Primary Prevention of Atherosclerosis
A Clinical Challenge for the Reversal of Epigenetic Mechanisms?

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Innovative advances in understanding the pathogenesis of atherosclerosis have been achieved over the past 25 years. Although elevated levels of serum low-density lipoprotein cholesterol (LDL-C) are the major cause of onset of the disease, as established by a large number of superb epidemiological, clinical, and experimental studies, important novel factors have entered the arena of the atherogenic process. Besides the historical oxidative hypothesis that states that oxidized LDL, by escaping the homeostatic mechanism, strongly accelerates plaque formation, more recent evidence has given credit to vascular inflammation and apoptosis as crucial players in the progression of atherosclerosis. The disease has also been linked to the subintimal infiltration of immune cells and endothelial dysfunction induced by cardiovascular risk factors. Currently, endothelial dysfunction is considered one of the first stages of vascular damage and an early event in atherogenesis. Etiologic and pathogenetic factors, of both genetic and environmental origin, act together to promote local and systemic effects that lead to the onset, progression, and final outcome of the atherosclerotic disease. The clinical sequelae of atherosclerosis, myocardial infarction, stroke, and peripheral arterial disease depend on the affected vascular district, which in turn depends on complex gene-environment interplay.

Despite the sudden occurrence of clinical symptoms, however, the evolution of atherosclerosis is very slow, which provides an opportunity for early diagnosis. In fact, a breakthrough in the field has been to recognize that although atherosclerosis generates severe diseases that most frequently affect middle-aged to old people, atherosclerosis begins very early in life, even at the fetal stage. Primary prevention of any disease is more effective if started sooner. Therefore, it is of paramount importance to identify high-risk individuals and to initiate primary prevention in a timely manner, especially for atherosclerosis, which can begin its slow but relentless damage of the arterial wall even before birth. Epigenetic mechanisms have been recognized recently as possible modifiers of the risk of developing premature atherosclerosis. Therefore, the recognition of epigenetic markers that reveal a tendency to vascular diseases could be an effective aid in the early detection of at-risk individuals. These people can then be targeted for both lifestyle changes and pharmacological treatments to prevent or delay atherosclerotic disease. Although the pharmaceutical industry has provided invaluable preventive and therapeutic tools, general rules of best practice dictate that life habits be modified first, and only if intermediate markers of the disease do not normalize should at-risk individuals be given drug therapy. This approach also allows reducing the health care expenditure on the primary prevention of atherosclerosis-related disease.

Early Onset of Human Atherogenesis
The prodromal stages of human atherosclerotic lesions are already set during fetal development. A seminal observation was that maternal hypercholesterolemia is associated with increased formation of fatty streaks in fetal arteries, which suggests that hypercholesterolemia is atherogenic even before birth. Fetal lesions occur in the same arterial districts as those of adolescents and adults and are histologically similar to lesions that occur later in life. Moreover, there is evidence that fetal lesions can partially regress during the final stages of pregnancy or early infancy, when cholesterol levels are low. Atherosclerosis is significantly accelerated in children of mothers with high serum cholesterol compared with children of mothers with normal serum cholesterol. The mechanism by which maternal hypercholesterolemia can promote development of lesions in offspring has been explored in animal models. Indeed, lesions doubled in a litter of hypercholesterolemic mother rabbits compared with normcholesterolemic mother rabbits, and there was a linear correlation between maternal cholesterol and vascular injuries at birth. Consistently, a similar correlation was observed in LDL receptor–deficient mice. Maternal chole-
terol levels increase physiologically from the first trimester and throughout the pregnancy, even in mothers with normal serum cholesterol levels; this increase is much greater in mothers who are already hypercholesterolemic before pregnancy. Microarray analysis of aortas has shown that many genes are elevated or inhibited in the offspring of hypercholesterolemic mothers.

Children and young adults are also vulnerable to the effects of cardiovascular risk factors and show early signs of atherosclerosis, which becomes a complex process driven by conventional risk factors. Among cardiovascular risk factors, body mass index, systolic and diastolic blood pressure, serum total cholesterol, triglycerides, LDL-C, and high-density lipoprotein cholesterol are strongly associated with the extension of lesions in the aorta and coronary arteries. In addition, the severity of aortic and coronary artery disease in young people increases in proportion to the number of cardiovascular risk factors they face.

### Epigenetic Mechanisms

Epigenetics deals with the mitotically and/or meiotically heritable variations of gene function that cannot be explained by changes of DNA sequence. The major consequence of epigenetic modifications is related to the packaging, and therefore the function, of the genetic material. Epigenetic modulation of gene expression is a central mechanism in development and differentiation of higher eukaryotes. The inherited cellular epigenetic setup directs the development of >250 cell types in humans; indeed, although the sequence of the DNA of the different cell types is identical within a single individual, the epigenetic modifications are quite different in the genomes of the different cells.

The most frequent epigenetic modifications in mammals include DNA methylation and histone modifications, which result in changes in chromatin structure (Figure), and RNA-based alterations that involve microRNAs (miRNAs) and long intergenic noncoding RNAs (lincRNAs). Epigenetic mechanisms alter the accessibility of chromatin (a protein-DNA complex that consists of DNA, histones, and nonhistone proteins) by modifying DNA and nucleosomes, including posttranslational modifications of histones after interaction with environmental factors. Altered chromatin accessibility implies an increased or decreased possibility of interaction between gene regulatory regions and the transcription machinery, thus leading to variations of gene expression (Figure). In epigenetic processes, DNA methylation is generally associated with lower gene activity and is localized at the C5 position of cytosine residues in CpG dinucleotides as a result of the action of DNA methyltransferases, which are capable of both methylation and demethylation, thus rendering the modification reversible. In vertebrates, CpG dinucleotides are concentrated in short interspersed DNA sequences that are called CpG islands. CpG islands are more often nonmethylated and favor a transcriptionally permissive chromatin state; however, they can undergo intense CpG methylation, thus silencing expression of surrounding genes. Moreover, posttranslational modifications of the N-terminal tails of histone proteins are pivotal events during the epigenetic regulation of genes.

To date, many modifications have been identified, including acetylation and methylation of lysine residues. Acetylation/deacetylation of lysines is correlated with chromatin accessibility and gene activation, whereas the role of histone methylation depends on the precise methylated residue and the number of added methyl groups. In addition, trimethylation of histones H3K9 and H3K27 might occur in inactive genes, whereas trimethylation of histones H3K4 and acetylation of H3/H4 are connected to active transcription. This chromatin plasticity is essential to maintain DNA in an open, permissive state. Thus, high levels of acetylation, together with trimethylation of H3K4, H3K36, and H3K79, have been found in transcribed genes; conversely, low levels of such modifications have been associated with inactive genes. Modifications of histones and DNA methylation are functionally linked activities. Throughout semiconservative DNA replication, the methylation of the daughter strand and recruitment of histone-modifying proteins retain the epigenome configuration in the next cell generation. Epigenetic modifications are naturally reversible, mainly because of the counterbalancing actions of the enzymes taking part in the maintenance of epigenome. The action of these enzymes restores a repressive or active chromatin state depending on specific sites. Therefore, these epigenetic events link mechanisms for genetic information integrity and epigenetic reprogramming. Other epigenetic mechanisms may involve acetyltransferases/deacetylases and methyltransferases/demethylases that also target nonhistone proteins, such as nuclear factor-κB. Further histone modifications other than lysine methylation and acetylation are known, such as the specific methylation and acetylation of arginine residues and the sumoylation and ubiquitination of histones; however, the function of these modifications remains unclear.

### Epigenetics-Environment Interaction

A major concept pertaining to epigenetics is its sensitivity to environmental stimuli. Interestingly, many of the environmental factors that are known to influence cardiovascular risk have been shown to also be associated with epigenetic modifications. Among others, nutrition can markedly affect epigenetic status, and this influence can also occur before birth and hence refer to the conditioning of the fetus by maternal nutrition. Important information came from studies on DNA methylation conducted in individuals exposed in utero to the Dutch Hunger Winter (Dutch famine of 1944–1945). Those people, in fact, were found to have significantly different methylation of several genes involved in metabolic regulation compared with control subjects. Most interestingly, people with the same gestational exposure were at increased sex-specific risk of hyperlipidemia, obesity, and mortality from myocardial infarction and ischemic stroke during adult life compared with control subjects who were not exposed prenatally to famine. Consistently, low birth weight was associated with increased incidence of coronary artery disease later in life.

In addition, experimental data support the thesis that maternal diet can profoundly affect the epigenetic status of the fetal genome and that maternally induced epigenetic modifications are maintained throughout adulthood.
In some cases, altered histone methylation and expression of lysine methyltransferase were observed in atherosclerotic tissues, thus suggesting a direct involvement of chromatin structure in the atherogenic process. Additional data demonstrated that prenatal protein restriction was associated with altered DNA methylation of genes involved in lipid metabolism. In addition, zinc deficiency has been associated with cardiovascular disease (CVD), and dietary zinc restriction in rats during fetal life, lactation, and the postweaning stage causes hypertension and renal impairment in adult life.

Similar to maternal diet, maternal tobacco smoking induces epigenetic changes in the fetal genome. Several loci were hypomethylated in children exposed to maternal smoking during gestation compared with controls, and the same children developed increased risk of diseases later in life.

Finally, environmentally induced epigenetic modifications do not take place exclusively during prenatal life but can occur at any age, including during adult life. For example, 2 conditions that introduce contaminated air into the lungs, cigarette smoking and exposure to pollution from traffic, have been associated with modification of DNA methylation in exposed individuals.

**Epigenetics and Atherosclerosis**

In light of the accumulating evidence, epigenetic mechanisms have been recognized as important mediators of the atherogenic process at its earliest stages and of consequent CVD. Alterations of epigenetic programming are not limited to CVD but have been implicated in several common human diseases and conditions, such as cancer, diabetes, neurological disorders, imprinting disorders, autoimmune diseases, and aging. Fetal exposure to maternal hypercholesterolemia has been associated with increased risk and progression of atherosclerosis both in humans and in experimental models, and...
hypomethylation of DNA is a landmark of advanced atherosclerotic lesions in humans and laboratory animals. Moreover, it has been shown that global hypomethylation of DNA extracted from human aortic lesions is caused in part by the near-complete demethylation of the subset of CpG islands that are hypermethylated in control aortas. In contrast, globally hypermethylated DNA from peripheral blood leukocytes was associated with the prevalence of CVD as well as specific hypermethylation, and decreased expression of the tissue factor pathway inhibitor 2 gene was detected in atherosclerotic plaques.

In addition to methylation of large genomic regions, more specific epigenetic modifications can provide an essential contribution to the development of atherosclerosis. A sound hypothesis states that hypomethylation of atherosclerosis-susceptibility genes and hypermethylation of atherosclerosis-resistance genes cause overexpression and underexpression of these genes, respectively, thus favoring plaque formation.

Among others, the DNA methylation of antiproliferative genes, such as that coding for estrogen receptor-\(\alpha\), was demonstrated in human atherosclerotic plaques, which suggests that this mechanism could be responsible for the proliferative events of atherogenesis, especially with reference to smooth muscle cells. As is always the case, a simple association between 2 events does not distinguish the cause from the consequence. For example, a CpG island of extracellular superoxide dismutase, which is overexpressed in experimental atherosclerosis, was reported to be hypomethylated in atherosclerotic lesions of rabbits. In contrast with the previous example, this epigenetic modification could be the nuclear response strategy of the diseased tissue to fight the progression of atherosclerosis.

Epigenetics also plays an important role in controlling inflammatory processes that are activated within the vascular wall by atherogenic stimuli and, in a vicious circle, aggravate the atherosclerotic degeneration of the involved arteries. Epigenetic mechanisms can also affect CVD by influencing the expression of atherosclerosis-related genes via modulation of transcription factors. These proteins can be divided into 4 classes (I through IV) classified by structural elements that mediate their DNA binding activity but also determine the classes of drugs that can affect their activity. It is known that statins modulate the activation of sterol responsive element-binding protein, a class I transcription factor whose target genes are involved in cholesterol and fatty acid metabolism. Similarly, insulin-like drugs target the nuclear receptor peroxisome proliferator-activated receptor-\(\gamma\) (PPAR-\(\gamma\), a class II transcription factor), and several anti-inflammatory drugs inhibit the activation of nuclear factor-\(\kappa\)B (a class IV transcription factor), whereas others (eg, flavopiridol, rapamycin, and paclitaxel) regulate cell-cycle proteins. In addition, the Mediator complex (comprising of \(\approx 30\) subunits), which regulates transcription by RNA polymerase II, has been suggested to play a role in epigenetic mechanisms because it is involved in gene expression and chromatin architecture.

Alterations of lipid metabolism have been associated with aortic methylation patterns in hyperlipidemic mice, whereas it has been shown recently that the triglyceride-rich very low-density lipoprotein (VLDL) causes de novo DNA methylation in human cultured macrophages. A possible interface between lipid molecules, epigenetic modifications, and atherosclerosis is provided by PPARs (including the previously mentioned PPAR-\(\gamma\)), which are nuclear receptors that control lipid and glucose metabolism, as well as adipogenesis and some inflammatory pathways. In fact, PPARs bind lipid molecules and regulate transcription of target genes by recruiting coactivators and corepressors, typically histone acetyltransferases or histone deacetylases, respectively. Furthermore, PPAR activity is sensitive to resveratrol, a dietary polyphenol with antioxidant and atheroprotective potentials that also activates sirtuins, a class of \(\text{NAD}^+\)-dependent deacetylases. Therefore, PPARs, and PPAR-\(\gamma\) in particular, are good candidates to be at the interface between nutritional stimuli and epigenetic modifications.

Not only is there a vast knowledge base about specific genes involved in CVD, but more information is available concerning chromosomal loci that could be relevant for the onset and evolution of this disease. Recent genome-wide association studies have identified multiple loci that are associated with atherosclerotic phenotypes. Among these, the chromosomal 9p21 locus shows one of the strongest associations with CVD. The locus encompasses multiple single-nucleotide polymorphisms that are in strong linkage disequilibrium, forming a risky haplotype that is associated with a 20% to 30% increase in coronary artery disease risk. Variants in the chromosomal 9p21 region are associated with various pathological events, including stroke, abdominal aortic aneurysm, peripheral vascular disease, diabetes, and dementia, as well as with nonatherosclerotic phenotypes such as intracranial aneurysm, endometriosis, and cancers. The association of 9p21 variants with atherosclerosis has been confirmed in multiple white cohorts and populations of different ethnicities; however, the mechanisms through which the 9p21 locus influences atherosclerosis susceptibility remain incompletely understood. The identification of entire genomic regions involved in CVD is particularly relevant to the study of epigenetic modification and CVD itself.

More recently, it has been shown that noncoding RNAs influence epigenetic changes of DNA methylation and histone code in CVD. Many microRNAs have already been identified, and some of them are also involved in vascular inflammation and atherosclerosis. Of note, miR-33, an intronic microRNA, has been found to modulate the expression of genes involved in cellular cholesterol transport. The epigenetic mechanisms involved in CVD are described in Table 1.

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### Table 1: Epigenetic and Therapeutic Insights

The relatively reversible nature of epigenetic alterations has inspired the development of therapeutic strategies targeting various epigenetic components. Epigenetics-based therapies are being developed and represent a promising strategy for many diseases and areas of medicine. In regenerative medicine, it is possible to generate pluripotent cells from human somatic cells by modifying epigenetic profiles; however, there are several preliminary issues that need to be addressed before epigenetic therapies become a clinical routine. First, in

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and increased high-density lipoprotein levels in healthy volunteers.\textsuperscript{16} In a rat model of heart failure, consumption of curcumin inhibited p300 histone acetyltransferase activity, prevented ventricular hypertrophy, and preserved systolic function.\textsuperscript{76} Additionally, after interleukin-1\textbeta exposure, the transcription of early growth response factor-1 in human vascular smooth muscle cells was stimulated via the acetylation of histone H3 and prevented by garcinol, thus reflecting the efficacy of histone acetyltransferase inhibition during thrombus formation.\textsuperscript{77–79} Interestingly, the pleiotropic effects of statins may be linked to epigenetic mechanisms, as indicated by several studies investigating the treatment of CVD and stroke.\textsuperscript{80} In spite of the evidence supporting a potential therapeutic exploitation of epigenetic modulation, there is still a long way to go before epigenetically active compounds can be used clinically.\textsuperscript{16}

### Early Detection of CVD for Primary Prevention: A Role for Epigenetics

The main goal of primary prevention is to prevent the first episode of coronary heart disease or stroke. Most studies have described the association of traditional risk factors such as hypercholesterolemia, hypertension, male sex, family history, diabetes, and smoking with cardiovascular events. Subjects with a healthy lifestyle were reported to have an 84\% reduction in cardiovascular risk. This advantage was obtained by following simple guidelines such as diet, exercise, smoking cessation, and reduced alcohol consumption, which indicates that these lifestyles are associated with successful primary prevention of CVD.\textsuperscript{81} However, in many cases, primary prevention must be pursued by pharmacological intervention in addition to the adoption of healthy habits. Some of the most common drugs for primary prevention, such as statins, exert a beneficial effect on cholesterol levels and may have pleiotropic effects on the cardiovascular system.\textsuperscript{80,81} One of the most effective therapies for primary prevention is aspirin, but its effect is restricted to myocardial infarction and is very small for the prevention of ischemic stroke.\textsuperscript{82}

As a general rule, the earlier the intervention for primary prevention starts, the better it is in terms of risk reduction. For example, it is crucial to start a primary prevention plan for the increasing number of obese adolescents with or without diabetes as early as during the second or third decade of life. However, the real challenge is to identify at-risk individuals who do not present evident risk factors. By translating established research findings into clinical practice, it should be possible to use novel markers of cardiovascular risk that can allow an earlier prevention strategy. For example, endothelial dysfunction is a very early event in the atherogenic process that could alert a healthcare provider to the initial steps of plaque formation. Evaluation of the functional capacity of the endothelium includes measurement of the release of nitric oxide or peripheral markers of endothelial cell activation associated with inflammation and progressive functional failure, such as C-reactive protein, intercellular adhesion molecule-1, and interleukins.\textsuperscript{82}

One of the first structural changes generated by atherosclerosis is intimal thickening of the carotid artery wall. The

<p>| Table 1. Epigenetic-Regulated Genes Involved in Cardiovascular Disease |
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<table>
<thead>
<tr>
<th>Gene</th>
<th>Epigenetic Mechanism</th>
<th>Risk Factor for CVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>DNA methylation and histone modification</td>
<td>Persistent pulmonary hypertension of the newborn</td>
</tr>
<tr>
<td>Fads2</td>
<td>DNA methylation</td>
<td>Hyperhomocysteinemia</td>
</tr>
<tr>
<td>ER\textalpha</td>
<td>DNA methylation</td>
<td>Aging and atherosclerosis</td>
</tr>
<tr>
<td>ER\textbeta</td>
<td>DNA methylation</td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td>P66Shc</td>
<td>DNA methylation</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>EC-SOD</td>
<td>DNA methylation</td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td>H19/lgf2</td>
<td>DNA methylation</td>
<td>Hyperhomocysteinemia</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>DNA methylation</td>
<td>Cardiac dysfunction</td>
</tr>
<tr>
<td>SREBF-2</td>
<td>miRNA</td>
<td>Cholesterol transport</td>
</tr>
<tr>
<td>ApoE</td>
<td>DNA methylation and histone modification</td>
<td>Hypercholesterolemia and atherosclerosis</td>
</tr>
<tr>
<td>LDLR</td>
<td>DNA methylation and histone modification</td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td>LXR</td>
<td>DNA methylation</td>
<td>Prenatal protein restriction/birth weight</td>
</tr>
<tr>
<td>PPARs</td>
<td>DNA methylation</td>
<td>Metabolic syndrome</td>
</tr>
<tr>
<td>GR</td>
<td>DNA methylation and histone modification</td>
<td>Protein-restricted diet during pregnancy</td>
</tr>
</tbody>
</table>

\textsuperscript{*}eNOS indicates endothelial nitric oxide synthase; Fads2, fatty acid desaturase 2; ER, estrogen receptor; EC-SOD, superoxide dismutase 3, extracellular; Igf2, insulin-like growth factor 2; TIMP-3, tissue inhibitor of metalloproteinase 3; SREBF-2, sterol regulatory element binding factor 2; ApoE, apolipoprotein E; LDLR, low-density lipoprotein receptor; LXR, liver X receptor; PPARs, peroxisome proliferator-activated receptors; and GR, glutathione reductase. Modified from Napoli et al.\textsuperscript{18}
development of ultrasound technology has allowed monitoring of these structural changes at a very initial stage.\textsuperscript{83–85} Therefore, the use of ultrasound and other techniques (magnetic resonance imaging, multidetector computed tomography, and molecular imaging) may help identify a wide range of high-risk patients.\textsuperscript{85}

The epigenetic bases of CVD could provide novel and early markers of cardiovascular risk. The detection of epigenetic modifications that are known to increase the tendency to develop atherosclerosis could allow the identification of people prone to CVD even before the manifestation of classic risk factors. This possibility will be strongly favored by large-scale initiatives such as the International Human Epigenome Consortium, aimed at mapping 1000 reference epigenomes within a decade.\textsuperscript{86} Similarly, the National Institutes of Health’s Roadmap Epigenomics Mapping Consortium is ongoing. Such substantial coverage of the human epigenome will be instrumental in identifying the epigenetic variations that are specifically associated with major pathological states, including CVD. Of relevance, it is possible to list several conditions that result in specific epigenetic profiles that will identify high-risk individuals at a very early stage, thus allowing timely and efficacious primary prevention.

As mentioned previously, fetal calorie deprivation has been associated with epigenetic modifications and with intrauterine growth restriction.\textsuperscript{87} Children and adults who were exposed to intrauterine growth restriction display a lower DNA methylation of the imprinted insulin-like growth factor II (IGF2) gene and are predisposed to metabolic disturbances, which in turn leads to overweight, metabolic syndrome, type 2 diabetes mellitus, and CVD.\textsuperscript{88} With regard to prenatal exposure to tobacco, it can be more complex to track the epigenetic signs of an acquired increased risk for CVD later in life so that a primary prevention program can be started. In fact, smoking-related methylation of long interspersed element-1 is dependent on the activity of glutathione S-transferase (GSTP1), a detoxifying enzyme, once again providing a molecular mechanism for the epigenetic mediation (methylation of long interspersed element-1) of a gene (GSTP1)-environment (smoking) interaction.\textsuperscript{40} In addition, prenatal exposure to cocaine or to hypoxia causes methylation of the activator protein-1 binding site of the protein kinase C-\(\varepsilon\) gene (PKC-\(\varepsilon\)); this, in turn, leads to decreased expression of the kinase, which has a cardioprotective function after cardiac ischemia–reperfusion injury.\textsuperscript{89,90} However, this evidence was obtained in rats; the same issues must be studied in humans before the epigenetic modifications of PKC-\(\varepsilon\) can be used to identify people eligible for primary prevention measures.

The possibility of recognizing an individual predisposition to develop hypertension by means of epigenetic traits is another thing that connects the epigenome and the primary prevention of CVD. The renin-angiotensin-aldosterone system is a major pathway in the control of blood pressure in mammals. Hydroxysteroid dehydrogenase-11B2 (HSD11B2) is a crucial enzyme that degrades cortisol to cortisone, thus preventing the stimulation of mineralocorticoid receptors.\textsuperscript{91} Elevated methylation of the HSD11B2 promoter is associated with hypertension in glucocorticoid-treated patients, and individuals with essential hypertension are reported to have increased methylation of the HSD11B2 promoter.\textsuperscript{92} It has been shown that 3 days of water deprivation in pregnant rats caused an increase in angiotensin II levels in fetal liver and increased blood pressure after angiotensin II administration in adolescent offspring.\textsuperscript{93} Although specific epigenetic mechanisms for maternal water deprivation are not yet known, it is likely that the described maternal in utero influence on the offspring is caused by chromatin epigenetic remodeling. Interestingly, hypomethylation of the proximal promoter of the angiotensin 1b receptor (AT1bR) in the murine adrenal gland is associated with increased expression of the receptor that causes hypertension in response to salt.\textsuperscript{94} Thus, salt-sensitive hypertension could have an epigenetic origin. Taking into account the high prevalence of hypertension, the possibility of identifying hypertension-prone individuals to whom primary prevention measures could be applied is extremely desirable.

The future challenge for improving the primary prevention of CVD will be to characterize epigenetic signatures for conditions that predispose to CVD and that are likely to already be present at the fetal stage or early in postnatal life, and then to link those markers with modified gene expression and disease incidence. Once these points have been addressed in future studies, primary prevention can be begun in humans with epigenetic marks of cardiovascular risk as early as during prenatal life or during childhood. Most importantly, it will also be possible to monitor the efficacy of the adopted primary prevention measures to revert the altered epigenetic configuration toward a physiological one. This restored “healthy” epigenetic status should, in turn, reduce the incidence of diseases, which would indicate a significant risk reduction and the success of primary prevention.

**Clinical and Therapeutic Implications in Primary Prevention**

Primary prevention of diseases is a major goal of modern medicine, and this is true not only for CVD but also for other noncommunicable diseases, such as cancer and neurodegenerative diseases, and for communicable diseases, such as infections. The first breakthrough in CVD prevention dates back to \(\approx60\) years ago and is credited to Ancel Keys, who was the first to understand the relationship among dietary fats, serum cholesterol levels, and CVD.\textsuperscript{94,95} The results of the world-famous Seven Countries Study confirmed his intuition, and this dogma has held true in the third millennium.\textsuperscript{96} Following Keys’ legacy, the public cholesterol-lowering campaign of the last 30 years in the United States has significantly reduced mortality from CVD. This decline was related to both primary prevention (79%) and secondary prevention (21%); cholesterol lowering accounted for a 42.7% reduction in the death rate in asymptomatic people and 34% among CVD patients.\textsuperscript{97}

Of almost equal importance as the Seven Countries Study, the Framingham Heart Study, from the US National Heart, Lung, and Blood Institute, represents the other milestone of twentieth century cardiovascular epidemiology.\textsuperscript{98,99} In this prospective study, clear-cut evidence demonstrated that hyperlipidemia, hypertension, obesity, diabetes, and smoking...
were the leading determinants of atherosclerosis and consequent major forms of CVD, including primarily myocardial infarction and ischemic stroke. Most important, disorders of lipid and glucose metabolism, elevated blood pressure, and increased body mass were recognized as having a prevalent nutritional origin, thus laying the basis for a public health strategy to prevent CVD. Undoubtedly, the remarkable scientific achievements of the second half of the last century in defining the major cofactors that cause CVD form the essence of the primary prevention measures that are still the most reliable at the present time. Indeed, lifestyle measures, including a low-fat, low-salt, calorie-controlled diet, poor in simple refined sugars and rich in fibers and that substitutes animal fat with vegetable fat (always within the limits of recommended fat allowances), avoidance of alcoholic beverages and tobacco smoking, and physical activity are fundamental for the primary prevention of CVD. 

In addition, factors that are traditionally recognized to cause CVD, such as elevated LDL-C, thrombogenic mechanisms, and hypertension, provide the most rational explanation for the efficacy of statins, aspirin, and antihypertensive drugs in the primary prevention of CVD. Yet the use of aspirin is seriously hindered, because aspirin reduces the risk for myocardial infarction in men and strokes in women but increases the risk for serious bleeding events. The protective effect of statins for primary prevention of CVD has also been confirmed in women. The most recent major clinical trials on the primary prevention of CVD that had major cardiovascular events as the primary outcome are shown in Table 2. Earlier clinical trials on the primary prevention of CVD are reviewed elsewhere. A fraction of people acquire CVD without having traditional risk factors. This puzzling observation has prompted scientists to look for other causes or mechanisms for the atherogenic process, yet it is quite difficult to find convincing evidence in the scientific literature of novel etiologic and pathogenic factors of CVD. Inflammation is, perhaps, the most celebrated factor that has been associated with atherosclerosis in the last 20 years. However, although the finest scientists have defined atherosclerosis as an inflammatory disease, it is conceivable that inflammatory mechanisms, including the recently identified inflammasomes, are more a pathogenic response of the vascular wall to irritating noxae, such as cholesterol crystals deposition within the subintimal space. This interpretation could explain at least a fraction of those cases of CVD in the absence of marked alteration of the traditional risk factors; possibly, those individuals could react with an abnormally powerful inflammatory response to borderline levels of atherogenic factors. Therefore, because of the role of inflammation in CVD, anti-inflammatory drugs could offer an early intervention strategy to prevent or blunt atherosclerosis consequences even without eliminating any of the causative cardiovascular risk factors. Indeed, an association between primary prevention of CVD and drug treatment has been found with a statin, rosuvastatin, that caused a marked decrease in C-reactive protein but also lowered plasma LDL-C. No similar data exist for exclusively anti-inflammatory medications that do not also modify major causative factors for CVD, such as LDL-C or thrombogenic mechanisms.

If inflammation is an important pathogenic factor in atherogenesis that can be triggered by modified LDL and causes infiltration of the plaque with activated macrophages and T lymphocytes, then it is tempting to speculate that any other inflammatory stimuli could be atherogenic. According to this view and to some evidence in the literature, antigens derived from intracellular pathogens such as cytomegalovirus and Chlamydia pneumoniae have been proposed as promoters of atherosclerosis via a proinflammatory action. However, although some authors put specific infective agents and...
elevated plasma levels of LDL-C on the same level in terms of risk factors for atherosclerotic disease, it is difficult to accept this idea if you compare the solidity and amount of evidence related to LDL-C with the much less and sometimes controversial data supporting the causative role of microbes in atherosclerosis. Furthermore, atherosclerosis can develop in germ-free animals, which suggests that the inflammatory component of the degenerative process is a response to endogenous substances and not to microbes.\textsuperscript{116} Also, it is useful to consider that in China, where various infectious diseases are endemic, atherosclerosis was almost unknown until a few years ago, when the outbreak of obesity and its metabolic sequelae took place. This fact clearly does not associate pathological modification of the arterial wall with infections but rather with the classic risk factors for CVD that are linked to obesity: Dyslipidemia, diabetes, and hypertension. Regardless of this, if \textit{C pneumoniae} were an atherogenic agent, then a specific anti-\textit{Chlamydia} treatment should reduce the incidence of CVD-related events and could therefore provide a new means of primary or secondary prevention of CVD. The analogous thesis has been proved for plasma cholesterol. Unfortunately, several large, prospective intervention clinical trials did not ascertain any significant risk reduction for cardiovascular events after treatment with specific antibiotics.\textsuperscript{117–119} Consequently, it is unlikely that pathogens serve as etiologic agents for atherosclerosis.

Prevention, either primary or secondary, even more than a cure, requires actions based on solid proof, even during fetal development.\textsuperscript{5,7–9,16,110} Advances in the epigenetics of CVD could allow better identification not only of at-risk individuals but also of individuals who are responsive to specific lifestyle changes and long-term drug treatments during life.\textsuperscript{16,120,121}

Future Directions and Conclusions

According to the scientific evidence, the primary prevention of CVD is solidly anchored to the classic risk factors for CVD, established in the last half century: Elevated LDL-C, low high-density lipoprotein cholesterol, diabetes, hypertension, obesity, cigarette smoking, male sex, and positive family history, which indicates a general genetic predisposition to atherosclerosis-based diseases. This strong paradigm directs both the identification of apparently healthy high-risk individuals and their (possibly in utero) preventive treatment.\textsuperscript{5,7–9,16,110} Preventive treatment is pursued by lifestyle interventions first and then by the addition of pharmacological treatment for those people whose risk profiles are not normalized after they modify their daily habits. In some patients with mild hyperlipidemia, hypertension, hyperglycemia, overweight, and/or addiction to tobacco consumption, quitting smoking and/or adopting a healthy diet that limits the intake of calories, saturated fat, and salt and includes large amounts of fruit, vegetables, and fibers, in association with physical exercise, can significantly ameliorate metabolic disturbances, reduce blood pressure, and reduce body weight without wasting the muscular mass. This approach has been demonstrated to significantly reduce the morbidity and mortality related to CVD. However, if LDL-C, blood pressure, and glycemia are not normalized, there is no doubt that it is necessary to start specific drug treatments.

Can we envision improvements to this strategy? As stated previously, atherosclerosis can begin at the earliest stages of fetal life and progress silently for many years, until unexpected severe clinical consequences occur in the absence of a clearly altered risk profile. Furthermore, primary prevention is only effective if it is performed early and intensively.\textsuperscript{115} To this end, epigenetics poses a novel challenge: The possibility of characterizing the physiological and atherogenic pattern of epigenetic modifications and consequent chromatin structure that could allow much earlier prediction of individual cardiovascular risk. Once the pathological epigenetic setting is defined, it will be possible to develop innovative epigenetic therapies that will be intimately intertwined with lifestyles by either potentiating or counterbalancing their effect. Conceivably, the individual safety level of exposure to some established risk factors could vary if the epigenetic outcome is different within different people, and even more fascinating, this outcome could be therapeutically modulated. Very interesting and recent in vivo data demonstrated the feasibility of specific targeting of genetically engineered DNA methyltransferases to preferentially methylate a predetermined group of genes.\textsuperscript{120} Nevertheless, although epigenetics-based therapies might remain in the pipeline for a longer time, perhaps an epigenetics-based risk profile for CVD might be close at hand.

In conclusion, in the near future, epigenetics could provide novel specific markers of cardiovascular risk that could improve the quantification of environmental risk and enable earlier and more sensitive detection of those who would benefit from a timely and comprehensive primary prevention regimen, as well as reversible epigenetics-based therapies.

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Disclosures

None.

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