A-638G polymorphism of the RGS2 gene was a predictive factor for therapeutic performance of a CCB, azelnidipine. Other classical candidate genes including genes associated with the renin-angiotensin system, sodium handling, vasodilatation and vasoconstriction, and the sympathetic nervous system did not show significant association between their polymorphisms and the effect of a Ca channel blocker or ACE inhibitor. In addition, there was no significant relationship between RGS2 polymorphisms and

Table 3: Genotype frequencies of 22 SNPs.

SNP Name	Major Homo (*n*)		Hetero (*n*)		Minor Homo (*n*)		Hardy-Weinberg Expectation (o value)
ACE Ins/Del (I/D)	II	15	ID	23	DD	6	0.54
ADD1 Gly460Trp (G/T)	GG	10	GT	24	TT	10	0.55
ADIPOQ Ile164Thr (T/C)	TT	44	TC	0	CC	0	-
ADRB1 Ser49Gly (G/A)	GG	1	GA	6	AA	37	0.24
ADRB1 Arg389Gly (G/C)	GG	3	GC	14	CC	27	0.53
ADRB2 Gly16Arg (A/G)	AA	14	AG	18	GG	12	0.23
ADRB2 Glu27Gln (C/G)	CC	41	CG	3	GG	0	0.81
ADRB3 Trp64Arg (T/C)	TT	29	TC	13	CC	2	0.73
AGT Met235Trp (T/C)	TT	31	TC	12	CC	1	0.90
AGTR1 A1166C (A/C)	AA	36	AC	7	CC	1	0.38
ALDH2 Glu487Lys (G/A)	GG	29	GA	13	AA	2	0.73
ARHGAP8 Arg338Gly (C/G)	CC	9	CG	20	GG	15	0.63
BDKRB2 T-58C (T/C)	TT	7	TC	23	CC	14	0.63
FASL G-670A (G/A)	GG	16	GA	17	AA	11	0.15
GRK4 Ala486Val (C/T)	CC	12	CT	20	TT	12	0.55
GRK4 Arg65Leu (G/T)	GG	33	GT	9	TT	2	0.21
hOGG1 Ser326Cys (G/C)	GG	7	GC	24	CC	13	0.46
MTHFR C677T (C/T)	CC	16	CT	24	TT	4	0.24
RGS2 A to G in promoter (A/G)	AA	9	AG	22	GG	13	0.96
RGS2 A-638G (A/G)	AA	9	AG	23	GG	12	0.74
SLC12A3 Arg904Gln (G/A)	GG	39	GA	5	AA	0	0.69
TGFB1 Leu10Pro (T/C)	TT	12	TC	24	CC	8	0.51

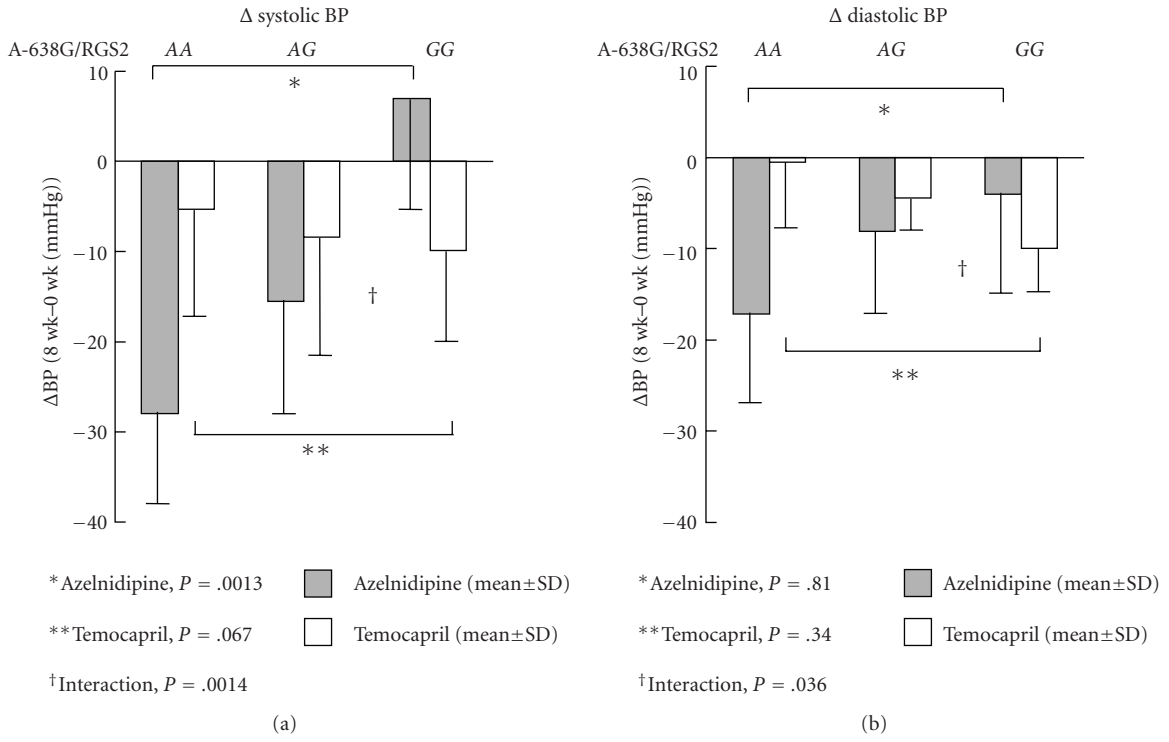

Figure 3: Relationship between A-638G polymorphism of RGS2 gene and BP change.

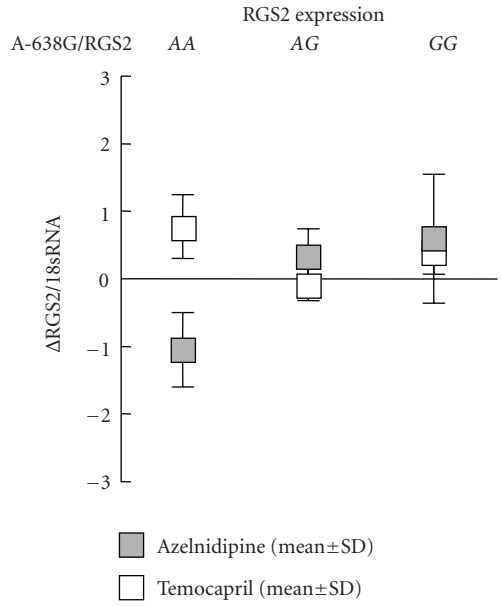

Figure 4: Relationship between A-638G polymorphism of RGS2 gene and RGS2 gene expression in peripheral blood.

BP-related phenotypes in this study, unlike previous reports. It has not yet been reported that the promoter variant A-638G of the RGS2 gene may change RGS2 function; however, this polymorphism has the possibility to change RGS2 protein production because this polymorphism is in the promoter region. There has been no evidence supporting the idea that RGS2 could be involved in the effect of CCBs. In several previous reports, genes without a drug-metabolizing effect, such as ACE [3] or AGTR1 [4], showed a significant association between their polymorphisms and response to antihypertensive medication; however, the mechanisms have not been clarified in any of these reports. In terms of the present study, RGS2 is suggested to function as a switch to turn on or off the G protein-associated pathway, and RGS2 can regulate blood pressure through smooth muscle cells. From this viewpoint, RGS2 might have the possibility of changing the effect of CCBs, because those mainly act through SMC; however, the detailed mechanism merits further investigation in the future.

Peripheral blood mainly contains white blood cells, red blood cells, platelets, and other circulating hormones, so the distribution of genes expressed in peripheral blood has to be investigated to clarify the significance of disease susceptibility gene expression in peripheral blood. In the present study, 11 genes were expressed in peripheral blood. There was no significant relationship between gene expression including the RGS2 gene and the effect of antihypertensives and phenotypes. From this viewpoint, RGS2 expression in peripheral blood does not seem to do anything and might not be a marker for BP regulation. However, further study is needed on its clinical application as a marker of drug efficacy.

As the limitation of this study, we could not exclude the possibility of the false positive association (type I error) due to the use of small number of subjects. However, this study was carried out under the strict protocol of a clinical trial so that the reliability of the results obtained seems to be high. To confirm the effect of RGS2 gene in the tailored medicine of hypertension, further study using another panel of hypertensive subjects should be required.

In conclusion, the A-638G polymorphism of the RGS2 gene could be a predictive factor for therapeutic effectiveness of CCBs such as azelnidipine. Further research is needed to determine the optimal approach for personalizing antihypertensive medication treatment regimens according to genotype information and for achieving the best blood pressure control.

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Research Article

Genetic Determinants of Osteoporosis: Common Bases to Cardiovascular Diseases?

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Osteoporosis is the most common and serious age-related skeletal disorder, characterized by a low bone mass and bone microarchitectural deterioration, with a consequent increase in bone fragility and susceptibility to spontaneous fractures, and it represents a major worldwide health care problem with important implications for health care costs, morbidity and mortality. Today is well accepted that osteoporosis is a multifactorial disorder caused by the interaction between environment and genes that singularly exert modest effects on bone mass and other aspects of bone strength and fracture risk. The individuation of genetic factors responsible for osteoporosis predisposition and development is fundamental for the disease prevention and for the setting of novel therapies, before fracture occurrence. In the last decades the interest of the Scientific Community has been concentrated in the understanding the genetic bases of this disease but with controversial and/or inconclusive results. This review tries to summarize data on the most representative osteoporosis candidate genes. Moreover, since recently osteoporosis and cardiovascular diseases have shown to share common physiopathological mechanisms, this review also provides information on the current understanding of osteoporosis and cardiovascular diseases common genetic bases.

1. Introduction

Osteoporosis is the most common age-related skeletal chronic disorder, characterized by reduced bone mass, deterioration of bone micro-architecture and increased risk of low-trauma fractures. Fragility fractures are the endpoint of osteoporosis and represent the major cause of morbidity and mortality. With the constant growing age of population, not only in developed countries but also in South America, Asia, and Africa, osteoporosis is becoming more and more a worldwide major public health problem. Today over two hundreds millions people worldwide and the thirty per cent of all postmenopausal women in USA and Europe are affected by osteoporosis. At least 40% of all affected women and 15–30% of all affected men will suffer a fragility fracture during their lifetime. The individuation of factors responsible for osteoporosis predisposition and development is fundamental for disease prevention and for setting of novel therapies.

According to the International Osteoporosis Foundation (IOF) guidelines, osteoporosis risk factors can be divided into two main classes: (1) modifiable risks that depend principally on lifestyle and nutrition habits and can be modified and (2) fixed risks that are innate and cannot be modified. Main osteoporosis risk factors are depicted in Table 1.

Today Scientific Community agrees that osteoporosis is a complex multifactorial disorder caused by the interaction between environmental factors and genes that singularly exert modest effects on bone metabolism and fracture risk. Studies on osteoporosis sibs and families demonstrated that genetic factors are responsible for about 60–85% of inter-individual variability of bone mineral density (BMD) [1, 2], and this effect appears to persist even in the late decades of life. BMD heritability varies between different skeletal sites [3]. Also fragility fracture risk seems to have a genetic component; family history of fracture has been shown in some epidemiological studies as a risk factor for

Table 1: Main risk factors for osteoporosis.

Osteoporosis modifiable risks	Osteoporosis fixed risks
(i) Alchool	(i) Age
(ii) Smoking	(ii) Ethnicity
(iii) Low body mass index	(iii) Female gender
(iv) Poor nutrition	(iv) Family history of fractures
(v) Eating disorders	(v) Previous fractures
(vi) Insufficient physical activity	(vi) Menopause/hysterectomy
(vii) Low dietary calcium intake	(vii) Hormonal status
(viii) Vitamin D deficiency	(viii) Long-term glucocorticoid therapy
(ix) Frequent falls	(ix) Primary/secondary hypogonadism in men

fractures [4, 5]. Interestingly, the heritability of fractures has been shown to be independent of BMD and maybe influenced by other factors such as bone geometry, bone turnover or the risk of falling. However, the heritability of fractures seems to decrease with age maybe as environmental factors become more important. Other bone features such as quantitative ultrasound properties, femoral neck geometry, bone turnover markers range have been demonstrated to be under the control of genetic factors [6, 7]. Except for some rare Mendelian monogenic inherited osteoporosis forms (osteoporosis associated with estrogen deficiency due to inactivating mutation of the aromatase gene (*CYP19*) [8] or associated with estrogen resistance due to inactivating mutation of the estrogen receptor alpha ($ER\alpha$) gene [9], and autosomal recessive osteoporosis pseudoglioma caused by inactivating mutation of lipoprotein receptor-related protein 5 (*LRP5*) [10], classic age-related osteoporosis is a multifactorial heterogeneous disorder and, to date, its exact genetic bases are still unknown. In fact, bone metabolism is regulated by several genes, some exerting a high degree of influence (major genes) and other, even more numerous, exerting minor effects (minor genes). Due to the complex biology of the skeleton, putative osteoporosis candidate genes are very numerous and the number of genes identified to be involved in bone metabolism is constantly increasing. They include genes involved in the regulation of bone and calcium metabolism, such as those encoding for calciotrophic and sex hormones and their receptors, for bone matrix proteins, for cytokines, growth factors and local mediators and their receptors, and for proteins involved in molecular pathways of bone cells. Principal osteoporosis candidate genes are described in Table 2.

Moreover, the presence of epigenetic regulative factors, gene-gene and gene-environment interactions complicate the situation. Different genetic and environmental factors may result in the same osteoporotic phenotype and it is also possible that some individuals having one or more predisposing alleles and genetically at risk of osteoporosis never become osteoporotic or, controversially, individuals with no predisposing alleles may develop osteoporosis with age due to non-genetic factors.

Candidate gene association studies have identified several polymorphisms associated to BMD, bone characteristics and fragility fracture risk. However, these association studies generated conflicting results may be due to inadequate population sampling, ethnicity, gender, age, confounding factors, lack of standardized genotyping methods, gene-gene interactions, linkage disequilibrium with other trait-causing polymorphisms in a nearby locus, epigenetic and/or post-transcriptional gene expression regulation (i.e., microRNAs) and gene-environment interactions. Retrospective meta-analyses, including several different association studies, and multicentric studies on large and well characterized populations are both helpful in reducing these issues and increasing the power of statistical associations. Several reviews on genetics of osteoporosis tried to summarize the most representative osteoporosis association studies [11–15].

Some of the most important and most studied osteoporosis candidate genes will be briefly discussed below, with specific focus on the results of the European Genomos study. The Genomos (Genetic Markers for Osteoporosis) study is an European multicentric consortium that collected over 20 000 Caucasian subjects (women and men) from several European centers, using prospective genotyping with cross-center standardization, for the study of osteoporosis candidate genes.

For years osteoporosis and cardiovascular diseases (CVDs) have been considered as two independent consequences of aging, however, recent evidences support an association between these two diseases, indicating common physiopathological mechanisms, and maybe genetic bases.

2. Osteoporosis and Cardiovascular Diseases

Some studies have reported associations between age-related CVDs and bone loss and have indicated common etiologies for CVDs and osteoporotic fractures with a substantially increased risk of hip fractures in women after the diagnosis of a CVD [16–19].

More than 90% of atherosclerosis fatty plaques undergo calcification. Now it is well assessed that calcium metabolism has a central role both in bone mineralization and on the risk of arteriosclerosis development and progression, since regulatory factors of bone cells functions can also affect vascular calcification. Osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) regulate osteoclast activation and function but are also involved in the vascular calcification process and atherosclerosis [20, 21]. Bone morphogenetic protein (BMP2) is involved in osteoblastic differentiation by the stimulation of Runx2 expression; in humans, atherosclerotic lesions show an increased expression of BMP2 and Runx2 with respect to normal arteries [22] and this may be responsible for arteries wall calcification. Some others biological and environmental factors seem to be involved both in altered bone mineralization and in vascular calcifications, such as vitamin D insufficiency, low calcium intake, estrogen deficiency, chronic inflammation, oxidative

Table 2: Main osteoporosis candidate genes.

Putative candidate genes	Chromosome location	Biological functions in bone and mineral metabolism	Main polymorphisms analyzed in osteoporosis association studies
Calciothropic and sex hormones and their receptors			
(i) Vitamin D receptor (*VDR*)	12q12-14	Calcium and phosphatase homeostasis; regulation of osteoclasts and osteoblasts functions.	*Cdx2* (promoter region); *FokI* (exon 2); *BsmI*, *ApaI*, *EcoRV* and *TaqI* (3′ UTR)
(ii) Parathyroid hormone (*PTH*) and PTH receptor (*PTHR*)	11p15 and 3p22-21	Calcium homeostasis, endogenous vitamin D synthesis and regulation of bone cells activity	*BstBI* (intron 2 of *PTH* gene); $(AAAG)_n$ repeat (P3 promoter of *PTHR* gene)
(iii) Estrogen Receptor Alpha and Beta (*ERα* and *ERβ*)	6q25 and 14q22-24	Control of bone remodelling, reduction of bone resorption	$(TA)_n$ repeat (promoter region) and *XbaI* and *PvuII* (intron 1) (*ERα*); $(CA)_n$ repeat (promoter region) and AluI (exon 8) (*ERβ*)
(iv) Calcitonin (*CT*) and its receptor (*CTR*)	11p15 and 7q21	Increasing of osteoblast activity, retaining of calcium in bones and prevention of phosphorus and calcium loss	$(CA)_n$ repeat (5′ flanking region of *CT* gene); *AluI* (nucleotide 1377 of *CTR* gene)
(v) Aromatase (*CYP19A1*)	15q21	Catalyzation of androgens conversion to estrogens	$(TTTA)_n$ repeat (intron 4)
(vi) Androgen receptor (*AR*)	Xq11-12	Regulation of osteoblast function and suppressive action on bone resorption	$(CAG)_n$ repeat (exon 1)
(vii) Calcium-sensing receptor (*CaSR*)	3q13-21	Regulation of calcium homeostasis at parathyroid, kidney, bowel and bone level	$(CA)_n$ repeat (5′ flanking region at the *CaSR* locus); T/C Ala986Ser (exon 7)
(viii) Glucocorticoid receptor (*GR*)	5q31	Inhibition of bone formation, suppression of calcium absorption	Asp363Ser (exon 2)
Cytokine, growth factors and local regulators			
(i) Interleukin-6 (*IL6*)	7p21	Effect on osteoclastogenesis and bone resorption	$(CA)_n$ repeat (5′ flanking region at the *IL6* locus); −634 C/G, −572G/C and −174G/C (promoter region).
(ii) Insulin-like growth factor 1 (*IGF-I*)	12q22-24	Stimulation of bone formation, recruitment of pre-osteoblasts, growth factor for osteoblasts	$(CA)_n$ repeat (promoter region)

Table 2: Continued.

Putative candidate genes	Chromosome location	Biological functions in bone and mineral metabolism	Main polymorphisms analyzed in osteoporosis association studies
(iii) Transforming growth factor β1 (*TGFβ-1*)	19q13	Osteoclast and osteoblast activity	−1348C/T and −509C/T (promoter region), 29T/C Leu10Pro and 74G/C Arg25Pro (exon 1), 869T/C and 788C/T Thr263Ile (exon 5), 713-8delC (intron 4), 861-20T/C (intron 5)
(iv) Bone morphogenetic protein 7 (*BMP7, OP1*)	20q13	Promotes mesenchymal cells into osteoblastic differentiation	—
(v) Bone morphogenetic protein 4 (*BMP4*)	14q22-q23	Involved in bone and cartilage development and in fracture repair	−5826G/A (promoter region); 3564C/T (intron 2); 6007C/T Ala147Val (exon 4)
(vi) Bone morphogenetic protein 2 (*BMP2*)	20p12	Stimulates the differentiation and/or activity of osteoclasts	Ser37Ala (exon 2); A/G Ser87Ser (exon 2); Arg190Ser (exon 3)
Bone matrix proteins			
(i) Collagen type I alpha1 (*COLIA1*)	17q21-22	Encode for collagen type I alpha1 chain	Sp1 (binding site of the transcription factor Sp1 in the intron 1)
(ii) Collagen type I alpha2 (*COLI-A2*)	7q22	Encode for collagen type I alpha2 chain	*PvuII*, *RsaI*, $(ACT)_n$ repeat (intron 12)
(iii) Osteopontin (*OPN, SPP1*)	4q21-25	Anchoring osteoclasts to the mineral matrix of bones	Intragenic $(CA)_n$ repeat
(iv) Osteocalcin (*OCN, BGLAP*)	1q25-q31	Role in bone matrix mineralization and calcium homeostasis	C/T and $(CA)_n$ repeat (promoter region)
(v) Osteonectin (*ON, SPARC*)	5q31.3-q32	Binds calcium, initiates mineralization and promotes mineral crystal formation	Intragenic $(CA)_n$ repeat
Miscellaneous			
(i) Low-density lipoprotein receptor-related protein 5 (*LRP5*)	11q13	Regulates osteoblasts proliferation and bone formation	Val667Ala (exon 9); Ala1330Val (exon 18)
(ii) Low-density lipoprotein receptor-related protein 6 (*LRP6*)	12p13.3-p11.2	Regulates osteoblasts proliferation and bone formation	Ile1062Val (exon 14)
(iii) Receptor activator of nuclear factor kappa B (*RANK*)	18q22.1	Receptor of RANKL expressed on osteoclast precursors and osteoclasts. The association of RANK-RANKL regulates osteoclast formation, activation and survival in normal bone modelling	421C/T, 575C/T

Table 2: Continued.

Putative candidate genes	Chromosome location	Biological functions in bone and mineral metabolism	Main polymorphisms analyzed in osteoporosis association studies
(iv) RANK ligand (*RANKL*)	13q14	Ligand of RANK expressed on osteoblasts and stromal cells. The association of RANK-RANKL regulates osteoclast formation, activation and survival in normal bone modelling	−290C/T, −643C/T, −693G/C and −1594G/A (promoter region)
(v) Osteoprotegerin (*OPG*)	8q24	Soluble "decoy receptor" of RANKL, inhibits osteoclast development by blocking the RANK-RANKL interaction	163A/G, 209G/A, 245T/G and 1181G/C (exon 1)
(vi) Sclerotin (*SOST*)	17q11.2	Potent osteocyte expressed negative regulator of bone formation in vitro	−9247T/C, −7859C/G, −1605C/T and −1396delGGA (promoter region); 27G/A (exon 1)
(vii) Chloride channel 7 (*CLCN7*)	16p13	Encodes a Chloride channel highly expressed in osteoclasts and essential for acidification of the resorption lacuna	Ala390Ala (exon 14), Val418Met (exon 15)
(viii) Methylentetrahydrofolate reductase (*MTHFR*)	1p36.3	Involved in collagen synthesis	677C/T (exon 4)

stress, dyslipidemia, high dietary fat intake, smoking, low physical activity. Elderly people present with calcium and vitamin D deficiency that could contribute to calcium mobilization from bones with consequently higher risk of fractures and of severe vessels and arteries calcification. At the same time the age-related estrogen deficiency may induce the increase of pro-inflammatory cytokines (IL1, IL6 and TNFα) that enhances the expression of adhesion molecules on leukocytes and endothelial cells favoring the progression of atherosclerosis plaques. Estrogen deficiency also induces the decrease of OPG with subsequent calcium mobilization from bones and risk of calcification of atherosclerosis plaques. Last, estrogen deficiency induces a reduction of production of nitric oxide which has athero-protective effects but also plays a role in osteoblast function and regulates the endothelial function of bone microcirculation. Moreover, elevated LDL and low HDL cholesterol, suspected to be responsible for atherosclerosis, are associated also with low BMD and with vertebral fractures in postmenopausal women [23]. The altered lipid metabolism is associated with both bone remodeling and atherosclerosis process [22] and this may explain, in part, the coexistence of atherosclerosis and osteoporosis in patients with dyslipidemia.

Animal, clinical and epidemiological studies suggest that high blood pressure is associated with abnormalities of calcium metabolism, leading to increased calcium loss, increased movement of calcium from bone and long-term risk of bone demineralization and osteoporosis [24–26]. Metabolic studies in hypertensive rats showed that associated hypercalciuria and secondary activation of parathyroid glands induced a reduced growth and a decreased bone mineral content later in life [27, 28]. The mechanisms by which this occurs are probably due to a defect in the kidney ability to handle calcium. Moreover, cross-sectional studies [29, 30] in humans have shown an inverse positive association between blood pressure and bone mineral density, supporting a possible correlation between hypertension and osteoporosis.

Last, recent evidences of the action of bone antiresorptive drugs also on the reduction of CVDs risk and evidences of the positive effect of statins, antihypertensive drugs and insulin on bone mass increase [22, 31–33] suggest that osteoporosis and CVDs share common physiopathological molecular pathways. Bisphosphonates are potent antiresorptive agents widely used in osteoporosis treatment and in prevention of fracture risk. Experimental studies on animal models demonstrated that bisphosphonates also inhibit arterial and cardiac calcification in mice [34] and prevent foam cell formation by inhibiting LDL uptake and macrophages replication [33]. Raloxifene is a selective estrogen receptor modulator prescribed for both the prevention and treatment of osteoporosis and with a proven efficacy in the reduction of risk of fragility fractures. Raloxifene seems to have favorable effects on LDL cholesterol level and risk of coronary heart disease and it improves vascular endothelial function in postmenopausal women [35, 36]. The results from the MORE (Multiple Outcomes of Raloxifene Evaluation) randomized trial made possible to decipher the effects of raloxifene on cardiovascular events in osteoporotic postmenopausal women, evidencing that raloxifene therapy for 4 years did not significantly affect the risk of cardiovascular events in the overall studied population but it significantly reduced this risk in the subpopulation of women with increased cardiovascular risk [37]. Statins reduce cardiovascular mortality through the regression of coronary calcification and the reduction of LDL cholesterol levels in patients with dyslipidemia. These hypolipidemic drugs have also been associated to increased bone mineralization in mice [38] and in patients with osteoporosis [39] and with a reduction of fracture incidence. Last, recent clinical studies indicated that beta blockers and antihypertension drugs would reduce the risk of fragility fractures in the elderly population [40]. Moreover, in a rodent model angiotensin II type 1 and 2 receptor blockers (ARB) widely used antihypertensive agents, were shown to enhance bone mass through both the increase of osteoblast activity and the suppression of osteoclast activity [41]. Recently, a preventive effect of angiotensin II type 1 receptor blocker on osteoporosis has been reported but these data need confirmation [42]. The fact that all these drugs are effective on both osteoporosis and CVDs suggests a possible link between vascular and skeletal systems. Therefore, it is priority to establish to what extent treatments for osteoporosis are effective and beneficial for CVDs and vice versa, as well as to comprehend the exact physiopathological mechanisms shared by these diseases.

According to the current knowledge, further specific studies are necessary to better define the relationship between osteoporosis and CVDs and to identify common risk factors and genetic determinants.

3. Vitamin D Receptor Gene (*VDR*)

Since the important role of vitamin D in the regulation of calcium homeostasis and bone metabolism, vitamin D receptor (*VDR*) gene has been the first candidate gene to be analyzed in association studies by Morrison at al. in 1994 [43] and it was proposed as a major locus for genetic effects on bone metabolism. Principal analyzed polymorphisms are the *BsmI*, *ApaI*, and *TaqI* polymorphisms in the 3′ UTR of the gene, the *FokI* polymorphism in exon 2 (that creates an alternative initiation traduction codon) and the *Cdx2* polymorphism in the promoter region of the gene. During the last two decades, several association studies have been performed but with conflicting data. Recent data seem to indicate that association between *VDR* polymophisms and bone mass is rather weak and the clinical impact of these variants remains unclear. Given the extent of published data on this gene and the non-concording results, interest now focuses on meta-analyses rather than single association studies alone. A haplotype meta-analysis by Thakkinstian et al. [44] evidenced that *VDR* single polymorphisms were not significantly associated to osteoporosis while *Bat* and *BAt* haplotypes were significantly associated, demonstrating the importance of haplotype studies rather than single polymorphism studies.

The Genomos study collected 26,242 Caucasian participants (18,405 women) and evaluated the association between

Cdx2, *FokI*, *BsmI*, *ApaI,* and *TaqI* polymorphisms with the DEXA-measure femoral neck and lumbar spine BMD and fractures, concluding that the *FokI*, *BsmI*, *ApaI,* and *TaqI* polymorphisms are not associated with BMD variation or with fractures while the "A" allele of the *Cdx2* polymorphism is associated with a reduced risk of vertebral fractures [45].

VDR is involved in vascular smooth muscle cell growth and in the regulation of calcium homeostasis and could therefore be involved in vascular plaques instability and calcification, thus, *VDR* polymorphisms may be associated with different risk for CVDs. In a study by Van Shooten et al. [46] the *bb* genotype of the *VDR* gene appeared to be predictive of severe coronary artery disease (CAD). The bb genotype is associated with low levels of circulating active form of vitamin D (calcitriol) [43], thus, results from this study seem to agree with previous finding of an inverse association between circulating calcitriol and CAD [47, 48]. The genetic association between VDR polymorphisms and CAD risk was not confirmed by a study in a Chinese population [49].

4. Estrogen Receptors Alpha and Beta Genes (*ERα* and *ERβ*)

Estrogens exert important effects on bone mass acquisition and maintenance. A rare case of a 28-year-old man with estrogen resistance and juvenile osteoporosis due to a nonsense inactivating point mutation in exon 2 of the *ERα* gene has been described [9]. Patient presented with unfused epiphyses and continuing linear growth in adulthood, indicating that estrogen is important for normal skeletal growth and bone development and mineralization in men as well as women. Genes encoding for estrogen receptors have been widely investigated in osteoporosis association studies. Most studies have focused on the $(TA)_n$ repeat microsatellite in the promoter region and on the *PvuII* and *XbaI* single nucleotide polymorphisms (SNPs) in the intron 1 of the *ERα* gene. However, these studies generated conflicting results, suggesting the needing of large-scale investigations and analysis standardization. A meta-analysis by Ioannidis et al. [50] seemed to indicate no significant association between single *ERα* polymorphisms with BMD, while a significant reduction of fracture risk was associated to the *XX* genotype. Conversely, when *ERα* polymorphisms are analyzed together as haplotypes [51], significant associations of haplotypes with spinal BMD, decreased vertebral spine bone area and increased risk for spinal fracture were found in women, but not in men. This study also evidenced that *ERα*-dependent fracture risk is independent of BMD and bone area.

The Genomos study collected 18 917 individuals (14,622 women) and evaluated the association of the three common *ERα* polymorphisms with the DEXA-measured femoral neck and lumbar spine BMD and with fractures [52]. The study evidenced that *ERα* polymorphisms exert only small effects on BMD. Conversely, the *XX* genotype resulted to be associated with a 20% reduction in fracture risk by mechanisms independent of BMD.

However, to date most association studies on *ERα* gene have evaluated the association of its polymorphisms with BMD and fracture risk but not with bone structural and geometric properties. Recently, Cepollaro et al. [53] have evaluated the influence of *XbaI* and *PvuII* polymorphisms on structural and geometric bone parameters assessed by pQCT at the tibia in 541 Italian women and 449 Italian men. The study evidenced a significant association between the *PP* genotype of *PvuII* and higher values of tibial cortical thickness in male subjects. This result indicated a role for *ERα* gene in the control of tibia bone geometry and could explain the mechanism by which *ERα* gene polymorphisms influence fracture risk independently of BMD.

ERβ gene has been less studied in osteoporosis association studies since its role on bone metabolism is not yet completely known. *ERβ* seems to have a role in mediating estrogen effect on bone growth and bone size but not on BMD [54]. A $(CA)_n$ repeat microsatellite polymorphism in the promoter region has been associated with BMD in Asian [55, 56] and Caucasian [57] populations. No meta-analysis of *ERβ* association studies is available so far.

Estrogens have vasodilatatory, antiinflammatory and antiproliferative effects on the cardiovascular system and they have been reported to provide protection against CAD in postmenopausal women [58]. Lu et al. [59] associated the novel −1989T/G polymorphism in the *ERα* promoter B with CAD risk, concluding that the G/G genotype may be an independent predictor for CAD in patients with familiar hypercholesterolemia. In addition, they found that the long number (>17) of $(TA)_n$ repeat in the *ERα* promoter region was significantly higher in postmenopausal women with CAD than in those without CAD, but not in men. Pollak et al. [60] confirmed this result and found an association between homozygote genotype for long alleles (>18) and a significantly higher angiographic severity of CAD in young patients. Alevizaki et al. [61] found that *ERα PuII* and *XbaI* polymorphisms may influence the severity of CAD in women, associating the C allele of *PvuII* and the G allele of *XbaI* with a higher number of arteries with a significant stenosis in the coronary angiography.

5. Collagen Type I α1 Gene (*COLIA1*)

COLI1A1 gene encodes for the α1 chain of collagen type I that is the principal proteic component of bone extracellular matrix, thus, this gene is an important candidate for osteoporosis risk. Several association studies have been conducted on a polymorphism in intron 1, a G/T substitution that creates a binding site for the transcription factor *Sp1*. The *ss* (T/T) genotype has been associated with reduced BMD [62, 63], increased age-related bone loss [64, 65], increased femoral neck angle [66], an impaired ability of osteoblast-like cells to form mineralized bone nodules in vitro and with abnormalities of bone mineralization in vivo [67] and a higher risk of fracture due to altered bone density and quality [68]. In general, association studies on this gene demonstrated a positive correlation between *SS* (G/G) genotype and reduced fracture risk even with lack of

association with BMD values. A meta-analysis evidenced that different *Sp1* alleles are associated with modest variation in BMD but with significant changes in fracture risk [69]. The T allele is associated with an abnormally increased synthesis of collagen I α1 chain generating an imbalance between the α1 and α2 chains and a reduction of bone strength and bone matrix mineralization [67].

The Genomos study collected standardized data on 20,786 individuals and evaluated association of the *Sp1* polymorphism with the DEXA-measured femoral neck and lumbar spine BMD and with fractures [70]. The *s* allele resulted to be associated with recessive-inherited reduced BMD. However, this association was quite weak. Authors also found a modest association of the *s* allele with vertebral fractures, particularly in women, and hypothesized that this allele could predispose to incidental vertebral fractures independently of BMD.

6. Transforming Growth Factor β1 (*TGFβ1*)

*TGFβ1*gene encodes a local growth factor that is widely expressed by bone cells and it is involved in the regulation of bone turnover [71–73]. This gene has been analyzed as a possible osteoporosis candidate gene; principal investigated polymorphisms are located in the promoter region (*−1348C/T* and *−509C/T*) and in the exon 1 (*29T/C Leu10Pro* and *74G/C Arg25Pro*). Several studies have associated variants in this gene with BMD variation and/or fragility fractures risk [74–80], but none of them have investigated the effects of *TGFβ1* haplotypes.

The Genomos study performed a wide standardized analysis including 28 924 participants from 10 European centers [81]. The study genotyped five polymorphisms of *TGFβ1* gene (two located in the promoter region, two located in the exon 1 and one located in exon 5) and associated them with DEXA-measured BMD of lumbar spine and femoral neck and with fractures. None of the polymorphisms or haplotypes resulted to be associated to BMD variations or to affect the overall risk of fractures. A weak association was detected between carriers of the rare *788T* allele of the *788 C/T* polymorphism (*Thr263Ile*) in exon 5 and the risk of incident vertebral fractures.

TGFβ seems to have contrasting functions on cardiovascular system. Some studies reported a protective effect of TGFβ by reducing the risk of CVDs [82–85], while others described TGFβ as inducing or facilitating CVDs such as vascular stenosis and thrombogenesis [86, 87]. The *Arg25Pro* polymorphism in exon 1 has been associated with different risk of essential hypertension in Russian male individuals [88], with variation in systemic blood pressure in essential hypertensive patients [89] and with different risk of myocardial infarction or hypertension in Caucasian patients [90], but no correlation has been found between this polymorphism and the risk of myocardial infarction and stroke [91], the risk of CAD in Caucasian patients [92] and with the severity of CAD and the occurrence of myocardial infarction or hypertension in Australian patients [93]. The Leu10Pro polymorphism in exon 1 has been associated with susceptibility to myocardial infarction in a Japanese population [94], with clinical characteristics of hypertension [95] and with the risk of stroke in an elderly Caucasian population [91], but not with the risk of myocardial infarction in the same elderly Caucasian population [91].

7. Low-Density Lipoprotein Receptor-Related Protein 5 and 6 (*LRP5* and *LRP6*)

LRP5 and its related homologue *LRP6* are cell-membrane coreceptors for Wnt proteins an the Wnt/beta catenin signalin pathway, that controls osteoblast activity and bone formation. *LRP5* gene has a clear role in rare bone diseases and also in normal variation in peak BMD.

Inactivating mutations of *LRP5* lead to osteoporosis pseudoglyoma [96] while activating mutations result in high bone mass phenotypes [97, 98].

Point mutations in LRP6 gene cause abnormal formation of the axial skeleton and a low bone mass phenotype in mice [99].

Thus, common variants in *LRP5* and *LRP6* genes may contribute to normal population variance in human bone metabolism, and these two genes have been recently proposed and analyzed as putative osteoporosis candidate genes. Two studies [100, 101] have demonstrated an association between *LRP5* variants and bone mass in human populations. Particularly, Ferrari et al. [100] found an association between *2047 G/A* substitution (*Val667Met*) in exon 9 and bone mineral content at lumbar spine, bone area and with stature in men, but not in women.

A study by van Meurs et al. [102] analyzed the role of four variants of the *LRP5* gene and one aminoacid variant of the *LRP6* gene in determining BMD, bone geometry and fracture risk. Authors found that the *1330Val* allele (*Ala1330Val* polymorphism) of the *LRP5* gene was associated with a decreased BMD at lumbar spine and femoral neck and with reduced vertebral body size and femoral neck width in men. Male carriers of the *1330Val* allele had a 60% increased risk for fragility fractures. A borderline association of the *LRP6 Ile1062Val* polymorphism with height and vertebral body size was observed in men. Males carriers of the *1062Val* allele had a 60% higher risk of fractures. In women all these association were weaker than in men.

All these studies indicated that *LRP5* and *LRP6* associations with bone phenotypes are sex specific.

The Genomos study collected and analyzed 37,534 Caucasian individuals from Europe and North America and associated DEXA-measured BMD at lumbar spine and femoral neck and fracture risk with *Val667Met* and *Ala1330Val* polymorphisms of the *LRP5* gene and with *Ile1062Val* polymorphism of the *LRP6* gene [103]. The *Met667* and *Val1330* alleles were both associated with reduced spinal and femoral BMD and with increased risk of vertebral or total fractures. Haplotype analysis indicated that *Met667* and *Val1330* variants affected BMD independently. Conversely, the *LRP6 Ile1062Val* polymorphism was not associated with any osteoporotic phenotype.

No studies on the association of *LRP5* and *LRP6* polymorphisms with CVDs risk are available so far.

8. Aromatase Gene (*CYP19A1*)

Aromatase enzyme catalyses the conversion of androgens to estrogens. Inactivating mutations of the *CYP19A1* gene have been associated, in both sexes, with an increased bone turnover and consequently with a decreased BMD [8].

A microsatellite tetranucleotide $(TTTA)_n$ repeat polymorphism in intron 4 was associated to osteoporosis risk [104]. Analysis of polymorphism in a postmenopausal cohort of Italian women associated the $(TTTA)_{12}$ allele with a protective action versus osteoporosis development. Women with high number of repeats (>11) showed higher lumbar BMD values than women with low number of repeats (from 8 to 11). However, the molecular mechanism that can explain association between aromatase activity and $(TTTA)_n$ repeat is still unknown.

Aromatase has been hypothesized as one of the factor affecting blood pressure and maybe a susceptibility gene for hypertension. A study by Shimodaira et al. [105] found an association between the rs700518 and rs10046 polymorphism, as well as a haplotype constructed with rs1870049 and rs10046 polymorphisms, of *CYP19A1* gene with variation in systolic blood pressure and diastolic blood pressure. Interestingly, the at risk genotypes of rs700518 and rs10046 showed a sex-dependent inverse relationship, suggesting the possibility to use them as genetic markers for gender-specific essential hypertension risk. Ramirez-lorca et al. [106] confirmed that the rs10046 polymorphism may be involved in the genetic regulation of blood pressure in women. Another study by Letonja et al. [107] concluded that in Caucasian subjects the $(TTTA)_n$ repeat polymorphism does not contribute to the genetic susceptibility to CAD.

9. Insulin-Like Growth Factor I Gene (*IGF-1*)

IGF-1 exerts anabolic effects on BMD increasing synthesis of collagen type I and osteocalcin and stimulating alkaline phosphatase activity that results on both proliferation and differentiation of osteoblasts. It is an essential factor during childhood and adulthood in the regulation of trabecular and cortical bone formation.

Association studies evaluated a $(CA)_n$ repeat in the promoter region of the gene but with controversial results. The presence of the 194-bp allele has been associated to higher BMD and increased level of circulating IGF-1 in osteoporotic Korean women with respect to healthy controls [108]. Low levels of IGF-1 and a reduced BMD were associated to the homozygote 192 bp allele in men with idiopatic osteoporosis [109] and in Caucasian postmenopausal women [110]. However, a study on Japanese postmenopausal women [111], a study on premenopausal Chinese women [112], and a study on premenopausal Caucasian and Afro-American sibling pairs [113] did not confirm the precedent results.

10. Interleukin 6 (*IL6*)

After menopause the increase of IL6 and other pro-inflammatory cytokines production related to the estrogen decrease has been associated to the extent of bone loss [114]. The *IL6* gene locus (*7p21*) has been associated with BMD variations in postmenopausal women [115]. Two polymorphisms (*−572G/C* and *−174G/C*) in the promoter region of the *IL6* gene have been associated to markers of bone resorption in postmenopausal women [116, 117]. A study of Ferrari et al. [118] evidenced that the G/G genotype of the *−174G/C* polymorphism was associated to a lower BMD with respect to the G/C and C/C genotypes in over 15-year postmenopausal women but not in premenopausal women and in men. These results seem demonstrate that *IL6* polymorphisms regulate bone mass only after menopause with a molecular mechanism dependent on the estrogen deficiency.

IL6 is a key mediator of inflammation and it has been demonstrated to play an important role in the pathogenesis of atherosclerosis and vascular diseases. Elevated concentrations of IL6 are predictive of future coronary events in healthy individuals and of mortality in patients with acute coronary disease. The *−174G/C* polymorphism in the promoter region has been associated with variation in IL6 production. Several studies have demonstrated that this polymorphism is associated with risk of coronary hearth disease in men [119–122], with the risk of ischemic carebrovascular events [123], with carotid artery compliance, systolic and diastolic blood pressure and serum high-density lipoprotein cholesterol in men [124], ischaemic stroke [125], number of severely stenosed coronary arteries [126]. However, despite of numerous studies the role of this polymorphism as a risk factor for CVDs remains inconsistent [127, 128] and needs for further researches.

11. Other Candidate Genes

Other osteoporosis candidate genes have been studied even if less extensively and with less conclusive results. They include calcitonin receptor (*CTR*) [129–133], calcium sensing receptor gene (*CaSR*) [134–136], androgen receptor (*AR*) [137], parathyroid hormone receptor (*PTHR1*) [138], sclerotin (*SOST*) [139], bone morphogenetic protein 2 (*BMP2*) [140], osteoprotegerin (*OPG*) [141, 142], and so forth. All these gene require confirmation on larger cohorts.

12. Genome-Wide Association Studies

The complete sequencing of human genome [143], the results of the HapMap project [144] and the development of novel chip technologies have opened novel avenues for the identification of genetic loci, genes and/or polymorphisms associated with complex diseases such as osteoporosis, through the application of genome-wide association analyses. This approach has permitted the simultaneous analysis of hundreds loci/genes along the human genome and the identification of novel osteoporosis subsceptibility loci

Table 3: QTLs identified and replicated in genome-wide linkage studies for osteoporosis [145]. Asterisks indicate genetic region associated also to clinical CVD events through genome-wide association studies [147].

QTLs replicated in at least two studies	QTLs replicated in at least three studies	QTLs replicated in at least five studies
- 2p21-24	- 13q31-34	- 1p36
- 2q33-37*	- 17p11-13	- 1q21-24
- 3q12-26		- 4q31-34
- 4p15-16		- 12q23-24*
- 5q33-35		
- 6p21		
- 8q24-qter		
- 10q26		
- 11q23-24		
- 14q12-24		
- 14q31-32		
- 16p13		
- 19p13-q13		
- 21q22-qter*		

and/or genes that were not candidates based on the current knowledge of the phatophysiology of bone metabolism and osteoporosis. Over 60 quantitative trait loci (QTLs) have been associated with bone metabolism and they were located in all but chromosome Y [145]. A list of genetic loci identified to be linked to bone metabolism through genome-wide approaches, and replicated in at least two different studies, are reported in Table 3. The first osteoporosis genome-wide association study [146] associated 100,000 SNPs with BMD values, bone ultrasound properties and hip geometry index. Some weak associations with genetic markers within or near known osteoporosis candidate genes (i.e., *ERα*, *CYP19*, *COLIA1,* and *LRP5*) were detected.

Particularly, the application of genome-wide association scan has allowed the identification of bone morphogenetic protein 2 (*BMP2*) as a candidate gene for osteoporosis, through the analysis of 207 osteoporotic families (1323 individuals) in Iceland and the subsequent followup association analysis [140]. Recently, also the latent transforming growth factor beta binding protein 2 (*LTBP2*) [148] and the signal transducer and the activator of transcription 1 (*STAT1*) [149] genes have been associated with osteoporotic phenotypes.

Given the extensive number of identified candidate loci to date, and given the potential large number of genes within these loci, the next step will be the refinement of the significant loci and the identification of putative candidate genes. Novel identified candidate genes have to be confirmed by follow-up population-based association studies and functional studies. Caution should be taken in the interpretation of replication/confirmation of the results since some genomic region could eventually be proven to be a false positive.

The past few years have seen a significant increase in the number of genetic loci associated with CVDs through genome-wide association studies. Significant results have been reported in a recent review [147].

13. Animal Models

Comparative genetics is helpful in the comprehension of molecular mechanisms of bone remodeling and in the searching for osteoporosis candidate genes. Studies in animals are essential because they allow breeding strategies that cannot be performed in humans and they also provided extreme bone strength phenotypes that cannot be measured in vivo in humans. Rodents and primates are the most suitable models. Linkage studies in rats [150], mice [151, 152] and primates [153] have permitted the identification of numerous QTLs that regulate BMD and other bone quality properties (shape, microstructure and strength). Particularly, linkage analysis in mouse has allowed the identification of *Alox15* gene as a negative regulator of BMD. The *Alox15*-knock-out mice presented an increased BMD and the inhibition of *Alox15* expression compensated the ovariectomy-induced bone loss [154]. Recent studies have shown that genetic variations in a human homologue of *Alox15* (*ALOX12*) accounted for approximately 3% of the bone mass variation in humans [155, 156]. Moreover, the using of knock-out and transgenic animal models for the study of bone monogenic diseases has helped in the identification of biologically relevant osteoporosis candidate genes for association studies. These approaches also helped in delivering biological function of a specific gene in bone metabolism. An example is the *LRP5* gene whose activating or inactivating mutations have been demonstrated to be responsible for opposite extreme bone phenotypes [10, 96–98], using specific *LRP5*-mutated animal models.

14. Future Perspectives: the Pharmacogenetics of Osteoporosis

A very novel area of the genetics of osteoporosis is the pharmacogenetics of osteoporosis. Pharmacogenetics is the application of genetic studies to predict the outcome of drug treatments with respect to both beneficial and adverse events, and it is particularly important in chronic diseases, such as osteoporosis, that require long-term drug treatments and for which alternative effective drug therapies are available. Potentially pharmacogenetics of osteoporosis will allow clinicians to choose in advance the best treatment and the most effective drug regimen based on patient genotype. However, to date only few studies on the pharmacogenetics of osteoporosis have been published and no clinical applications are available. Pharmacogenetic studies associated osteoporosis candidate genes (*VDR, ERα, ERβ*, and *COLIA1*) with the response to antiresorptive and antifracture agents such as hormone replacement therapy, raloxifene and bisphosphonates [157] and found weak associations. Recently a study by our Research Group [158] associated the *A/C rs2297480*

polymorphism of the *FDPS* gene with the response to aminobisphosphonates in a cohort of Danish osteoporotic women.

All these studies suggested the possibility to use genetic screening to tailor decisions about osteoporosis antifracture treatment choice; however, all these preliminary data need to be confirmed and validated by large-scale studies, by prospective well-designed clinical trials and by functional analyses.

Data from studies on pharmacogenetics of osteoporosis would be useful also in the field of CVDs since there are scientific evidences of the positive action of antiresorptive drugs, such as bisphosphonates and raloxifene, on the reduction of CVDs risk [22, 31–33].

15. Conclusions

The contribution of genetic factors to age-related chronic diseases such as osteoporosis and CVDs is important. The identification of genes that contribute to the pathogenesis of such disorders has potential public health, clinic and therapeutic implications. However, since osteoporosis is a complex multifactorial disease, the association studies performed to date presented with non-concluding or conflicting results. Now it is clear that a single SNP exerts less than 1–3% of effect in the determination of bone metabolism. Only large-scale standardized studies in well-characterized and homogenous populations and the study of haplotypes and/or multiple SNPs and/or genes might help in a better understanding of genetic factors underlying bone phenotypes heritability and calcium and lipid metabolism regulation. Moreover, technological advances such as genome-wide association scan will help in identifying novel candidate loci and/or genes, in validating the role of candidate gene polymorphisms and in analyzing hundreds of SNPs simultaneously.

However, genetic epidemiology association studies did not tell how genes contribute to the disease, thus, functional genomic studies, large-scale gene expression studies and proteomic studies are fundamental for the comprehension of molecular and cellular mechanisms regulating bone and cardiovascular pathophysiology. The aim of functional genetics is not only to collect information about single gene functions but also to understand how biological component work together to regulate bone metabolism and cardiovascular system functionality. The great challenge of genetics of osteoporosis and cardiovascular diseases will be not only identifying the responsible genes but also understanding how these genes act and how they are influenced by other biological and/or environmental factors.

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Research Article

Gene/Environment Interaction in Atherosclerosis: An Example of Clinical Medicine as Seen from the Evolutionary Perspective

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Evolutionary medicine is the application of evolution theory to understanding health and disease. It provides a complementary scientific approach to the present mechanistic explanations that dominate medical science, and particularly medical education. The chronic multifactorial disease of atherosclerosis clearly illustrates the Darwinian paradigm. Recent research, combining the effects of genes and environment, has provided surprising clues to the pathogenesis of this major public health problem. This example makes a strong case for recognizing evolution biology as a basic science for medicine.

1. Introduction

"Nothing in Biology Makes Sense Except in the Light of Evolution" is a 1973 essay by the great evolutionary biologist Dobzhansky [1]. He writes: "Seen in the light of evolution, biology is—perhaps—intellectually the most satisfying and inspiring science. But without this light, it becomes a pile of sundry facts, some of them interesting or curious but making no meaningful picture as a whole."

Evolution's role is also of paramount importance in the sub-discipline of biology that addresses health and disease in humans.

The co-evolution of man and pathogenic microorganisms, as well as man's persistent vulnerability to chronic diseases should all be viewed in the context of continuing evolution. These subjects form the core of "evolutionary medicine," also known as "Darwinian medicine" [2].

Evolutionary medicine is not a form of "alternative medicine." Actually, it is the opposite, complementing scientific evidence-based medicine by not only seeking the immediate causes of disease—the "how"—but also the longer-term reasons for the existence of these diseases—the "why."

While the practice of medicine by itself aims at counteracting natural selection, the following pledge for evolutionary medicine is not paradoxical. Indeed, it is by improving the knowledge on evolution biology of clinicians, learning them to see health and disease from an evolutionary perspective, that more effective treatment and preventive strategies can be developed.

Atherosclerosis is an example of the contribution of Darwinian insights into the aetiology of a major chronic disease. Recent research, combining the effects of genes and environment, has provided new clues to the cause of this leading cause of global morbidity and mortality.

2. Genetic Factors

2.1. Alleles of the Apo E Gene. Genetic factors have an unquestionable role in atherosclerosis [3].

The apo E gene [4] is located at chromosome 19q13.2. Among the variants of this gene, the alleles epsilon2, epsilon3 and epsilon4 constitute the common polymorphism found in most populations, the epsilon3 allele being the most frequent (>0.60) in all populations studied. The polymorphism however is not neutral but has functional effects on lipoprotein metabolism, mediated through the hepatic binding, uptake, and catabolism of chylomicrons, chylomicron remnants, very low density lipoproteins and high density lipoproteins. Epidemiological studies have demonstrated that the primary genetic risk factor predisposing to atherosclerosis is the epsilon4 allele of the apo E gene [5].

2.2. Population Genetics and Evolution. The apo E epsilon3 allele is the most frequent in all studied human populations, its frequency being highest (0.85–0.90) among the populations of the Mediterranean basin. Correspondingly, the apo E epsilon4 allele has the lowest frequencies (0.05–0.13) in Mediterranean populations, who were also the first to establish agricultural economy. On the other hand, the frequency of the epsilon4 allele remains higher in populations like Pygmies (0.41), Khoi San (0.37), aborigines of Malaysia (0.24) and Australia (0.26), Papuans (0.37), some Native Americans (0.28) and Lapps (0.31), where an economy of foraging still exists or food supply is (or was until the recent past) scarce and sporadically available [6].

From the evolutionary perspective, these observations lead to the conclusion that the epsilon4 allele must be the ancestral allele [7]. As an evolutionary relic from the pre-agricultural history of *Homo sapiens*, it is not adapted to a modern, highly nutrient-rich culture [8]. Through the process of natural selection, other, better adapted alleles are replacing the epsilon4 allele, generation after generation. Still, the allele has at present, 10 000 years after the discovery of agriculture, still a high frequency. The mechanism of natural selection eliminating the epsilon4 allele after the introduction of agriculture, is supported by the fact that in contemporary populations with a longer tradition of agriculture the allele is less frequent than in populations that have shifted more recently to agriculture, and even much less frequent than in "primitive" populations with a hunter-and-gatherer lifestyle today [9].

3. Environmental Factors

3.1. Multiple Factors. Aside from genetic factors, there are also environmental—other than dietary—factors to consider in atherosclerosis.

It is widely known that cigarette smoking constitutes a major environmental risk factor for atherosclerosis. Gene/environment interaction has also been demonstrated in this regard. Indeed, a synergistic effect between cigarette smoking and carrier state of the apo E epsilon4 allele increases the risk of atherosclerosis to a large extent [10].

3.2. Role of Chlamydia Pneumoniae. An environmental factor of recent interest in the pathogenesis of atherosclerosis is bacterial infection.

First, histopathological studies in humans have shown—contrasting with what was traditionally assumed—that triglycerides and cholesterol do not simply accumulate on the inner surface of the artery, but within the arterial wall itself. Within atherosclerotic lesions—somewhat surprisingly—bacteria have been demonstrated, especially *Chlamydia pneumoniae* [11]. This is an intracellular pathogen, known as a leading cause of human respiratory tract infections worldwide. In order to play a causative role in chronic disease, *C. pneumoniae* would need to persist within infected tissue for extended periods of time, thereby stimulating a chronic inflammatory response. *C. pneumoniae* has been shown to disseminate systemically from the lungs through infected peripheral blood mononuclear cells and to localize in arteries where it may infect endothelial cells, vascular smooth muscle cells, monocytes/macrophages and promote an inflammatory atherogenous process [12].

Also using an animal model, *C. pneumoniae* have been demonstrated in atherosclerotic lesions but not in normal arteries [13]. Apo E knockout mice, which spontaneously develop atherosclerosis, and C57BL/6J mice, which only develop atherosclerosis on an atherogenic diet, were evaluated. Following intranasal inoculations of apo E knockout mice, *C. pneumoniae* were detected in lung, aorta, and spleen for 20 weeks in 25 to 100% of mice. In the aorta, *C. pneumoniae* were detected within the atherosclerotic lesion. In C57BL/6J mice on a nonatherogenic diet, *C. pneumoniae* were detected in the aorta within 2 weeks after intranasal inoculation. The persistence of *C. pneumoniae* in atheromas suggests a tropism of *C. pneumoniae* to the lesion. These mouse models should be useful for studying the pathogenic role of *C. pneumoniae* in atherosclerosis.

The synergistic effect between the genetic and the environmental risk factors has also been elucidated [14]. The gene product of the epsilon4 allele of the apo E gene functions as a receptor protein permitting *C. pneumoniae* to penetrate the endothelial cells of the arterial wall. Thus, all pieces of the puzzle come together and we get close to proof that *C. pneumoniae* is causally involved in atherosclerosis [15].

3.3. Resolving the "Passive Smoker's Paradox". When a respiratory infection is viewed as the primary cause of atherosclerosis, the paradox of "passive smoking" [16] is resolved. Traditionally, the risk of atherosclerosis by passive smoking is attributed to the direct effect of toxic compounds in the smoke. Still, the link between second-hand exposure to smoke and the increased atherosclerosis risk appears to be quantitatively problematic. Compared with a nonsmoker, a heavy smoker increases his risk by 100%. Living with a smoker increases the risk by 30%, although the exposure to smoke is 100 times smaller in the passive smoker [17]. Thus, the relative effect of passive smoking seems out of proportion to the small amount of smoke inhaled. This paradox goes up in smoke if the primary cause of atherosclerosis is infection, spread by airway transmission. Smoking indeed increases the susceptibility to respiratory infection and living together with a smoker increases the risk of acquiring the same infection.

The insight that a frequent and potentially mortal disease could be caused by a bacterium opens new doors for treatment and prevention.

4. Synthesis and Conclusion

The consideration of apo E alleles and smoking provides two illustrations of a comprehensive approach to the causation of atherosclerosis. Instead of accumulating risk factors, this approach seeks a unified theory of causation. Infection as a primary cause of atherosclerosis provides a conceptually

cohesive framework for understanding the role of noninfectious environmental risk factors, such as smoking and diet.

The example of atherosclerosis witnesses the progress made at the intersection of medicine and evolution, of both humans and pathogenic microorganisms. The involvement of *C. pneumoniae* in atherosclerosis has been shown by seroepidemiological and pathological studies, in vivo and in vitro studies and in clinical antibiotic treatment trials. This factor can be identified, modified and may be a therapeutic target.

Prospective interventionist studies on humans to prove the relation between genetic evolution and an environmental risk factor are difficult because of ethical and—above all—practical reasons (long generation time, limited number of offspring). Therefore, continued basic research on apo E knockout mice as well as C57BL/6J mice is needed for the future [18].

The key questions in evolutionary medicine are centred around which aspects of modern environment and lifestyle are pathogenic. That is what we ultimately need to know and it should be clear to all: Medicine Needs Evolution [19].

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