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Identification of High Risk Coronary Artery Disease Patient by Molecular Techniques: The MEF-2A Paradigm

Direttore della Scuola : Ch.mo Prof. Achille Cesare Pessina

Supervisore :Ch.mo Prof. Gian Paolo Rossi

Dottoranda: Dr.ssa Stefania Colonna

A Denis

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ABSTRACT

Background. The coronary artery disease (CAD) and its clinical manifestations (angina and myocardial infarction, MI) are the first cause of death in most industrialized societies. Current guidelines used to detect those susceptible to heart attack fail to identifying many high-risk individuals. In recent years, a number of new candidate risk factors or markers have been proposed. Among risk factors, family history is one of the most significant independent risk factor for CAD/MI. Only one disease-causing gene, *myocyte enhancer factor-2 (MEF2A),* encoding a member of the MEF2 family of transcription factors, has been identified for primary CAD and MI without other accompanying clinical feature

Aim of the Study. Available evidences suggest that MEF2A plays a role in vascular ontogeny and shows its predominant expression in the coronary artery endothelium. Considering the pivotal role played by the latter in atherogenesis, we investigated:

1. the prevalence of MEF2A deleted gene in a large case-control study (GENICA Study)
2. if the deletion might be associated with coronary artery structural and functional abnormalities;
3. if it might be associated with widespread endothelial dysfunction;
4. if either one or the other or both alterations might eventually result into clinically relevant coronary artery disease.

Methods. We developed techniques suitable high throughout genotyping based on FRET () and HMRA (). After validation of these techniques vs sequencing, we prospectively genotyped 2 cohorts of healthy subjects, a cohort of primary hypertensive patients and the vast cohort of the GENICA Study.

Results. In the present study, the prevalence rate of the MEF2A deletion resulted 0 individuals in healthy subjects of 2 cohorts (n= 170 pts) and in primary hypertensive patients (n=131 pts); to be very low in the vast majority of the GENICA Study (n=1141 pts) cohort. In this study we found the MEF2A deletion in only one of 1142 consecutive patients referred for coronary artery angiography. Therefore, the prevalence in the latter was 8.7 per 10.000 (<1 ‰) patients.

Then we investigated of his pedigree and found that the deletion has been transmitted to one of the subject of the third generation. The MEF2A deleted-gene patient showed a clear endothelial impairment, and, at cardiac Magnetic Resonance a first passage hypoperfusion in the postero-lateral wall with a late enhancement as a post-ischemic fibrotic tissue.

Discussion. To our knowledge, our pedigree is the first to be identified in Europe. The significance of identification of *MEF2A* as the first disease-causing gene for CAD and MI makes genetic testing possible for many individuals with a very high risk for CAD and MI. and wise to extend screening to pedigree of subjects with acute myocardial infarct or acute coronary syndrome and few or no risk factors.

RIASSUNTO

Premesse. La coronaropatia e le sue manifestazioni cliniche (angina ed infarto miocardico) sono la prima cause di morte nei paesi industrializzati. Le attuali linee guida utilizzate per l'identificazione dei soggetti predisposti ad un attacco cardiaco, falliscono nell'identificazione proprio di quelli ad alto rischio. Negli ultimi anni sono emersi dei nuovi fattori di rischio, tra i quali la familiarità è uno di quelli più significativi. Solamente un gene-malattia è stato finora riconosciuto essere associato alla coronaropatia e all'infarto, il *myocyte enhancer factor-2 (MEF2A),* che codifica per un fattore di trascrizione della famiglia dei MEF2.

Obiettivo dello studio. Le evidenze disponibili in letteratura suggeriscono che MEF2A ha un ruolo predominate nell'ontogenesi e mostra la sua espressione nell'endotelio coronarico. Considerato il ruolo centrale svolto da quest'ultimo nell'aterogenesi, scopo dello studio è stato:

1. valutare la prevalenza della delezione del gene MEF2A in un ampio studio caso-controllo (GENICA)
2. valutare se la delezione poteva essere associata ad anomalie coronariche strutturali e funzionali;
3. valutare se la delezione poteva essere associata ad una diffusa disfunzione endoteliale
4. valutare se le due precedenti ipotesi potevano essere clinicamente rilevanti

Metodi. Abbiamo sviluppato alcune tecniche di alta genotipizzazione basate su metodologie FRET (fluorescence resonance energy transfer) e HMRA (high resolution melting amplicon). Dopo la convalida di queste tecniche con il sequenziamento, abbiamo prospetticamente genotipizzato 2 coorti di soggetti sani, una coorte di pazienti con ipertensione essenziale ed una più ampia coorte (GENICA Studio) di soggetti reclutati per angiografia coronarica.

Risultati. Nel presente studio, il tasso di prevalenza della delezione del gene MEF2A ha mostrato l'assenza di soggetti affetti nelle 2 coorti di soggetti sani (n = 170 pts) ed in quella dei soggetti ipertesi essenziali (n = 131 pts), ed è risultata molto bassa nella coorte dello Studio GENICA (n = 1142 pts) coorte. In questo studio abbiamo trovato la delezione del gene MEF2A solo in uno di 1142 pazienti consecutivi. Pertanto, la prevalenza in questo ultimo gruppo è stata di 8,7 per 10.000 (ossia <1 ‰).

Abbiamo quindi esaminato il pedigree del paziente affetto e riscontrato che la delezione è stata trasmessa ad uno dei due figli. Il soggetto con la delezione ha inoltre mostrato un chiaro danno endoteliale, ed alla Risonanza Magnetica cardiaca, un'ipoperfusione di primo passaggio nella parete postero-laterale ed un late-enhancement del tessuto fibrotico post-ischemico

Discussione. A nostra conoscenza, il nostro pedigree è il primo ad essere identificato in Europa. L'identificazione del MEF2A come primo gene-malattia per la coronaropatia e l'infarto miocardico rende possibile l'analisi genetica per quei soggetti ad alto rischio. Potrebbe essere saggio in futuro estendere lo screening alle famiglie di soggetti con infarto acuto del miocardio o sindrome coronarica acuta e con pochi o nessun fattore di rischio.

INTRODUCTION

The coronary artery disease (CAD) and its clinical manifestations (angina and myocardial infarction) are the first cause of death in most industrialized societies (AHA Statistical Report (1)) and constitute a complex pathological and clinical entity that is determined by a number of environmental and genetic factors (2). The environmental factors are certainly the best known, thanks to the much epidemiologic evidence, and essentially relate to lifestyle. Indeed, our current potential to prevent the development of CAD is most exclusively based on the corrections of wrong lifestyles.

With regard to genetic factors, the studies conducted so far have explored the existence of specific genetic determinants primarily through a case-control type approach in selected populations. This kind of analysis has not yet managed to reach conclusive data on the genetic basis of molecular-CAD, although many have been accumulated evidence of the existence of genes responsible for atherosclerosis and coronary heart disease (3;4).

The identification of genetic factors responsible for complex diseases as CAD represents an important tool to acquire new knowledge on the mechanisms responsible for the disease and especially on possible new target for therapeutic approaches, and for effective strategies of prevention. The relevance of these statements justifies the investigative efforts that are continually made in this area.

The classic genetic association study conducted in cases vs. controls analyzes distributions of a specific DNA structural variation (genetic marker or SNP) located within a few genes or candidate, in order to highlight significant differences in the risk of develop a specific disease in relation to the status of a bearer of an allele or a genotype changed.

The subsequent complex statistical analysis allows obtaining information on the existence of chromosomal areas (genetic loci) that are associated with a significantly higher incidence of suspected phenotype. The locus extension is defined by genetic markers (SNP). The method is defined more exactly "genome-wide association study" (5). (Figure 1)

In such studies, the next step is represented by the analysis of potential candidate genes mapped within the locus of interest. This includes the search for sequences that are located in close correspondence, or in the immediate vicinity, of a locus corresponding to the greater statistical significance. The *in vitro* characterization of functional properties of the protein than normal and changed data can be very useful for a better understanding of the real etiopathogenetic link between these two candidate genes and the presence of atherosclerosis and therefore coronary heart disease. At the same time there are still many limitations.

First of all, the complex statistical analysis of thousands of SNPs on thousands of subjects and the consequent chance of false positive results associated with multiple testing, second the need to check on other population the veracity of the results, leaving anyway a certain degree of doubt.

Therefore, in order to identify the gene responsible, it is necessary to take a long journey in search of candidate genes within the chromosome, followed by their structural analysis looking for mutations responsible, and their functional analysis both in vitro and in vivo using knock-out or overexpression studies.

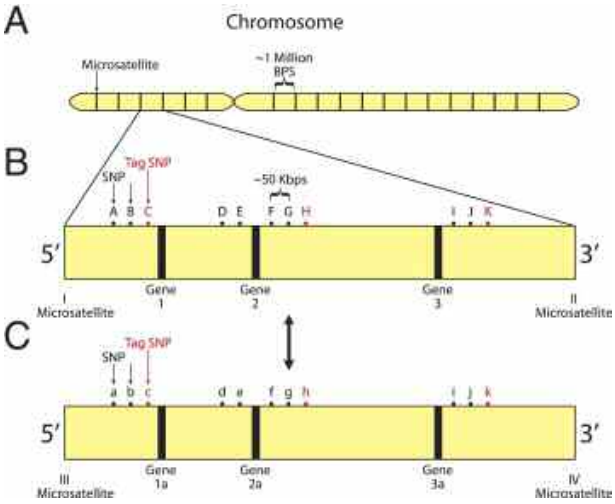


Figure 1. Genomic Markers.**(A)** Autologous chromosome with evenly spaced microsatellites. **(B)** Segment of deoxyribonucleic acid (DNA) between microsatellite markers. Single nucleotide polymorphisms (SNPs) are noted (A,B,C…) within the DNA segment. Tag SNPs (C,H,K) travel with other noted SNPs as blocks (haplotypes) and can serve as a surrogate for these haplotypes and, more importantly, disease-causing genes in close proximity. **(C)** A DNA segment with alternative alleles and genomic markers of the same genes designated in part **B** of the figure. Note that the microsatellite markers are not as close in proximity to the genes as the noted SNPs.

Prevalence of cardiovascular Disease from Heart Disease and Stroke Statistics—2008 Update (1).

Coronary artery disease (CAD) is the leading cause of death in developed countries. The prevalence of CAD is rising rapidly in developing countries due to increased exposure to CAD risk factors. CAD is the most common heart disease that is believed to be caused by multiple genetic factors. An estimated 80700 000 American adults (1 in 3) have 1 or more types of cardiovascular disease (CVD). Of these, 38 200 000 (47%) are estimated to be age 60 or older (extrapolated to 2005 from national Center for Health Statistics and National Health and Nutrition Examination Survey (NCHS NHANES 1999–2004).

- High blood pressure (HBP)—73 000 000 (90%). (Defined as systolic pressure 140 mm Hg or greater and/or diastolic pressure 90 mm Hg or greater, taking antihypertensive medication, or being told at least twice by a physician or other health professional that one has HBP.)
- Coronary heart disease (CHD)—16 000 000 (20%).
- Myocardial infarction (heart attack)—8 100 000 (10%)
- Angina Pectoris (chest pain)—9 100 000 (11%).
- Heart Failure—5 300 000 (6.5%)
- Stroke—5 800 000 (7.1%)
- Congenital cardiovascular defects—650 000 to 1 300 000 (0.8-1.6%)

The prevalence rate of CVD is directly associated with age (Figure 2) and therefore it is expected to grow further with aging of the population worldwide.

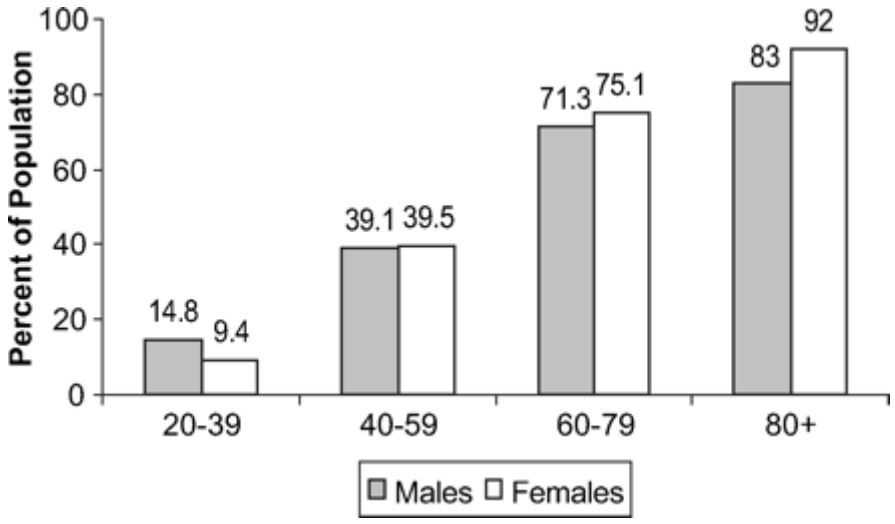


Figure 2. Prevalence of CVDs in adults age 20 and older by age and sex (NHANES: 1999–2004). These data include CHD, heart failure (HF), stroke, and hypertension.

Incidence of CVD

The National Heart, Lung and Blood Institute's Framingham Heart Study (FHS) in its 44-year follow-up of participants and the 20-year follow-up of their offspring showed that the average annual rates of first major cardiovascular events rise from 3 per 1000 men at ages 35 to 44 years to 74 per 1000 at ages 85 to 94 years. For women, comparable rates occur 10 years later in life. The gap between gender narrows with advancing age.

Before age 75, a higher proportion of CVD events due to CHD occur in men than in women, and a higher proportion of events due to congestive heart failure (CHF) occur in women than in men.

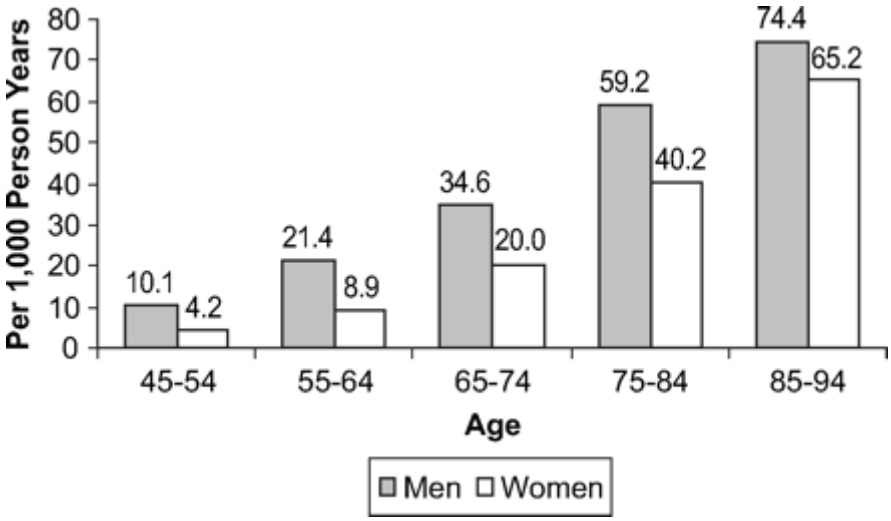


Figure 3. Incidence of CVD* by age and sex (FHS, 1980–2003). *CHD, HF, stroke, or intracerebral hemorrhage. Does not include hypertension alone.

Mortality of CVD

Mortality data show that CVD as the underlying cause of death accounted for 36.3% (869 724) of all 2 397 615 deaths in 2004, or 1 of every 2.8 deaths in the United States. CVD total mentions (1 357 000 deaths in 2004) constituted about 57% of all deaths that year.

In every year since 1900 except 1918, CVD accounted for more deaths than any other single cause or group of causes of death in the United States.

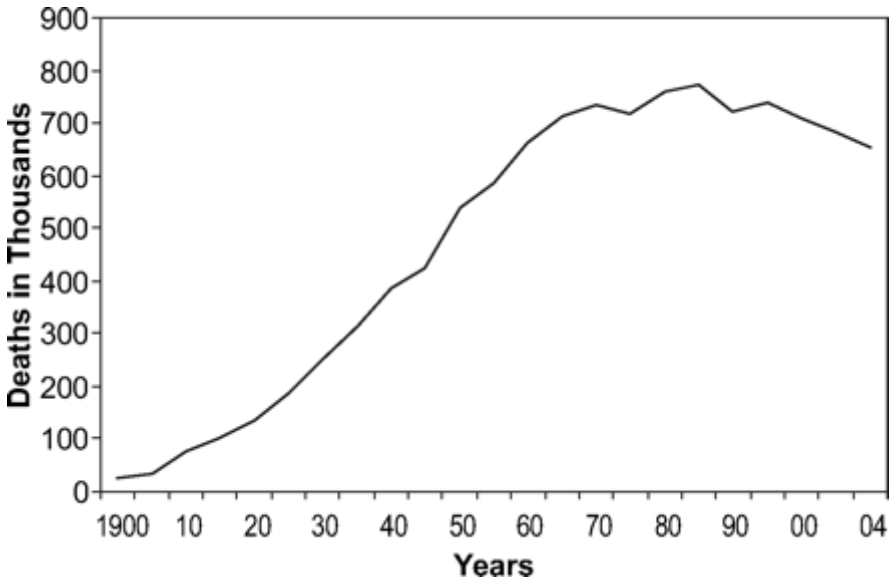


Figure 4. Deaths from diseases of the heart (United States: 1900–2004).

Prevalence of coronary heart disease (CHD)

Data from 2005 from the Behavior Risk Factor Surveillance System (BRFSS) survey of cardiovascular disease showed that 6.5 % of respondents reported a history of CHD. Men had a significantly higher prevalence of MI history than women (5.5% versus 2.9%), angina/CHD (5.5% versus 3.4%), and 1 or more of these conditions (8.2% versus 5.0%). Prevalence of these conditions increased with age and decreased with higher education.

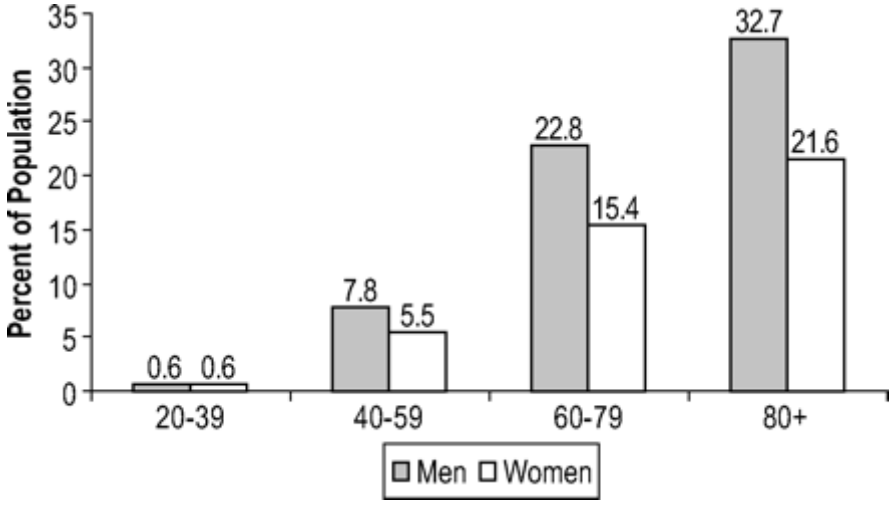


Figure 5. Prevalence of CHD by age and sex (NHANES: 1999–2004).

Incidence of CHD

This year, an estimated 770 000 Americans will have a new coronary attack and about 430 000 will have a recurrent attack. It is estimated that an additional 190 000 silent first MIs occur each year.

The estimated annual incidence of MI is 600 000 new attacks and 320 000 recurrent attacks annually

Average age at first MI is 64.5 years for men and 70.4 years for women.

CHD makes up more than half of all cardiovascular events in men and women > 75 years of age.

The lifetime risk of developing CHD after 40 years of age is 49% for men and 32% for women.

The incidence of CHD in women lags behind men by 10 years for total CHD and by 20 years for more serious clinical events such as MI and sudden death.

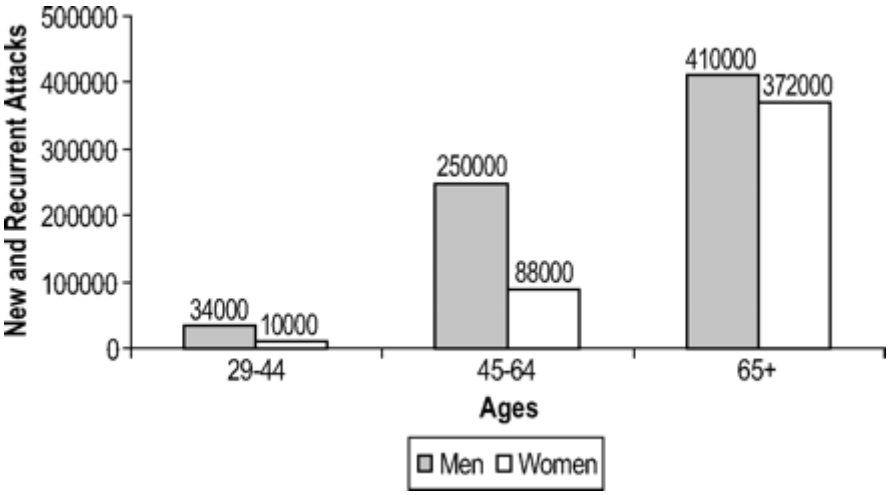


Figure 6 Annual number of Americans having diagnosed heart attack by age and sex

Mortality for CHD

CHD caused 1 of every 5 deaths in the United States in 2004. CHD mortality was 451 326. CHD total-mention mortality was 607 000. MI total-mention mortality was 196 000 (Vital Statistics of the United States, NCHS). CHD is the largest major killer of American males and females. About every 26 seconds, an American will suffer a coronary event, and about every minute someone will die from one. About 38% of the people who experience a coronary attack in a given year will die from it (AHA computation).

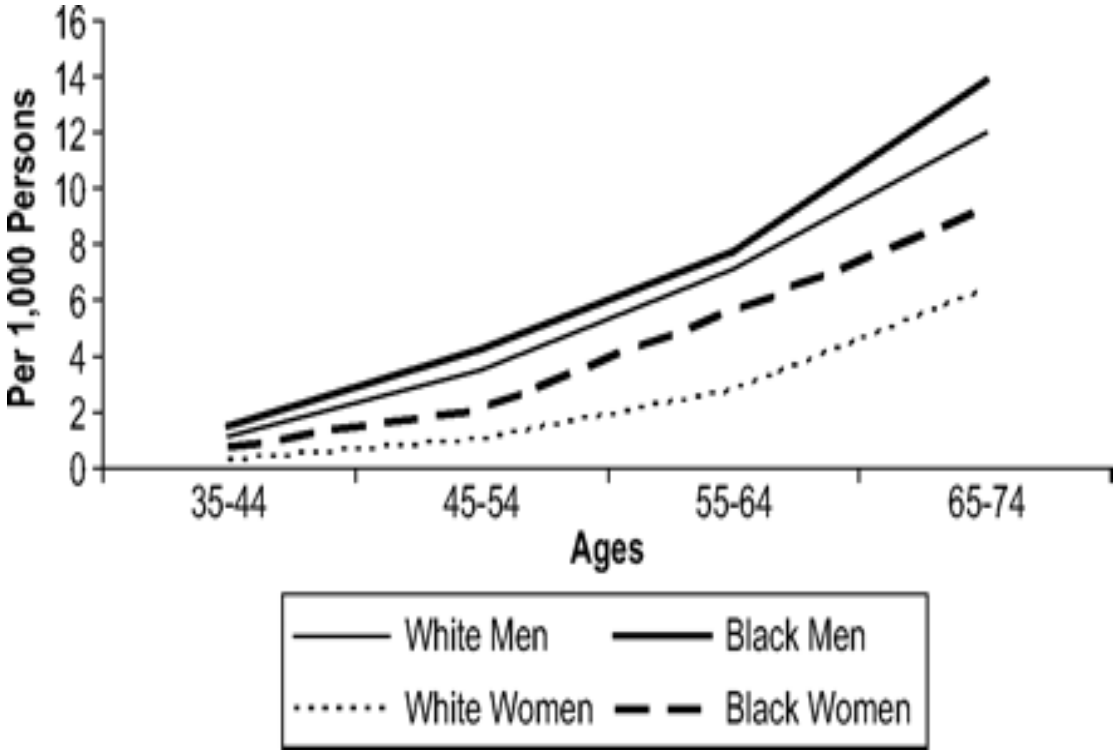


Figure 7 Annual rate of first MIs by age, sex, and race (Atherosclerosis Risk in Communities study: 1987–2000).

Traditional risk factors for Atherosclerotic Vascular Disease

The major reason for the limited success in the field of CAD and myocardial infarction (MI) genetics is that CAD is a complex disease and MI is very difficult to be predicted. Both are believed to be caused by many genetic factors and environmental and by an interactions among these.

Many risk factors have been identified for CAD/MI including smoking, advanced age, male gender, diabetes mellitus, high blood pressure, personal history of angina pectoris, family history of CAD/MI, high-fat diet, infectious agents, obesity, increased plasma total and low-density lipoprotein (LDL) cholesterol, increased plasma triglycerides, and decreased plasma high-density lipoprotein (HDL) cholesterol.

Smoking (6)

The association between smoking and cardiovascular mortality has been recognized for decades and an independent relationship between smoking and an increased cardiovascular risk has been widely documented. Smoking is directly responsible both for the deaths of approximately 350 000 men and 80 000 women yearly and for premature cardiovascular events. The recent World Health Organization report identified smoking as the fourth greatest global threat to health.

Smoking places a significant physiological stress on the vasculature by acutely decreasing coronary blood flow and myocardial oxygen delivery and by inducing profound, predominantly silent, regional disturbances in myocardial perfusion. Numerous mechanisms contribute to the increased cardiovascular risk in smokers, including increased activation of platelets and leucocytes, and adverse effects on lipids, blood pressure and insulin resistance. However, more recently damaging effects of tobacco smoke on the endothelium are of critical importance. Endothelial cells exposed to tobacco smoke have an irregular appearance with disturbances in membrane architecture, and develop marked functional change, including decreased activity of endothelial (e)NOS, enhanced expression of adhesions and dysregulation of the local thrombotic balance. Cigarette smoke is a complex mixture of chemical compounds containing a high concentration of free radicals. These reactive oxygen species (ROS) damage endothelial cells via several distinct pathways that include direct cellular damage and indirect effects on lipid peroxidation, and scavenging of nitric oxide with generation of the potent oxidant, peroxynitrite. Several studies have demonstrated dose-related impairment of endothelium-dependent vasodilatation in the peripheral and coronary circulation of smokers. Furthermore,

passive smoking also causes endothelial dysfunction in a dose-dependent fashion. Despite the compelling evidence for a causal relationship between smoking, endothelial dysfunction and atherosclerosis, important questions remain unanswered.

Dyslipidaemia

Hypercholesterolaemia

Hypercholesterolaemia is consistently associated with endothelial dysfunction. Oxidized low-density lipoprotein (LDL) is an important mediator of endothelial damage, but individuals with smaller LDL particles can also have endothelial dysfunction, independently of LDL plasma concentration. In both conduit and resistance coronary arteries, hypercholesterolaemia is associated with impaired endothelium-derived nitric oxide bioavailability, even when the coronary arteries are angiographically normal. In patients with hypercholesterolaemia, the decrease in the central augmentation index induced by inhaled salbutamol is blunted; in contrast, basal endothelial production of nitric oxide, measured as the reduction in forearm blood flow that occurs with the inhibition of NOS by L-N^G-monomethyl arginine (L-NMMA), is not impaired. In addition, release of tPA from the endothelium is normal and endogenous endothelin (ET)-1 activity in the forearm is not increased in hypercholesterolaemia .

Familial hypercholesterolaemia is associated with the development of endothelial dysfunction before the onset of clinically overt arterial disease. In children with familial hypercholesterolaemia in who total cholesterol and lipoprotein(a) concentrations are inversely related to the degree of flow-mediated dilatation (FMD).

Hypertriglyceridaemia

The impact of hypertriglyceridaemia on endothelial function is controversial. In patients with chronic hypertriglyceridaemia, vasodilatation to serotonin in the forearm circulation was impaired whereas that to acetylcholine was unimpaired. Brachial artery FMD was also diminished in young men with hypertriglyceridaemia. However, acute administration of triglycerides to normal individuals did not impair brachial artery FMD consistently. As hypertriglyceridaemia frequently coexists with insulin resistance, it is likely that it is the latter, rather than the former, that induces endothelial dysfunction .

High-density lipoprotein

Lower plasma concentrations of high-density lipoprotein (HDL) cholesterol might affect endothelial function independently of LDL cholesterol. Furthermore, both infusion of reconstituted HDL cholesterol to hypercholesterolaemic patients and restoring the HDL concentration in patients with familial hypoalphalipoproteinaemia improved endothelial dysfunction.

Diabetes mellitus

Cardiovascular disease is the major complication of diabetes (particularly type 2). Clustering of cardiovascular risk factors as part of the 'metabolic syndrome' may partly explain the excess cardiovascular risk associated with diabetes. The fact that many cardiovascular risk factors are present long before the development of diabetes has led to increasing support for the 'common ground' hypothesis in which type 2 diabetes and cardiovascular disease share common genetic and environmental antecedents. Although the mechanisms linking the metabolic syndrome, type 2 diabetes and atherosclerosis remain unclear at a cellular level, all share a decreased nitric oxide bioactivity - that is, endothelial dysfunction. The L-arginine/nitric oxide pathway may be impaired at a number of sites in individuals with diabetes. Endothelial damage may lead to a decrease in either basal or stimulated release of nitric oxide or, alternatively, there may be an increased breakdown of nitric oxide. VSMCs may exhibit a decreased sensitivity to the actions of nitric oxide. Finally, the build up of endogenous inhibitors of NOS, such as ADMA, may decrease the biological activity of eNOS and hence decrease the production of nitric oxide. Whatever the mechanisms, compelling evidence indicates that endothelial dysfunction contributes to the complications of diabetes.

Type 1 diabetes

The data concerning endothelial function in type 1 (insulin-dependent) diabetes mellitus are conflicting, although a number of investigators have demonstrated forearm endothelial dysfunction . In most studies, responses to endothelium-independent agonists were preserved, indicating decreased NO bioactivity. The coexistence of microalbuminuria can also influence responses to both endothelium-dependent and -independent agonists. In a recent study, responses to acetylcholine and glyceryl trinitrate were both impaired and a further study demonstrated an impaired FMD that correlated with both the duration of diabetes and the concentrations of LDL. Furthermore, endothelium-dependent microvascular responses have also been reported to be impaired, albeit not in all studies . None of these studies revealed abnormal responses to endothelium-independent vasodilators; in contrast, another study

demonstrated preserved responses to acetylcholine, but a reduced response to nitroprusside and a reduced effect of L-NMMA on basal blood flow, suggesting decreased basal release of nitric oxide. Similar findings have been reported by others, but only in diabetic individuals with microalbuminuria. A blunted constrictor response to L-NMMA in patients with poorly controlled diabetes has also been described.

The majority of studies have used venous occlusion plethysmography to study endothelial function and the variability of results may be explained by several factors. Various endothelium-dependent agonists have been used, which may differ in their degree of NO-mediated vasodilator activity. The selection of patients in terms of duration of diabetes , glycaemic control and presence or absence of microalbuminuria is also important. The clustering of other cardiovascular risk factors should be taken into consideration. The variable increase in basal forearm blood flow may also influence responses to vasoactive drugs. Finally, abnormal responses to agonists may be masked by other vasodilator pathways that are upregulated in response to a defective L-arginine/nitric oxide pathway.

Type 2 diabetes

Endothelial function has been subject to intense investigation in patients with type 2 diabetes, in whom an impaired dilatation in response to endothelium-dependent agonists was consistently shown. However, some studies also demonstrated impaired responses to endothelium-independent stimuli . Overall, the available results suggest that the decreased nitric oxide bioactivity may be the result of nitric oxide breakdown. It has been demonstrated, first, that type 2 diabetes is associated with increased generation of ROS and, secondly, that antioxidants can improve endothelial dysfunction under experimental conditions . Particular interest has, therefore, focused on oxidized LDL, a peroxidation product that damages the endothelium, has potent proatherogenic activity and triggers formation of autoantibodies. Oxidized LDL inhibits endothelium-dependent relaxation more potently than native LDL and the LDL particles from patients with type 2 diabetes are smaller, more dense and more susceptible to oxidation - that is, more atherogenic - than those from individuals without diabetes.

Arterial hypertension

Endothelial dysfunction is the hallmark of hypertension both primary (essential) and secondary. This has been documented in different vascular beds with receptor-operated (acetylcholine,

bradykinin, substance P), mechanical (increase in shear stress) or mixed (dynamic exercise and cold pressor test) stimuli. Endothelial dysfunction associated with essential (primary) hypertension is characterized by impaired nitric oxide bioactivity determined by ROS, which scavenge nitric oxide. ROS can be generated by non-enzymatic and enzymatic sources, including NAD(P)H oxidases or xanthine oxidase, COX and NOS-induced superoxide production caused by depletion of the cofactor, tetrahydrobiopterin. In the presence of reduced availability of nitric oxide, alternative pathways, including hyperpolarization, account for endothelium-dependent vasodilatation. Furthermore, part of endothelial dysfunction can be explained by variations in the *eNOS* gene.

An interaction of the NO and ET-1 systems may participate in the pathogenesis of endothelial dysfunction. Although inconclusive evidence exists of increased plasma concentrations of ET-1 in essential hypertension, the vasoconstrictor activity of the peptide is increased along with diminished availability of NO. In the presence of the latter, the endothelial ET_B-receptor-mediated inhibitory effect of nitric oxide on ET-1 production may be impaired. Thus imbalance between the two systems may enhance the vasoconstrictor and proliferogenic activity of ET-1.

The impaired endothelium-dependent vasodilatation in essential hypertension is often portrayed as a causal mechanism of the increase in blood pressure, or maintenance of hypertension. This is unlikely, because endothelial dysfunction is not specific to essential hypertension, but rather a hallmark of all the major cardiovascular risk factors, and because there is dissociation between the degree of endothelial dysfunction and blood pressure values. Therefore, as for diabetes mellitus there might be a 'common ground' determining susceptibility to endothelial dysfunction, atherosclerotic disease and events in patients with cardiovascular risk factors. Mounting evidence for an association of endothelial dysfunction with markers of vascular damage and cardiovascular events in patients with essential hypertension supports this contention. In patients with essential hypertension, an impaired forearm response to acetylcholine correlates with intima-media thickening of the carotid arteries. Moreover, in epicardial coronary arteries of normotensive individuals, the response to acetylcholine showed an inverse correlation with intramural plaque.

The presence of coronary endothelial dysfunction has also been associated with cardiovascular events in longitudinal studies. The authors of one paper suggested that forearm vasodilatation in response to acetylcholine might also predict cardiovascular events in patients with essential hypertension, but the reported rate of total cardiovascular events at follow-up was 4.9%

events/year, which is 10- to 40-fold greater than that (0.1-0.4%/year) expected from population surveys and from studies in high-risk patients, thus suggesting a serious selection bias. Consequently, further studies are required to establish a direct relationship between endothelial dysfunction and cardiovascular events in patients with essential hypertension.

Moreover, there seems to be a gap between the knowledge on ED that has been acquired in the last two decades and the investigation of ED in clinical practice. This is likely because of the unavailability of accurate indexes of ED that are simple, inexpensive and non-invasive.

Obesity

Endothelial function in obesity has not been investigated as intensively as in other conditions; nevertheless, recent contributions have provided substantial novel information.

Resistance arteries

In obese individuals, a reduced vasodilatation in response to stepwise intra-arterial infusion of muscarinic agonists has been found using different techniques and different study designs, although findings are not fully consistent. Measurements of leg blood flow with positron emission tomography demonstrated that a twofold greater rate of infusion of bradykinin was necessary to induce a 70% vasodilatation in obese individuals compared with that in lean individuals.

In another study, the leg blood flow response to metacholine was similarly impaired in obese individuals with or without type 2 diabetes, whereas endothelium-independent vasodilatation was normal. The insulin-induced enhancement of the response to metacholine was also impaired; these estimates of endothelial function correlated significantly with body mass index, suggesting no additive effect of obesity and diabetes. In contrast, hypertension and obesity do appear to be additive.

As for the mechanisms responsible for endothelial dysfunction in obesity, there is no evidence that the blunted response to muscarinic agonists depends on impaired nitric oxide synthesis; furthermore, endothelial dysfunction was only partially restored by either vitamin C or indomethacin, suggesting that part of the defect could be attributed to ROS or COX products, or both . Using a selective ET_A antagonist, an enhanced basal ET-1 tone was recently demonstrated in obese patients: when ET_A receptors were blocked, intra-arterial infusion of metacholine induced similar vasodilatation in obese and control individuals, implying that an imbalance of ET-1 tone contributed to the poorer endothelium-dependent dilatation in those who are obese. Some clues can also be gained from weight loss studies. Surprisingly, weight loss *per se* (in the range of 10% of initial body weight) achieved by diet alone did not significantly

influence the response of resistance arteries. The effect was more consistent when the diet was associated with either physical exercise or orlistat (which restrains lipid absorption). With the latter, the improved vascular reactivity correlated with the change in LDL cholesterol; with exercise, improvement in vascular reactivity and decrease in the indices of endothelial activation and inflammation were related to the change in plasma insulin concentrations. The studies in animal models of obesity are somewhat inconsistent. Overall, they suggest that endothelial dysfunction largely depends on the presence of insulin resistance, which has been shown to extend to the vascular tissues and to be selective for the phosphatidyl inositol 3-kinase/Akt pathway.

Conductance arteries

A severe reduction in FMD of brachial and femoral arteries with a preserved response to nitrates has been found in individuals with uncomplicated obesity. Interestingly, all the studies reported a stronger correlation with indices of central fat distribution than of overall adiposity. Scarce information is available, in patients with concomitant essential hypertension or diabetes. Weight reduction with diet and exercise improved FMD in proportion to the weight change and independently of the presence of diabetes or impaired glucose tolerance.

Novel Risk Factors for Atherosclerotic Vascular Disease (or limitations of the strategy for risk stratification based on the classical CV risk factor)

Current guidelines used to detect those susceptible to heart attack, fail to identifying many high-risk individuals. According to a recent study (7) 88% of heart attack victims would have been considered low to moderate risk if they were tested with current national guidelines the day before their heart attack. Moreover, most myocardial infarction occurs in less severe coronary artery stenoses.

It is apparent, however, that a substantial proportion of cardiovascular events occur in individuals without these established risk factors. The reasons for this are multifold; in particular, there is evidence that even modest elevations of blood pressure, cholesterol, and glucose levels combine to place individuals at risk for CVD (8;9). Hence, despite the fact that most cardiovascular events can be explained by conventional risk factors, the search for additional etiologic agents must continue. Moreover, while the population-attributable risk of the major vascular risk factors is substantial, it is often difficult to distinguish those individuals with a moderate baseline risk who might benefit from aggressive risk reduction strategies. Therefore, additional tests to assist in the prediction of risk in these individuals may be warranted.

In recent years, a number of new candidate risk factors or markers have been proposed as significant predictors of atherosclerosis and its complications (Box 1) We will herein highlight four important emerging risk predictors: C-reactive protein (CRP), lipoprotein(a) [Lp(a)], fibrinogen, and homocysteine. These risk predictors were selected because there is substantial evidence on their predictive abilities, there is a genetic basis for premature disease, modifying treatments are available, and/or these factors are the subject of ongoing or completed clinical trials (8).

Box 1. Novel Risk Factors for Atherosclerotic Vascular Disease

- ✓ Inflammatory Markers
- ✓ C-reactive protein
- ✓ Interleukins (eg, IL-6)
- ✓ Serum amyloid A
- ✓ Vascular and cellular adhesion molecules
- ✓ Soluble CD40 ligand Leukocyte count

Hemostasis/Thrombosis Markers

- ✓ Fibrinogen
- ✓ von Willebrand factor antigen

- ✓ Plasminogen activator inhibitor 1 (PAI-1)
- ✓ Tissue-plasminogen activator
- ✓ Factors V, VII, and VIII
- ✓ D-dimer
- ✓ Fibrinopeptide A
- ✓ Prothrombin fragment 1 + 2

Platelet-Related Factors

- ✓ Platelet aggregation
- ✓ Platelet activit
- ✓ Platelet size and volume

Lipid-Related Factors

- ✓ Small dense low-density lipoprotein (LDL)
- ✓ Lipoprotein(a)
- ✓ Remnant lipoproteins
- ✓ Apolipoproteins A1 and B
- ✓ High-density lipoprotein subtypes
- ✓ Oxidized LDL
- ✓ Lp PLA_2

Other Factors

- ✓ Homocysteine
- ✓ Lipoprotein-associated phospholipase A(2)
- ✓ Microalbuminuria
- ✓ Insulin resistance
- ✓ PAI-1 genotype
- ✓ Angiotensin-converting enzyme genotype
- ✓ ApoE genotype
- ✓ Infectious agents: Cytomegalovirus, *Chlamydia pneumonia, Helicobacter pylori*, Herpes simplex virus
- ✓ Psychosocial factors

C-Reactive Protein

C-reactive protein (CRP) is a circulating acute-phase reactant that is increased many-fold during the inflammatory response to tissue injury or infection. C-reactive protein is synthesized primarily in the liver and its release is stimulated by interleukin 6 (IL-6) and other proinflammatory cytokines. This protein has received substantial attention in recent years as a promising biological predictor of atherosclerotic disease. This stems in part from a recent shift in thinking about the pathogenesis of ASVD, an entity once primarily considered to be a bland lipid storage disease. Inflammation is now widely accepted as central to every aspect of the atherosclerotic process, from its initiation to its progression to plaque rupture, the latter being the quintessential event underlying the acute coronary syndromes. In particular, local inflammatory processes may trigger the occurrence of vascular events by mediating plaque instability.

An evolving body of work suggests that even small increases in CRP within the normal range are predictive of future vascular events in apparently healthy, asymptomatic individuals.

The predictive abilities of CRP seem to extend to patients with preexisting vascular disease as well. A number of prospective studies have demonstrated that CRP predicts recurrent events and/or increased mortality in patients with ischemic stroke, acute coronary syndromes, chronic stable angina, and peripheral vascular disease. An elevated CRP level before percutaneous coronary intervention also portends a worse prognosis, as it does among patients undergoing coronary artery bypass grafting. C-reactive protein is correlated with the presence of abdominal obesity and a raised level predicts the risk of developing type 2 diabetes. The predictive value of CRP is additive to that of several surrogate markers of atherosclerosis, including carotid intimal medial thickness, quantitative coronary calcium scoring, and the presence of the metabolic syndrome.

Whether or not CRP is a marker or mediator of inflammation is unclear. There is evidence that CRP may play a direct role in the pathogenesis of atherosclerosis. The protein is markedly up-regulated in atheromatous plaques, where it may promote low-density lipoprotein (LDL) cholesterol uptake by macrophages, a key step in atherogenesis. C-reactive protein may also induce the expression of intercellular adhesion molecules by endothelial cells, thereby facilitating the recruitment of circulating monocytes to plaque sites, and can bind to and activate complement in serum. These effects appear to be mediated through CRP-induced secretion of endothelin 1 and IL-6.

However, the utility of CRP as a tool in global risk assessment has some important limitations. These include CRP's poor specificity in the setting of coexisting inflammatory states (eg, rheumatoid arthritis, chronic pulmonary disease, and infections) and minimal data from nonwhite populations. In addition, CRP is strongly correlated with other cardiovascular risk factors such as fibrinogen; in studies examining CRP's incremental predictive value, its independence from fibrinogen has not been demonstrated. For example, in patients with established vascular disease, fibrinogen, but not CRP, was a significant independent predictor of recurrent cardiovascular events after adjustment for conventional risk factors.

Retrospective subgroup analyses have raised the possibility that statins and aspirin (which lower CRP levels) may lead to reduced cardiovascular events even among patients without overt hyperlipidemia (10). However, to date, no clinical trials have prospectively demonstrated that

targeting patients with elevated CRP lowers vascular event rates, or whether interventions aimed at lowering CRP translates into reduced vascular risk, although several are ongoing (11).

Lipoprotein(a)

Lipoprotein(a) is an LDL-like particle in which an apolipoprotein(a) [apo(a)] moiety is linked via a disulfide bond to apoB-100. Concentrations of Lp(a) are largely under genetic control and vary substantially between individuals depending on the size of the apo(a) isoform present; conversely, Lp(a) levels vary little with diet or exercise, unlike other lipoproteins such as LDL and high-density lipoprotein (HDL). The wide range of Lp(a) in plasma within a population is due in large part to a variable number of plasminogen-like kringle IV repeats, and an inverse correlation between the number of kringle IV type 2 repeats in the apo(a) gene and Lp(a) plasma concentration exists. The biological function of Lp(a) is still unclear, but there is strong evidence that its phylogenetic role may have been to respond to tissue injury and vascular lesions, prevent infectious pathogens from invading cells, and promote wound healing (12).

The use of Lp(a) as a screening tool has some limitations. No universally accepted, standardized method of determination for Lp(a) exists, although recently, a working group of the International Federation of Clinical Chemistry demonstrated the inaccuracy of Lp(a) values determined by methods sensitive to apo(a) size and recommended the widespread implementation of a proposed reference material for those Lp(a) assays that are validated to be unaffected by apo(a) size heterogeneity (13). The incremental predictive value of Lp(a) measurement additive to that of traditional screening methods for global risk assessment has not been formally studied.

Fibrinogen

Fibrinogen is a circulating glycoprotein that acts at the final step in the coagulation response to vascular and tissue injury (14). Cleavage by thrombin produces soluble fibrin fragments, which are the most abundant component of blood clots. Aside from its role in thrombosis, fibrinogen has a number of other functions that lend it biological plausibility as a possible participant in vascular disease, including the following: regulation of cell adhesion, chemotaxis, and proliferation; vasoconstriction at sites of vessel wall injury; stimulation of platelet aggregation; and determination of blood viscosity. Fibrinogen, like CRP, is an acute-phase reactant. Hepatic synthesis of fibrinogen can increase up to 4-fold in response to inflammatory or infectious triggers.

Epidemiological data support an independent association between elevated levels of fibrinogen and cardiovascular morbidity and mortality. Several factors other than inflammation have been shown to modulate fibrinogen levels. Smoking and smoking cessation are associated with an increase or decrease, respectively, in plasma fibrinogen (15). Furthermore, there is a dose-response relationship between number of cigarettes smoked and fibrinogen level. Fibrinogen levels tend to be higher in patients with diabetes, hypertension, obesity, and those with sedentary lifestyles. Fibrates and niacin lower fibrinogen levels (in addition to lipid parameters), whereas statins and aspirin do not. Further clinical trials are necessary before it can be determined whether fibrinogen has a causal role in atherothrombosis or is merely a marker of the degree of vascular damage taking place.

Homocysteine

Homocysteine is a highly reactive, sulfur-containing amino acid formed as a by-product of the metabolism of the essential amino acid methionine (16). Cells remetabolize homocysteine by a number of possible pathways involving several different enzymes; these enzymes variously use B vitamins as substrates or cofactors, namely folate, cobalamin (vitamin B_{12}), and pyridoxine (vitamin B_6).

In the 1960s and 1970s, 3 different inborn errors of homocysteine metabolism involving these enzymes were described (termed "homozygous homocystinurias"). Common to all 3 disorders are extremely high levels of homocysteine in the blood and urine of individuals homozygous for these mutations; half of affected individuals develop arterial or venous thrombosis by 30 years of age. This risk can be substantially ameliorated by the provision of high-dose B vitamins, which partially lower homocysteine levels back toward the normal range (17).

It has been postulated that mild to moderate elevations of homocysteine in the general population predispose to atherosclerosis in a manner akin to the classic risk factors. This is important because of the availability of an inexpensive, safe, and effective therapy for lowering homocysteine (B vitamins) and folicacid supplementation. Mechanistic studies have demonstrated that homocysteine may induce vascular damage by promoting platelet activation, oxidative stress, endothelial dysfunction, hypercoagulability, vascular smooth muscle cell proliferation, and endoplasmic reticulum stress (Figure 8).

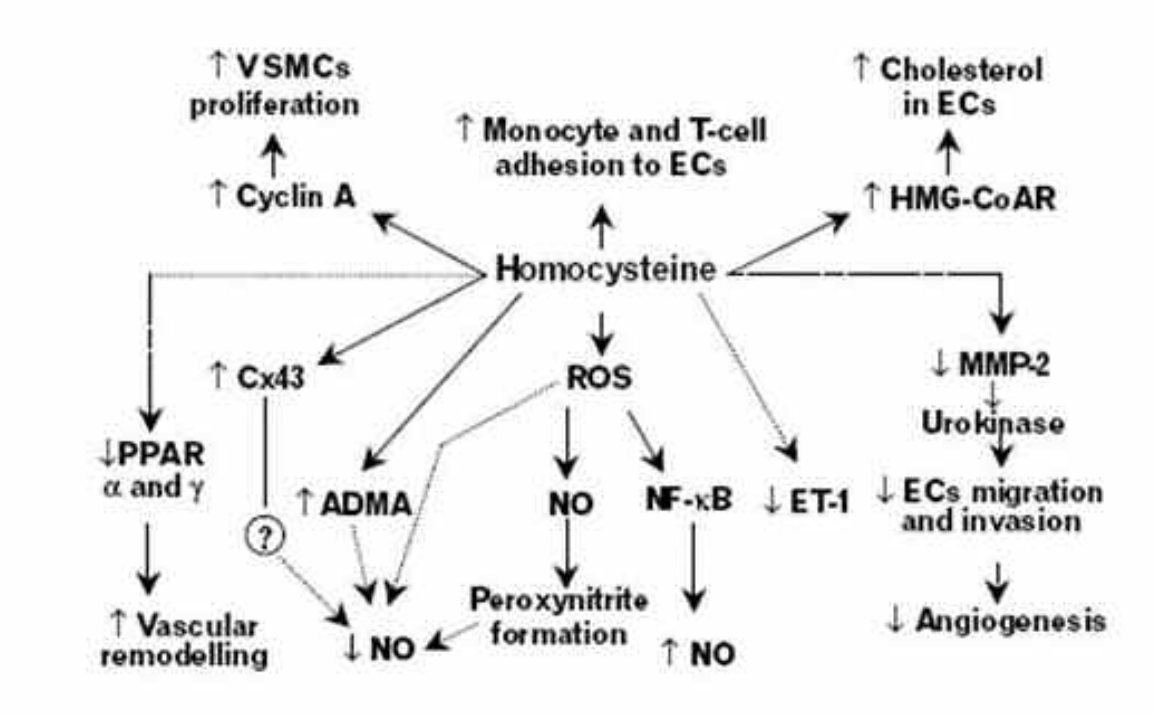


Figure 8 Schematic representation of the mechanisms by which hyperhomocysteinaemia can adversely affect endothelial function. Continuous and dotted lines indicate stimulation and inhibition, respectively. (6)

A common gene mutation encoding one of the enzymes that metabolizes homocysteine (5, 10-methylenetetrahydrofolate reductase [*MTHFR*]) leads to moderate increases in homocysteine levels on the order of 25%, particularly in the presence of low folate intake. Homozygosity for this *MTHFR* variant (677C→T) is present in up to 15% of the Caucasian population, thus providing a natural experiment by which the relationships between the abnormal *MTHFR* genotype, higher homocysteine levels, and vascular disease can be discerned (18).

Numerous observational studies have also reported on the association between homocysteine levels and vascular risk in both the general population and in those with preexisting vascular disease. Whether homocysteine is causative in the pathogenesis of atherosclerosis, is related to other confounding cardiovascular risk factors, or is a marker of existing vascular disease will have to await the completion of a number of large, randomized controlled trials studying the effect of homocysteine-lowering vitamins on cardiovascular end points (19).

But four large intervention trials testing the hypothesis that lowering homocysteine (tHcy) improves cardiovascular outcome have given negative results (19-23).

In a recent review, Rossi GP et al (24) underlines that, given the multifactorial and complex nature of atherosclerosis, it is probably naïve to assume that lowering tHcy is a "panacea" for all CV events and that this intervention could be proven to be beneficial, even in patients who do not have HHcy. In the HOPE trial (22) the patients were enrolled irrespective of their tHcy levels and more than two-thirds had tHcy levels less than 12.7 μmol/L, e.g., within the normal range.

Therefore, it is unclear why these patients should receive tHcy lowering treatment and why they would show any benefit from this treatment. Moreover, if the relationship between risk of CV events is linear across the range of normal to mildly elevated tHcy values (curve A in the figure 9) one might anticipate that lowering tHcy in the normal-to-mildly elevated range would decrease CV events; however, if the relationship has a shape as curve B (Figure 9) one would expect that no lowering of CV events can occur when tHcy are not overtly elevated. This implies that even very large clinical trials can be underpowered if the relationship of tHcy with CV outcome is that depicted by curve B. in addition, it might be that the investigation of a carefully selected cohort of patients with left ventricular dysfunction and HHcy might provide a streamlined approach to question whether the Hcy hypothesis of atherothrombosis is correct or not.

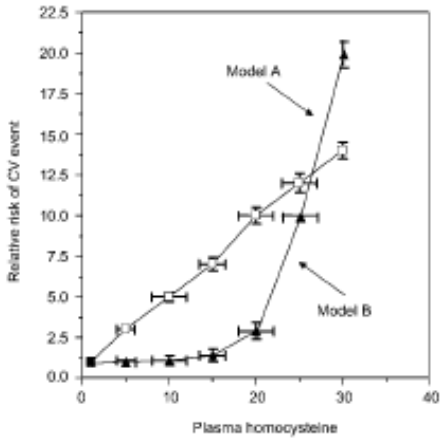


Figure 9. Hypothetical modeling of the relationship of homocysteine with risk of cardiovascular events (see text for details).

Is Endothelial Dysfunction the "common ground" of susceptibility to CAD?

From what already discussed in previously, it is apparent that an early trigger for the pathogenesis of CAD and MI may be dysfunction or abnormal development of the endothelium. This might be genetically determined and can translate into increased susceptibility of the coronary arteries to inflammation, leading the development of CAD and MI (25).

Given the profound involvement of endothelial dysfunction (ED) in the pathogenesis of CVD, it is not surprising that ED has been consistently associated with cardiovascular risk factors and also demonstrated in most disease conditions that predict high risk of cardiovascular events, as seen before.

Exercise normally increases coronary artery diameter; however, in patients with CAD increased blood flow (the major physiological stimulus for endothelial release of nitric oxide) leads to coronary vasoconstrictions, probably trough unopposed alfa-adrenergic mechanism (6).

Acetylcholine can also evoke a paradoxical vasoconstriction in atherosclerotic coronary arteries, through unopposed activation of muscarinic receptors on VSMCs. Furthermore, epicardial atherosclerosis is associated with impairment in endothelium-dependent dilatation of the coronary microvasculature (26).

Endothelium-dependent vasodilatation in the human forearm and coronary vascular beds are strongly correlated. This might reflect a "common ground", such as a genetic predisposition to ED and cardiovascular disease.

Modifications of cardiovascular risk factors that contribute to ED improve patient's outcome disproportionately to the improvement in coronary atherosclerosis, thus implying that these beneficial effects may be mediated in part through improvement in endothelial function. Suwaidi et al (27) evaluate the outcome of patients with mildly diseased coronary arteries and ED, demonstrating that patients with non-obstructive CAD and severe ED are at increased risk for cardiac events. The mechanism by which endothelial dysfunction leads to cardiac events may be multifactorial. One possible mechanism is myocardial ischemia secondary to endothelial dysfunction even in the absence of obstructive coronary artery disease (26). Indeed, Suwaidi et al. have demonstrated that the reduction in CBF response to acetylcholine resulting from coronary endothelial dysfunction was associated with myocardial perfusion defects.

Another possible mechanism by which coronary endothelial dysfunction may contribute to cardiac events is through acceleration of coronary atherosclerosis, as evidenced by the development of obstructive coronary artery disease. This is also supported by the observation in

cardiac transplant patients that coronary endothelial dysfunction precedes the development of coronary atherosclerosis (28). It may be hypothesized that endothelial dysfunction represents the stage of rapid progression of atherosclerosis, which may be secondary to the loss of various protective physiological roles of endothelial cells. The abnormal response to the endothelium-dependent vasodilator acetylcholine may represent a reduction in nitric oxide (NO) bioavailability (29). Pathophysiological states associated with a decrease in NO bioavailability and endothelial adhesion molecules for monocytes are upregulated. This could enhance local inflammation of the vessel wall, which may play a critical role in plaque rupture. This hypothesis is supported by the observation that L-arginine supplementation, the precursor of NO, improves endothelium-dependent vasorelaxation and attenuates the progression of atherosclerosis in an experimental rabbit hypercholesterolemia model. Indeed, medical intervention that increases NO bioavailability was shown to improve patient outcome.

Probably, ED represents the stage of rapid progression of atherosclerosis, which may be secondary to the loss of various protective physiological roles of endothelial cells.

Role of endothelial shear stress in the natural history of endothelial dysfunction (30)

Although the entire vasculature is exposed to the atherogenic effects of the systemic risk factors, atherosclerosis lesions form at specific regions of the arterial tree, such as in the vicinity of branch points, the outer wall of bifurcations, the inner wall of curvatures, where disturbed flow occurs. Local factors, such as hemodynamic forces, play a major role in the regional localization of atherosclerosis. These hemodynamic forces include flow-generated endothelial shear stress (ESS) and blood pressure-derived tensile stress, with ESS playing the most fundamental role in atherosclerosis.

Endothelial shear stress derived from friction of the flowing blood on the endothelial surface of the arterial wall; it is expressed in unit of force/unit area (N/m2) and is proportional to the product of blood viscosity (u) and the spatial gradient of blood velocity at the wall (ESS= u x dv/dy).

In geometrically irregular regions, where disturbed laminar flow occurs, pulsatile flow generates low and/or oscillatory ESS. Low SS refers to ESS that is unidirectional at any given point but has a periodically fluctuating magnitude that results in a significantly low time-average. Low ESS typically occurs at the inner areas of curvatures as well as upstream of stenoses, and is closely associated with atherogenesis. Endothelial cell (EC) surfaces (luminal, junctional, and basal) are equipped with numerous mechanoreceptors capable of detecting and responding to ESS stimuli (31) (Figure 10).

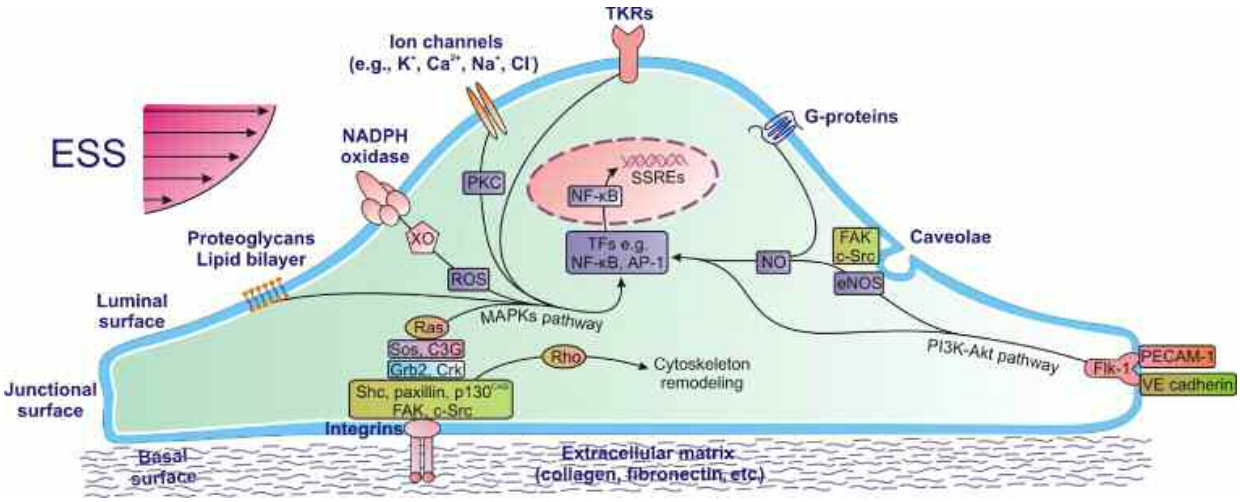


Figure 10 .Endothelial Mechanotransduction of ESS.

After activation of mechanoreceptors, a complex network of several intracellular pathways is triggered, a process known as mechanotransduction. These pathways are activated simultaneously and cross-talk with each other; the great majority of them converge into the mitogen-activated protein kinase (MAPKs) cascade at various levels. Cytoskeleton constitutes a central mediator in ESS signalling by providing a scaffold for the formation or translocation of various signalling molecules, serving as a bond between the luminal surface, where ESS is imposed, and several luminal, basal, or junctional formations, where the signalling pathways initiate (32). These pathways lead to phosphorylation of several transcription factors (TFs), which bind positive or negative shear stress responsive elements (SSREs) at promoters of mechanosensitive genes, inducing or suppressing their expression and, ultimately, modulating cellular function and morphology). In arterial regions with non-disturbed flow, where ESS varies within a physiologic range, the ECs express various atheroprotective genes and suppress several pro-atherogenic ones, leading eventually to stability and quiescence in that region. In contrast, in regions with low and disturbed flow where low ESS occurs, the atheroprotective genes are suppressed, whereas the pro-atherogenic genes are upregulated, thereby promoting the atherosclerotic process.

NO, a key component of normal vascular tone, also possesses strong anti-inflammatory, anti-apoptotic, anti-mitogenic, and anti-thrombotic properties (Table 1). Physiologic pulsatile ESS constitutes the most potent stimulus for continuous NO production by the endothelium, an effect that is regulated at either transcriptional level through upregulation of endothelial nitric

oxide synthase (eNOS) gene expression or at post-transcriptional level by eNOS protein phosphorylation and activation. In arterial regions with disturbed flow, low ESS reduces the bioavailability of NO by decreasing eNOS messenger ribonucleic acid (mRNA) and protein expression, thereby exposing the endothelium to the atherogenic effect of local and systemic risk factors (Figure 11).

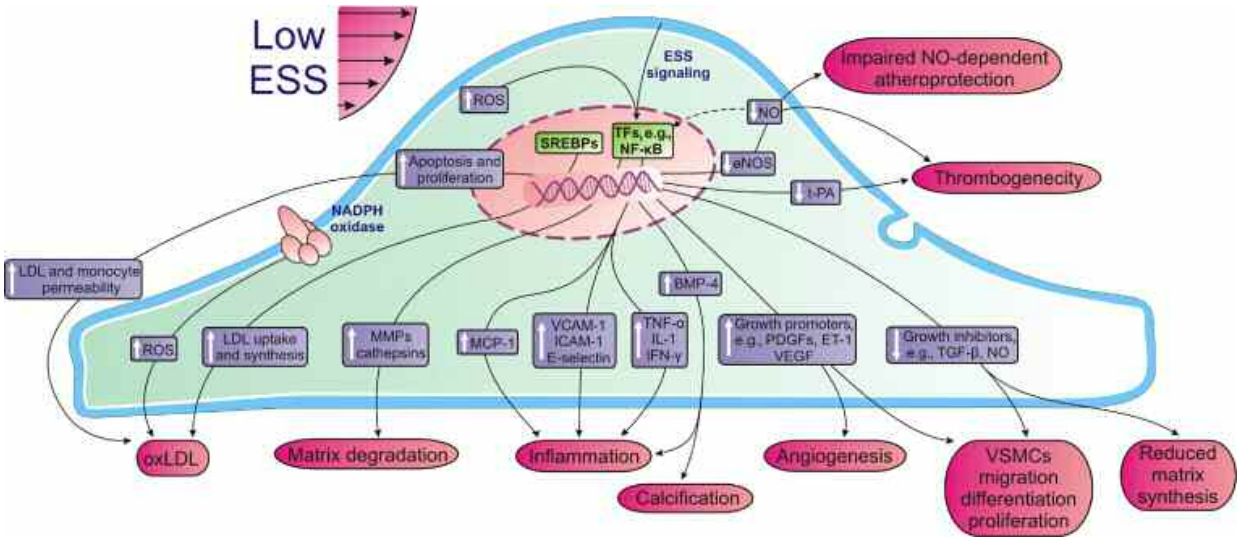


Figure 11. Role of Low ESS in Atherosclerosis.

In addition, low ESS down regulates prostacyclin, another endothelial vasodilatory substance, while upregulating endothelin-1 (ET-1) (33), a potent vasoconstrictive and mitogenic molecule, thereby precipitating atherosclerosis.

Low ESS promotes endothelial gene and protein expression of potent VSMC mitogens, such as platelet-derived growth factor (PDGF)-A and -B isoforms, ET-1, and vascular endothelial growth factor (VEGF) (Fig). Low ESS-induced formation of ROS and pro-inflammatory cytokines also promote the expression of these growth factors. Also, low and disturbed flow decreases the endothelial expression of plasminogen activator inhibitor (PAI)-1, an inhibitor of VSMC migration . Although the effect of low ESS on basic fibroblast growth factor (bFGF), angiotensin converting enzyme (ACE), and angiotensin II is yet unclear, potent suppressors of cell growth and migration, such as NO and transforming growth factor (TGF)-β are downregulated in areas with low and disturbed flow. Ultimately, low ESS-mediated over-expression of growth promoters and under-expression of growth inhibitors by the ECs stimulate VSMCs to migrate from media to intima through a regionally disrupted internal elastic lamina (IEL). Within the intima VSMCs acquire a "synthetic" phenotype, producing collagen

and other extracellular matrix (ECM) proteins, and proliferate. Over time, VSMCs along with the fibroblasts create a fibrous cap around the lipid core isolating the thrombogenic lipid material from the circulating platelets. The fibrous cap along with the lipid core constitutes the so-called early atherosclerotic plaque (fibrous cap atheroma; American Heart Association type IV lesion).

Table 1. Endothelial Genes and Vascular Functions Regulated by Low ED in Atherosclerosis

	Effect of Low ESS
Impaired flow-dependent vasodilation	?
Vasodilators	?
eNOS/NO	Downregulated
Prostacyclin	Downregulated
Vasoconstrictors	?
ET-1	Upregulated
Subendothelial accumulation of LDL	?
Endothelial LDL uptake and synthesis	Increased
Endothelial LDL permeability	Increased
Blood stagnation—accumulation of LDL close to the wall	Increased
ECs proliferation and apoptosis	Increased
Oxidative stress	?
Oxidative enzymes	?
NADPH oxidase	Upregulated)
Xanthine oxidase	Upregulated)
Antioxidative enzymes	?
Mn SOD	Downregulated
Glutathione	Downregulated
Inflammation	?
Chemoattractants (MCP-1)	Upregulated
Adhesion molecules (VCAM-1, ICAM-1, E-selectin)	Upregulated
Cytokines (TNF-α, IL-1, IFN-γ)	Upregulated
BMP-4	Upregulated
Leukocyte pseudopod projection	Increased
Blood stagnation—accumulation of monocytes close to the wall	Increased
VSMCs migration, differentiation, and proliferation	?
Growth promoters	?

	Effect of Low ESS
PDGF-A, PDGF-B	Upregulated
ET-1	Upregulated
VEGF	Upregulated
bFGF	Unclear
ACE	Unclear
Angiotensin II	Unclear
Growth inhibitors	?
eNOS/NO	Downregulated
TGF-β	Downregulated
PAI-1	Downregulated
Regulation of extracellular matrix content and composition	?
Increased matrix degradation	?
MMP-2, MMP-9	Upregulated)
Cathepsin L	Upregulated
Reduced matrix synthesis	?
IFN-γ	Upregulated
VSMCs apoptosis	Increased
eNOS/NO	Downregulated
TGF-β	Downregulated
Neovascularization	?
VEGF	Upregulated
Other angiogenic factors (e.g., angiopoietin-2)	Upregulated
Plaque calcification	?
BMP-4	Upregulated
Plaque thrombogenecity	?
eNOS/NO	Downregulated
Prostacyclin	Downregulated
Thrombomodulin	No effect
t-PA	Downregulated)
Blood stagnation— accumulation of blood thrombogenic factors close to the wall	Increased)

ACE = angiotensin-converting enzyme; bFGF = basic fibroblast growth factor; BMP = bone morphogenic protein; EC = endothelial cell; eNOS/NO = endothelial nitric oxide synthase/nitric oxide; ESS = endothelial shear stress; ET = endothelin; ICAM = intercellular adhesion molecule; IFN = interferon; IL = interleukin; LDL = low-density lipoprotein cholesterol; MCP = monocyte chemoattractant protein; MMP = matrix metalloproteinase; Mn SOD = manganese-dependant superoxide dismutase; NADPH = nicotinamide adenine dinucleotide phosphate; PAI = plasminogen activator inhibitor; PDGF = platelet derived growth factor; TGF = transforming growth factor; TNF = tumor necrosis factor; t-PA = tissue plasminogen activator; VCAM = vascular cell adhesion molecule; VEGF = vascular endothelial growth factor; VSMC = vascular smooth muscle cell.. ?=unknown

GENETIC DETERMINANTS OF CAD

Often the aforementioned traditional and novel risk factors are not enough to the manifestation of a clinical cardiovascular disease: patients with low risk score based on the traditional risk factors (PROCAM study and from ESC/ESH guidelines (34;35); may have a high genetic susceptibility for MI and CAD. Data on subclinical atherosclerosis indicate that this is hereditable with a percentage of variance due to genetic factors in different vascular beds (36-38).

Thus, information in both environmental and genetics factors are likely to be necessary to accomplish an accurate prediction of individual risk. Recent data (39-42)support this evidention.

GENETIC FACTORS

Among risk factors, family history is one of the most significant independent risk factor for CAD/MI. Twin studies also suggest the hypothesis that genetic factors contribute to the development of CAD and MI (43).

The most frequently used method for identifying the susceptibility genes for CAD and MI has been candidate gene case-control association studies. Methodologically, this is the easiest approach by which a candidate gene is selected based on its potential involvement in CAD/MI. Single-nucleotide polymorphisms (SNPs) are identified in the gene and genotyped in a group of patients (cases) and controls. The frequencies of SNP alleles or genotypes are then analyzed. An allele or genotype is associated with the disease if this occurrence in the cases is significantly different from that in the controls (Figure 12).

The results from the candidate gene association studies should be interpreted with caution as many of these studies are confounded by the selection bias of cases and controls, population admixture, imperfect matching of cases and controls, phenotyping errors, and small sample size. But significant progress has been made in mapping and identifying disease-causing genes and susceptibility genes for CAD and MI using genome-wide linkage analysis with large families of hundreds of small nuclear families and genome-wide association studies.

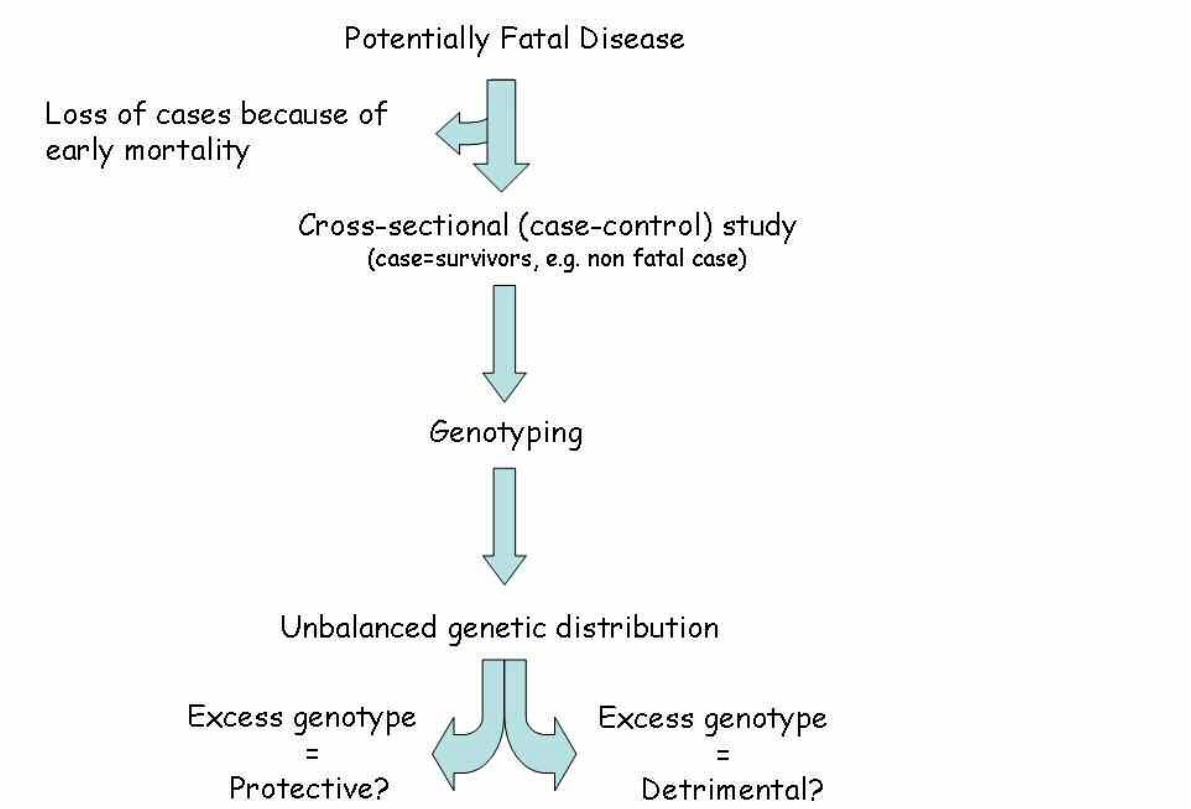


Figure 12.

Linkage analysis and association study

Two different types of linkage analyses have been used to map the chromosomal locations of genes for CAD and MI. In *linkage analysis*, the goal is to identify at least one polymorphic marker at a specific chromosomal location that co-inherits with the disease, which then suggests that the marker and disease gene are located close to each other, and the location of the marker is taken as the location of the disease gene.

- The first type of linkage analysis is model-bases linkage analysis using large families in which the inheritance pattern of the disease gene in the families is clearly defined. The family or families are generally genotyped with approximately 400 polymorphic markers that span the entire human genome every 10 cM. The genotyping data is then analyzed with a model-based linkage program, yielding a logarithm of the odds (LOD) score for each marker. The LOD score represent the log base 10 of the likelihood ratio favoring linkage. An LOD score of 3.0 or higher for a marker that co-segregates with the disease in a family or families is considered as significant evidence for identification of linkage.

- The second type of linkage analysis is model-free analysis using hundreds of small nuclear families with at least two affected siblings in each family. Similar to model-based linkage

analysis, genome-wide genotyping is usually performed with about 400 markers providing genome coverage. The amount of marker-allele sharing observed in the sibling pairs with that which would be expected if no linkage is present is then compared. A significant excess of shared alleles by concordant siblings, and a significant dearth of sharing by discordant siblings, is considered as evidence for linkage. *P* value or LOD scores are calculated without assumption of any inheritance model and a LOD score of 3.6 or a P value of 2.2x10-5 indicates significant linkage.

Disease-causing genes for coronary artery disease and myocardial infarction

When a genetic locus is mapped, candidates genes that are located in the region and potentially relevant to the disease physiology are selected for identification of apthogentic mutations that cause the disease (*disease-causing gene*, monogenic trait) or for identification of single nucleotide polymorphisms (SNPs) and SNP haplotypes that are associated with the disease (*susceptibility gene*, complex trait). *Disease-linked genes* are the genes that are connected to the disease by molecular biology, microarray, or proteomic analyses, but their relation to the disease as a cause or a consequence is not established. Some disease-linked gene may serve as biomarkers for the disease.

Disease-causing genes for disorders in lipid metabolism, a high risk factor for coronary artery disease (25)

Disease-causing genes have been identified for familial hypercholesterolemia and Tangier disease, both of which increase risk of premature arteriosclerosis and CAD. Hypercholesterolemia is characterized by elevation in levels of plasma cholesterol bound to LDL, tendon xanthomas, and atheroma and can be caused by mutations in the LDL receptor (*LDLR*) gene, the ApoB-100 gene, the proprotein convertase subtilisin/kexin-type 9 gene (*PCSK9*), the cholesterol 7-α-hydroxylase gene (*CYP7A1*)], and the *ARH* gene. Tangier disease is a rare disorder characterized by the absence of HDL cholesterol from plasma and deposition of cholesterol esters in the reticuloendothelial system, with splenomegaly and enlargement of tonsils and lymph nodes, hepatosplenomegaly, and peripheral neuropathy. The gene for Tangier disease has been identified as *ABCA1*, which encodes a member of the ATP-binding cassette transporter family involved in the efflux of lipids from peripheral cells to ApoA-I forming nascent HDL particles.

Susceptibility genes for coronary artery disease and myocardial infarction

As discussed above, numerous case-control studies have been carried out to identify susceptibility genes for CAD and MI. Genetic variants or SNPs in many candidate genes with physiologic relevance to CAD and MI have been found to be associated with increased or

decreased risks for CAD and MI. Numerous possible susceptibility genes have been identified for CAD and MI. Many of these studies need to be further validated or replicated as false-positive results can be generated easily in a case-control association study due to selection bias of cases and controls and population admixture. This article reviews only susceptibility genes identified by genome-wide association studies and by positional cloning based on genetic linkage analysis with small nuclear families.

Transcriptional factor gene *USF1* and familial combined hyperlipidemia .

Familial combined hyperlipidemia (FCHL) is present in about 20% of patients with CAD and is characterized by elevated serum total cholesterol or triglycerides. A major susceptibility locus for FCHL was mapped to chromosome 1q21–23 in a Finnish population. Recently, two synonymous SNPs in the *USF1* gene were found to be significantly associated with FCHL (P = 0.00002) (44). *USF1* encodes a transcriptional factor belonging to the basic helix-loop-helix leucine zipper family and regulates genes involved in glucose and lipid metabolism, including *ABCA1* and *apolipoproteins CIII*, *AII*, and *E*. The characteristics of the downstream genes regulated by *USF1* make it an attractive gene for the pathogenesis of FCHL; however, it remains to be determined whether the two SNPs associated with FCHL are the true causative variants, a common problem with association studies.

Cytokine gene lymphotoxin-α and myocardial infarction

Development of a high-density SNP map covering the entire human genome makes it possible to identify susceptibility genes for complex disease such as CAD and MI using genome-wide association studies. For a genome-wide association study, 50,000–100,000 SNPs are estimated to be sufficient to provide genome coverage, and a P value of $<5 \times 10^{-7}$ was proposed to be a cutoff value for achieving significance. The first genome-wide case-control association study for CAD and MI was carried out using 92 788 gene-based SNPs with 94 Japanese patients with MI. Positive SNPs with a nominal significance P value of 0.01 were then genotyped in 1133 MI cases and 1006 controls (45). Three SNPs in the *LTA* gene (exon 1 10G/A, intron 1 252A/G, exon 3 p.Thr26Asn) were found to be significantly associated with a high risk of MI when they were homozygous (odds ratio = 1.69–1.78; $P = 2.2 \times 10^{-5}$ to 3.3×10^{-6}). Lymphotoxin-α is a cytokine that mediates immune responses and inflammation. The functional effects of the three *LTA* SNPs associated with MI were examined with some biologic assays. SNP 252A/G in intron 1, but not SNP 10G/A in exon 1, increased expression of LTA by 1.5-fold. SNP p.Thr26Asn in exon 3 increased expression of adhesion molecules and cytokines including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, tumor necrosis factor,

interleukin-1A, interleukin-1B, and selectin E by twofold. The results from functional studies strengthen the conclusion that *LTA* variants increase susceptibility to MI.

Although the *P* value of 3.3×10^{-6} from the Japanese study does not reach the proposed cutoff value for significance for a genome-wide association study, it is one of the most impressive *P* values that was ever achieved for case-control association studies for CAD/MI. Interestingly, an independent case-control association study was carried out with 1891 MI patients and 1798 controls, also from a Japanese population, but failed to identify any association between SNP 252A/G in intron 1 or p.Thr26Asn in exon 3 of the *LTA* gene and MI. SNP 252A/G in intron 1 was also analyzed in a German population, and no association was detected with CAD or MI or with the risk of restenosis, death, or MI after coronary artery stenting. On the positive side, studies of more than 400 parental-proband trios families showed positive association of SNP p.Thr26Asn in exon 3 with CAD in white Europeans . Clearly, more research, both genetic and functional studies, is needed to further test the association between *LTA* SNPs and CAD/MI.

Lymphtoxin-α-regulatory gene *LGALS2* and myocardial infarction

Ozaki *et al.* (45) performed a study to identify proteins that interact with LTA and found that galectin-2 (encoded by the *LGALS2* gene), a member of the galactose-binding lectin family, binds to LTA and regulates the extracellular secretion of LTA. One SNP, 3279C/T in intron 1, of *LGALS2* was found to be significantly associated with MI (odds ratio = 1.57, $P = 2.6 \times 10^{-6}$), and the minor allele has a protective role against MI. Functional studies showed that the minor allele T reduced expression of *LGALS2* by 50%. Reduced expression of galectin-2 is expected to decrease the extracellular level of LTA, leading to less inflammation and reduced risk for MI. It will be interesting to test whether the association between *LGALS2* and MI can be replicated in another independent Japanese population or other populations.

ALOX5AP (encoding 5-lipoxygenase-activating protein) and myocardial infarction and stroke

A genome-wide linkage scan with 296 Icelandic families identified a suggestive linkage to MI on chromosome 13q12-13 in women — LOD score (log base 10 of the likelihood ratio under the hypotheses of linkage and nonlinkage) = 2.86 (46)– but not in men (LOD score = 1.51). *ALOX5AP* encoding 5-lipoxygenase-activating protein (FLAP) is located in the region and became a good candidate gene. In a case-control association study with 779 MI patients and 624 controls, a haplotype (HapA) involving four SNPs in *ALOX5AP* was found to be significantly associated with MI (relative risk of 1.80, $P = 2.3 \times 10^{-5}$, which remains significant, 0.005, after adjusted for the number of haplotypes tested) and also associated with stroke to a

lesser degree. A different haplotype, HapB, involving four other SNPs of *ALOX5AP* was significantly associated with MI in a British population (relative risk of 1.95, $P = 3.7 \times 10^{-4}$), which provides supportive evidence for the association between *ALOX5AP* and MI. Before the *ALOX5AP* report, the gene for 5-lipoxygenase (*5-LO*) was already shown to be involved in susceptibility to atherosclerosis in mice and was associated with carotid intimal-medial thickness in a cohort of 470 healthy, middle-aged women and men from the Los Angeles Atherosclerosis Study. 5-Lipoxygenase is an enzyme that mediates the production of leukotrienes, which are inflammatory mediators generated from arachidonic acid. All these studies suggest that the 5-lipoxygenase pathway plays an important role in increasing susceptibility to CAD and MI. It is important to note, however, that when the data from the *ALOX5AP* study were analyzed separately for women where the original linkage was identified, the significance level of the association with MI was reduced to 9.8×10^{-3}, which may become nonsignificant after adjusting for the number of haplotypes tested. This raises a possibility that there may be another gene that increases susceptibility to MI in women under the 13q12–13 linkage peak.

Phosphodiesterase 4D gene and stroke

A genome-wide linkage scan in an Icelandic population mapped a susceptibility locus for stroke on chromosome 5q12 (LOD score = 4.40). The susceptibility gene at this locus was identified as *PDE4D*, a gene that encodes the phosphodiesterase 4D (47). The strongest association was identified between different haplotypes of *PDE4D* and combined carotid and cardiogenic strokes, the forms of stroke related to atherosclerosis. The role of *PDE4D* in ischemic stroke is not clear, but the PDE4D protein degrades the second messenger cAMP, a key signalling molecule involved in inflammatory responses of vascular cells to oxidized lipids. The link between *PDE4D* and stroke is also supported by the finding that *PDE4D* is involved in susceptibility to ischemic brain damage in animal models. As with other case-control association studies, replication in an independent population will strengthen the association between *PDE4D* and stroke.

Two other genetic loci have been identified for MI on chromosomes 1p and 14q, and four significant linkages were reported for CAD on chromosomes 2q, 3q, 16p, and Xq (Table 2), but the specific genes at these loci remain to be identified.

Table 2 Major disease-causing and susceptibility genes for patients with coronary artery disease and myocardial infarction

	Chromosomal location	Gene (function)	Phenotype
Disease-causing genes			
adCAD1 (autosomal dominant CAD locus 1)	15q26	*MEF2A* (transcription factor in endothelium of coronary arteries)	CAD, MI
adCAD2	*?*	*?*	*?* *?*
Susceptibility genes			
1	1p34–36	?	MI
2	2q21.1–22	?	CAD
3	2q36–37	?	Acute coronary syndrome
4	3q13	?	CAD
5	5q12	*PDE4D* (phosphodiesterase 4D, cAMP signaling, inflammation)	Ischemic stroke
6	6p21	*LTA* (lymphotoxin-α, cytokine, inflammation, immune response)	MI
7	13q12–13	*ALOX5AP* (FLAP, generating leukotrienes, inflammatory mediators)	MI, stroke
8	14q	?	MI
9	16p13-ter	?	CAD
10	22q13.1	*LGALS2* (galectin-2, regulates secretion of LTA; inflammation)	MI
11	Xq23–26	?	CAD

CAD, coronary artery disease; LTA, lymphotoxin-α; MI, myocardial infarction; PDE4D, phosphodiesterase 4D.

Disease-linked genes for coronary artery disease and myocardial infarction

New genomic and proteomic approaches have begun to identify genes whose expression is linked to CAD and MI. Microarray analysis allows simultaneous analysis of expression of thousands of genes in CAD tissues *vs* non-CAD tissues. As an example, expression of 49 genes was newly linked to CAD, and these genes include intercellular adhesion molecule-2, PIM2, ECGF1, fusin, B cell activator (BL34, GOS8), Rho GTPase activating protein-4, retinoic acid receptor responder, β2-arrestin, and many others. Many other genes have been linked to CAD by microarray analysis and have been extensively reviewed (48).

The first proteomic study of CAD was reported recently (49). Proteins from CAD and non-CAD coronary arteries were separated by two-dimensional gel electrophoresis. Protein spots that showed different expression levels in two tissues were excised from the gels and identified by mass spectrometric analysis. The results from the two-dimensional gel analysis were confirmed by Western blot analysis. Expression of the ferritin light chain was found to be significantly increased in the diseased coronary arteries by about twofold. These results link the

ferritin light chain gene to CAD and supports the 'iron hypothesis' that proposes an association between excessive iron storage and a high risk of CAD. It remains to be determined whether an elevated ferritin level is a contributor or causative factor for atherosclerotic CAD or is merely associated with the disease process. Nevertheless, increased ferritin expression in coronary arteries may become a significant biomarker for atherosclerotic CAD and may be developed as a diagnostic marker for the disease with more studies in the future (40-42).

Disease-causing genes for coronary artery disease and myocardial infarction

Only one disease-causing gene, *myocyte enhancer factor-2 (MEF2A),* encoding a member of the MEF2 family of transcription factors, has been identified for primary CAD and MI without other accompanying clinical feature like hypercholesterolemia and Tangier disease .
(44;47;50-53).

MEF2A AND CORONARY ARTERY DISEASE

Mutation of MEF2A in an Inherited Disorder with Features of Coronary Artery Disease

In the 28 November 2003 issue of Science, Wang et al. describe a human pedigree with an autosomal dominant predisposition to CAD and early-onset MI (52). They studied a large family with 13 patients who displayed an autosomal dominant pattern of CAD (kindred QW1576 in Fig and Table). Multiple risk factors, including dyslipidemia, hypertension, and cigarette smoking, were present in some family members (Table S1). Nine of the 13 patients developed acute MI (Table 3). They carried out a genome-wide linkage scan with 382 markers that span chromosomes 1 to 22, with an average interval of 10 cM. The positive linkage was identified for marker *D15S120* with a lod score (logarithm of the odds ratio for linkage) of 4.19 at a recombination fraction of 0. Further haplotype analysis with markers *D15S1014, D15S212*, and *D15S87* verified the observed linkage (Figure 13). These data identified a significant linkage to autosomal dominant CAD/MI on chromosome 15q26; this locus is designated as *adCAD1* for the first autosomal dominant CAD and MI locus.

Table S1. Other risk factors for CAD/MI for the family members in Kindred QW1576.

ID#	Relevant Diagnosis	MEF2A Deletion	DLD	Smoking	HTN	DM	Other Risk Factors
I.1	CAD (MI)	Yes	No	N/A	No	No	Obesity
II.1	CAD (MI)	Yes	Yes	1pk/d	Yes	No	--
II.2	CAD (MI)	Yes	Yes	N/A	Yes	No	--
II.4	CAD MI)	Yes	Yes	No	No	No	--
II.5	Normal	No	No	No	Yes	No	--
II.6	CAD (MI)	Yes	No	3pks/d	Yes	Yes	--
II.8	CAD (MI)	Yes	Yes	<1pk/d	Yes	No	--
II.10	Normal	No	Yes	N/A	Yes	No	--
II.11	CAD	Yes	Yes	No	No	No	--
II.12	CAD (MI)	Yes	Yes	No	Yes	No	--
II.13	CAD	Yes	Yes	3pks/d	No	No	--
III.1	CAD	Yes	Yes	2pks/d	Yes	No	--
III.2	Uncertain	No	No	No	Yes	No	--
III.3	CAD	Yes	No	>1pk/d	No	No	--
III.4	CAD (MI)	Yes	Yes	No	Yes	No	--
III.5	Uncertain	No	No	No	No	No	--
III.6	CAD (MI)	N/A	No	N/A	Yes	No	Obesity
III.7	Normal	No	No	No	No	No	--
III.8	Normal	No	Yes	Occasional	Yes	No	--
III.9	Normal	No	Yes	No	Yes	No	---
III.10	Uncertain	No	N/A	N/A	N/A	N/A	---

CAD, coronary artery disease; MI, myocardial infarction; DLD, dyslipidemia (LDL >130 mg/dl); Smoking, cigarette smoking packs/day (pks/d); HTN, hypertension; DM, diabetes; N/A, data not available. Note that the 7 amino acid deletion of MEF2A co-segregates with CAD, but not with other CAD/MI risk factors including dyslipidemia, hypertension, or diabetes.

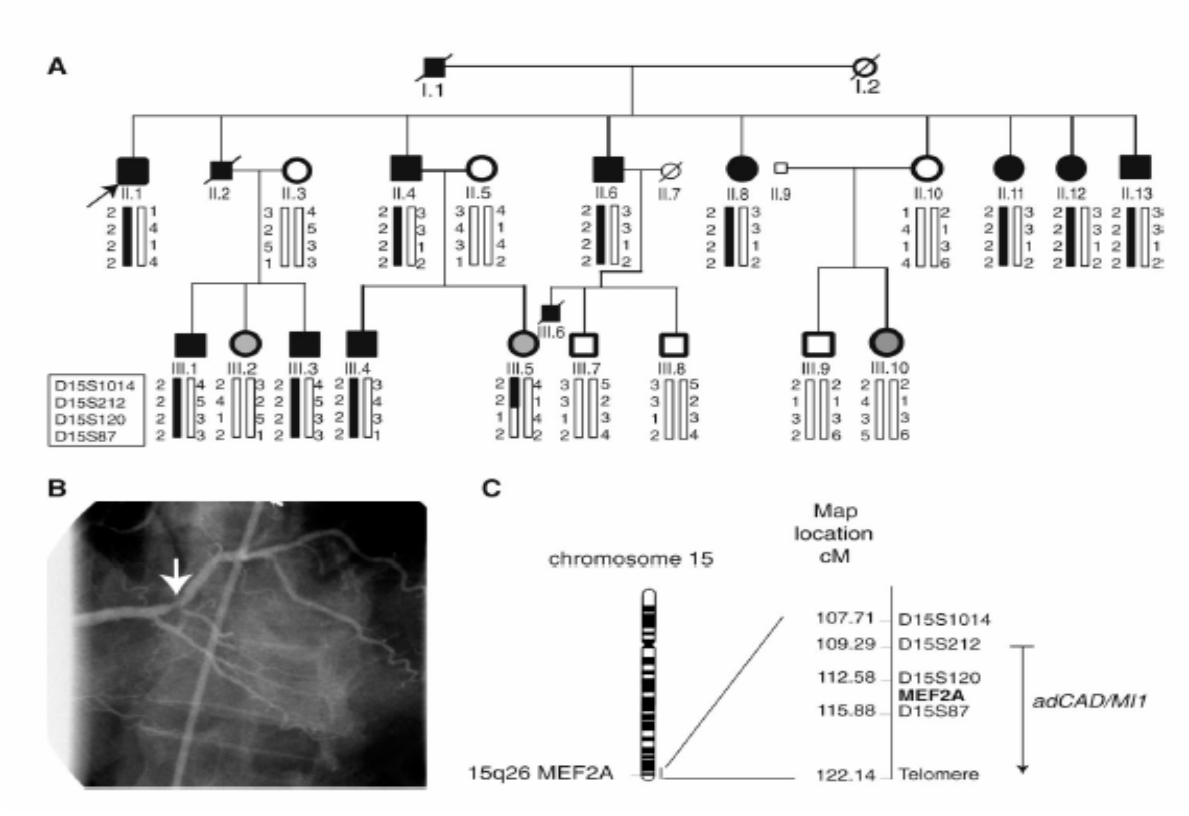


Figure 13. Genetic linkage of CAD/MI to chromosome 15q26 (*adCAD1*). (**A**) Pedigree structure and genotypic analysis of kindred QW1576. Individuals with CAD (Table) are indicated by solid squares (males) or solid circles (females). Unaffected individuals are indicated by open symbols. Normal males under the age of 50 years or normal females under 55 years are shown in light-gray as uncertain phenotype. Deceased individuals are indicated by a slash (/). The proband is indicated by an arrow. Genotypes for markers *D15S1014, D15S212, D15S120*, and *D15S87* are shown below each symbol. Initial linkage was identified with *D15S120*, which yielded a lod score of 4.19 at a recombination fraction of 0. Haplotype cosegregating with the disease is indicated by the black vertical bars. (**B**) Coronary angiogram from the proband, who experienced an inferior MI attributed to a plaque rupture lesion (arrow) with a 70% narrowing in the distal right coronary artery. This lesion is at a bifurcation site typical of the pattern of coronary atherosclerosis. The lesion was stented, and follow-up angiography of the site demonstrated wide patency, without any renarrowing. (**C**) Ideogram of chromosome 15 with the Geimsa banding pattern and localization of the *adCAD1* locus. The genetic map with chromosome 15q26 markers and the location of the *MEF2A* gene is shown on the right

Table 3 Clinical characteristics of the family members in kindred QW1576, a family with CAD and MI. PTCA, percutaneous coronary angioplasty; CABG, coronary artery bypass surgery; CATH, angiogram.

Individual ID no.	Age (years)*	Clinical diagnosis and treatment (age in years)†
I.1	-	MI (45)
II.1	63	MI (63), PTCA (63)
II.2	-	MI (65), CABG (65)
II.4	81	MI (80), CABG (65), CATH (>70% stenosis; 65)
II.5	81	Normal
II.6	61	MI (61), CABG (61)
II.8	77	MI (61), CATH (>70% stenosis; 61)
II.10	72	Normal
II.11	68	PTCA (68)
II.12	63	MI (63)
II.13	65	PTCA (64)
III.1	51	PTCA (35)
III.2	49	Uncertain (no symptoms, female, but ≤55 years of age)
III.3	47	PTCA (46)
III.4	49	MI (42), CABG (42)
III.5	50	Uncertain (no symptoms, female, but ≤55 years of age)
III.6	-	MI (40)
III.7	54	Normal (no symptoms, male, ≤50 years of age)
III.8	50	Normal (no symptoms, male, ≤50 years of age)
III.9	50	Normal (no symptoms, male, ≤50 years of age)
III.10	46	Uncertain (no symptoms, female, but ≤55 years of age)

The candidate *adCAD1* region contains ~93 genes, which consist of 43 known genes and 50 hypothetical genes. Among the known genes, *MEF2A,* which encodes a member of the myocyte enhancer factor–2 (MEF2) family of transcription factors (54), became a strong candidate, because *MEF2A* mRNA has been detected in blood vessels during mouse early embryogenesis (55).

Transcription factor MEF2A gene

MEF2 family belongs to the MADS (**M**CM1-**a**gamous-**d**eficiens-**s**erum response factor) supergene family of DNA binding proteins. In contrast to MRFs, MEF2 is expressed in a variety of adult tissues and established cell lines but at higher levels in skeletal muscle, cardiac muscle, smooth muscle, and neuronal cells. The MEF2 family in human and mouse comprises four members, MEF2A, B, C, and D. The RNA transcripts of all MEF2 members undergo alternative splicing processes with some of these being limited to specific cell types. The significance of these alternative splicing events is not yet clarified. MEF2 factors and other members of the MADS family share greater than 80% homology in the MADS domain (aa 2-57) (Figure 14) that determines the DNA-binding specificities of MADS proteins. MEF2 factors share an additional homology domain (aa 58-86) that is required for dimerization. The important role that MEF2 plays in the regulation of muscle development was indicated by the requirement of the MEF2 binding sites for the maximal activities of many skeletal muscle- and cardiac muscle-specific enhancers and promoters(56).

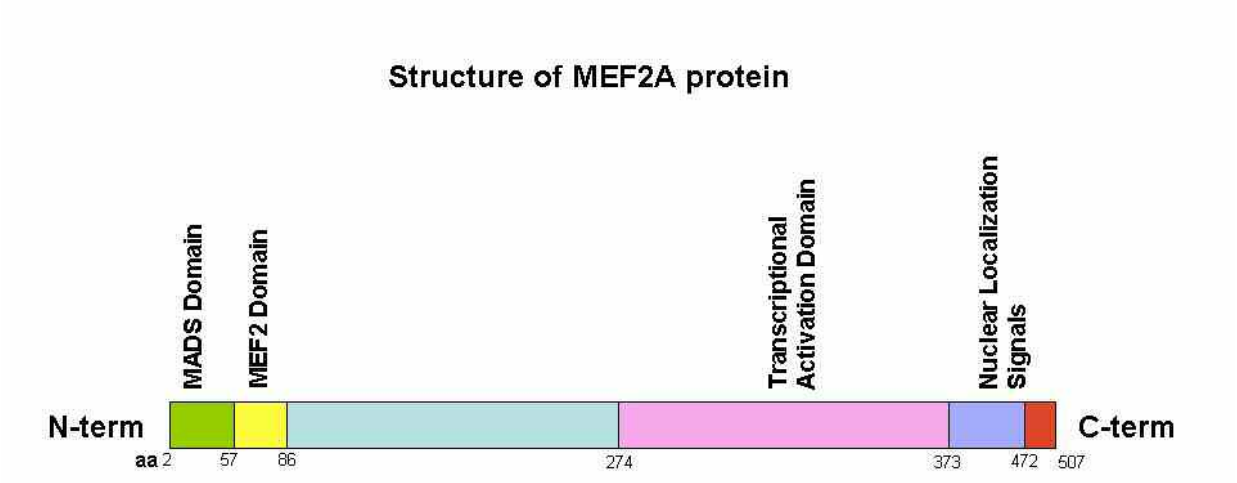


Figure 14. Structure of MEF2A protein. The MEF2A gene consists of 12 exons and encodes a 507 amino acid protein. The MADS domain and MEF2 domain at the N-terminal region are responsible for DNA binding, dimerization and interaction with other transcription factor. The transcription activation domain is located in the middle portion and the C-terminal region is responsible for nuclear localization site (NLS).

Furthermore, the level of MEF2A protein in quiescent vascular smooth muscle cells is markedly increased following serum stimulation, whereas the MEF2A mRNA levels remained unchanged, suggesting a translational control of MEF2A activity. Two other potentially important mechanisms for the regulation of MEF2A activity may lie in the control of its nuclear localization and its transcriptional activity. Transcription factors have modular structure, displaying distinct functional domains for various activities, including nuclear localization, DNA binding, ligand binding, dimerization, and transcriptional activation.

The nuclear localization signals (NLS) interacts with the NLS receptor, thus allowing transport through nuclear pore complexes in an ATP-dependent manner. The C-terminal 36-aa sequence of MEF2A contains a NLS that is capable of directing a normally cytoplasmic protein, β-galactosidase, to the nucleus. The identification of a NLS in MEF2A suggests that MEF2A can be transported to the nucleus by nuclear transport factors independently of a cofactor. The C-terminal 36-aa sequence of MEF2A (aa 472-507) does not contain any sequence with more than two consecutive basic amino acids. Therefore, it is likely that MEF2A contains a bipartite NLS.

MEF2A contains one dominant (aa 274-373) and two less active transcriptional activation domains (TADs). The activation domains of transcription factors have typically been classified on the basis of amino acid compositions, with groups comprising activators rich in acidic amino acids, glutamine (Gln), proline (Pro), or serine/threonine (Ser/Thr).

The hydrophobic amino acids may also play a role in the regulation of the transcriptional activity of MEF2A TAD via protein-protein interactions with the general transcription factors and/or coactivators. Each subdomain in the MEF2A TAD may mediate protein-protein interaction between MEF2A and one or more of its target proteins in the transcription initiation complex to regulate transcription efficiency. An alternatively spliced exon included in mRNA from skeletal muscle, heart, and brain, appears to play a greater role in the positive regulation of TAD activity in muscle cells. The two inhibitory domains also appear to have a cell type-specific effect on transcriptional activity.

These different interplays of subdomains may result from interactions of the subdomains with distinct coactivators in the transcription initiation complex in specific cell type. Since MEF2A is also expressed abundantly in cardiocytes, smooth muscle cells, and neuronal cells, the question whether MEF2A is regulated by distinct mechanisms in specific cell types remains to be addressed. As discussed below, several potential phosphorylation sites for various protein kinases are present in the TAD sequence. Therefore, it is likely that distinct signal transduction pathway(s) may operate in specific cell types to regulate the

activity of MEF2A (and other MEF2 factors). The MEF2A TAD contains multiple tyrosine, serine, and threonine residues. Potential phosphorylation sites for several protein kinases are present. It is conceivable that muscle cell differentiation may be mediated through MEF2 factors that are in turn regulated by various protein kinases.

Requirement of transcriptor factor MEF-2 for vascular development

Studies of MEF2 function initially focused on myogenesis and muscle-specific gene expression (57) (54). It is now apparent that these factors play many roles in various tissues and cell types, both during development and after terminal differentiation.

Mef2 protein contributes to the orchestration of skeletal muscle differentiation (58) and fiber type programming, cardiac development and hypertrophy, and vascular development and smooth muscle proliferation (59;60).

Formation of the vascular system is an essential early event in embryogenesis as well as wound healing and pathological processes associated with neovascularization, such as tumour angiogenesis. The process of vessel formation begins when angioblasts, which are derived from embryonic and extra embryonic mesoderm, become committed to an endothelial cell fate and organize into a primitive vascular plexus. This phase of vessel formation, referred to as vasculogenesis, requires endothelial cell migration and cell-cell interactions, lumen formation, and precise patterning of the vascular template. Smooth muscle cells (SMCs), which arise from multiple types of progenitors including neural crest cells, mesenchymal cells and endothelial cells, are then recruited to the endothelial cells network where they differentiate and form the contractile vessel walls (61). Subsequent proliferation and migration of endothelial cells within the primary vessels results in sprouting and remodelling of the vasculature, a process known as angiogenesis. Vessel formation also occurs within the embryonic yolk sac, however, in contrast to the vasculature of the embryo proper, the yolk sac vasculature does not contain SMCs and is composed solely of endothelial cells derived from blood islands.

While much has been learned about the roles of peptide growth factors and their receptors in vasculogenesis and angiogenesis, little is known of the transcription factors that regulate the formation, differentiation and patterning of endothelial cells or SMCs during vessel formation. Members of the myocyte enhancer factor-2 (MEF2) family of MADS (MCM1, Agamous, Deficiens, Serum response factor)-box transcription factors are expressed in endothelial cells and surrounding mesenchyme during embryogenesis. There are four vertebrate MEF2 genes, MEF2A, MEF2B; MEF2C, MEF2D, whose products bind as homodimers and heterodimers to an A/T-rich DNA sequence in the control regions of numerous of muscle-specific genes(54).

The phenotype of MEF2 mutant embryos (60) demonstrates that MEF2 plays multiple roles in formation of the vascular system by controlling patterning of endothelial cells into a primitive vascular plexus, as well as differentiation of SMCs. In the absence of MEF2, endothelial cells are specified, but they fail to become organized into a vascular network and SMCs do not differentiate. These defects suggest that MEF2 may function in developmental pathway for vascular development. The study of Lin et al. Show that MEf2 is expressed in the endothelial cell-derived vascular plexus and surrounding mesenchyme in the yolk sac. MEF2 is also expressed in endothelial cells, surrounding mesenchyme and smooth muscle cells of the developing vasculature of the embryo proper, as well as within neural crest cells in the brachial arches, which give rise to the brachial arch arteries and cardiac outflow tract (62).
In the absence of MEF2, the endothelial plexus is not stabilized and SMCs do not differentiate. Therefore, they propose that MEF2 is likely to play multiple roles in vascular development, being required for endothelial cell interactions and in mesenchimal cells surrounding the endothelial network for their responsiveness to endothelial cell signalling, their migration or their differentiation. The apparent failure of endothelial cells to interact properly to form a vascular plexus in MEF2 mutant embryos could reflect an underlying defect in cell adhesion.

The functions of MEF2 proteins in cardiovascular development and remodelling have been studied primarily in the mouse. Mice that lack the *MEF2C* gene die during embryogenesis from cardiac malformations, dysregulation of contractile protein gene expression, and an arrest in vascular development (see before)(59;60). In contrast, *MEF2A* knockout (null) mice die during the perinatal period from cardiac abnormalities that include fragmentation of the contractile apparatus, mitochondrial defects, arrhythmias, and pathological alterations in gene expression (58).

The phenotype of individuals that harbour the *MEF2A* mutation identified by Wang *et al.* (52) is clearly distinct from that of *MEF2A* null mice. The fact that coronary artery abnormalities have not been seen in heterozygous or homozygous *MEF2A* null mice supports the notion that the mutation in this affected pedigree creates a dominant negative version of the MEF2A protein that perturbs the activities of other MEF2 proteins, which might partially substitute for the lack of MEF2A in *MEF2A* null mice. However, it is also possible that mice are simply less sensitive than humans to the concentration of MEF2A protein or that the pathological consequences of MEF2A deficiency in humans are dependent on other genetic or environmental factors (diet, stress, age, and the like).

Consistent with its potential involvement in vascular function, MEF2A is expressed at high concentrations in the endothelial and smooth muscle layers of the coronary arteries. MEF2A and other MEF2 factors also have been shown to be up-regulated in smooth muscle cells within the vessel wall of balloon-injured rat carotid arteries (63).

MEF2: a calcium-dependent regulator of cell division, differentiation and death (64).

The three major decisions of a cell – whether to divide, differentiate or die – are profoundly influenced by signals from the environment. These signals culminate in the nucleus with the activation and repression of specific sets of genes whose products control the cellular processes. Intracellular calcium is a common currency among many of the signalling pathways that control cell division, differentiation and death. Recent studies have implicated myocyte enhancer factor-2 (MEF2) as a transcriptional effector of diverse calcium signalling pathways that control these fundamental cellular processes. The ability of a single transcription factor to govern mutually exclusive cellular decisions raises interesting questions about the mechanisms for transcriptional control and the integration of intracellular signals and cell identity. Here, we describe the diversity of mechanisms through which calcium signals regulate the transcriptional activity of MEF2, and the multiple roles of this transcription factor in the life and death of cells.

The MEF2 family of transcription factors

MEF2 was first described as a muscle-enriched transcription factor that bound to an A/T-rich DNA sequence in the control regions of numerous muscle-specific genes (reviewed in Ref. [1]). However, it soon became apparent that MEF2, while highly expressed in muscle cells, was also expressed at high levels in neurons and at lower levels in a wide range of cell types. There are four vertebrate *MEF2* genes, *MEF2A*, *-B*, *-C* and *-D*, which are expressed in distinct, but overlapping, patterns during embryogenesis, and in adult tissues. MEF2 proteins are nearly identical at their N-termini; where they have a MADS domain (present in MCM1, Agamous, Deficiens and serum response factor). This domain mediates dimerization and binding to the DNA sequence CTA(A/T)$_4$TAG/A. An adjacent MEF2-specific domain influences DNA-binding affinity and cofactor interactions, and the C-terminal regions of MEF2 proteins are required for transcriptional activation. Similar to other MADS domain proteins, MEF2 factors associate with a variety of transcriptional cofactors to control specific sets of downstream target genes. The target genes activated by MEF2 depend on the cell type and on the activation (or repression) of a variety of intracellular signaling pathways. As discussed in this article, MEF2

proteins are especially sensitive to calcium signals, which act through multiple post-translational mechanisms to modulate the transcriptional activity of these proteins.

Functions of MEF2 in muscle, neurons and immune cells

MEF2 has been studied most extensively in muscle cells.. MEF2 binds directly to the promoters or enhancers of the majority of muscle-specific genes and interacts with members of the MyoD family of basic helix–loop–helix (bHLH) proteins to activate the skeletal muscle differentiation program. Loss-of-function mutations of the murine *MEF2C* gene and of the *Drosophila melanogaster MEF2* gene have demonstrated an essential role for MEF2 in myogenesis and morphogenesis of striated and nonstriated muscle cell types. MEF2 factors have also been implicated in myocyte hypertrophy], in the formation of slow twitch skeletal muscle fibers (which maintain a relatively high intracellular calcium concentration compared with fast twitch fibers), and in vascular smooth muscle cell proliferation, differentiation and patterning.

An emerging body of evidence also supports roles for MEF2 as a calcium-dependent regulator of neuronal and immune cell differentiation and function. In post-mitotic neurons, MEF2 has been implicated as a calcium-dependent survival factor that protects from apoptotic cell death. In contrast to its anti-apoptotic activity in neurons, MEF2 serves a pro-apoptotic function during negative selection of thymocytes. In response to increased intracellular calcium concentration mediated by T-cell receptor signaling, MEF2 stimulates expression of the gene encoding the orphan nuclear hormone receptor Nur77 , also known as TR3, which is a potent activator of cytochrome *c*-mediated apoptosis. MEF2 binds directly to regulatory elements in the *Nur77* gene and cooperates with nuclear factor of activated T cells (NFAT) to drive *Nur77* expression.

Regulation of cell proliferation by MEF2

A large body of evidence implicates MEF2 as a key downstream effector of mitogenic signaling pathways. The connection between MEF2 and cell proliferation was made with the discovery that MEF2 regulates serum-inducible expression of *c-jun*, which positively regulates cell-cycle progression. MEF2 has since been shown to play a role in the induction of the *c-jun* promoter in response to signals emanating from multiple cell surface receptors, including G-protein-coupled receptors , the epidermal growth factor receptor, the lipopolysaccharide receptor, and the CD28 co-stimulatory receptor in T lymphocytes.

It seems paradoxical that MEF2 could control such diverse and opposing cellular decisions as differentiation, proliferation and apoptosis. An understanding of how MEF2 proteins perform

these diverse functions lies in the protein–protein interactions and signal-responsiveness of this family of transcription factors.

CaMK signaling

MEF2 proteins act as integrators of calcium signals, many of which are controlled by the intracellular calcium-binding protein calmodulin. The calcium/calmodulin-dependent protein kinase (CaMK) is a potent activator of MEF2 activity. CaMK regulation of MEF2 activity appears to be intimately involved in the stimulation of cardiomyocyte hypertrophy, which is associated with the transcriptional activation of an array of fetal cardiac genes. A variety of agonists can evoke a hypertrophic response in cardiomyocytes in culture, and pharmacological inhibitors of CaMK signaling block induction of MEF2 in response to these agents.

CaMK also activates MEF2 *in vivo*, as revealed by experiments using a line of transgenic mice, referred to as MEF2 indicator mice, that harbor a *lacZ* reporter gene linked to tandem copies of the MEF2 consensus DNA-binding site. Ectopic expression of activated CaMKIV in the heart led to a >100-fold increase in MEF2 transcriptional activity without changing MEF2 DNA-binding activity.

How does CaMK signaling activate MEF2? CaMK is capable of phosphorylating MEF2D, resulting in increased transcriptional activity. However, CaMK can also activate transcription mediated by MEF2A and -C, but there is no evidence that it can phosphorylate these MEF2 family members, raising questions about the universality of this mechanism for CaMK-mediated activation of MEF2-dependent transcription. Recently, we showed that the region of MEF2 that confers responsiveness to CaMK signaling maps to the MADS/MEF2 domains, which mediate DNA binding and cofactor interactions. We found no evidence for phosphorylation of this region of MEF2 by CaMK, suggesting that CaMK conveys a signal to MEF2 via an intermediary factor associated with the MADS/MEF2 domain. Indeed, this domain mediates interactions between MEF2 and histone deacetylases (HDACs), which act as transcriptional repressors. CaMK activates MEF2 by disrupting MEF2–HDAC interactions.

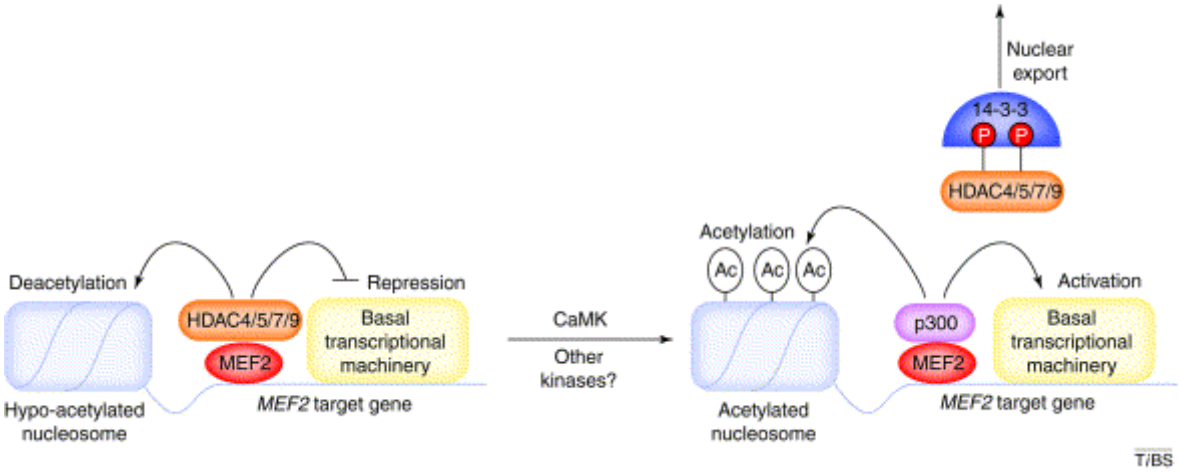


Figure 15. Activation of MEF2 by CaMK signaling.

CaMK signaling to HDACs

There appear to be at least 17 HDACs in humans, which are grouped into three classes (I, II and III) on the basis of their homology with three structurally and biochemically distinct yeast HDACs: Rpd3p, Hda1p and Sir2, respectively. HDACs -4, -5 and -7 are class II HDACs that interact with the MADS/MEF2 domains of all MEF2 family members through a unique 18-amino acid motif not found in other HDACs. This interaction does not affect MEF2 DNA-binding activity and results in recruitment of HDACs to MEF2-dependent gene regulatory regions. Recently, a fourth class II HDAC with the capacity to inhibit MEF2 function was described, HDAC9. In adult tissues, the most abundant mRNA transcript for HDAC9 encodes a truncated form of the protein, termed MEF2-interacting transcription repressor (MITR). MITR does not possess intrinsic HDAC activity and inhibits MEF2-dependent genes by associating with HDACs and CtBP, a ubiquitous co-repressor that associates with a variety of transcriptional regulators. Consistent with a role for class II HDACs and MITR in the regulation of MEF2, these repressors are expressed predominantly in heart, brain and skeletal muscle, mirroring the tissues in which MEF2 is expressed at highest levels.

HDACs repress transcription by deacetylating the N-terminal tails of core histones, which results in chromatin condensation. The repressive activity of HDACs is antagonized by histone acetyltransferases (HATs), such as p300 and PCAF, which acetylate histone tails, relaxing chromatin by weakening the electrostatic interactions between positively charged histone tails and negatively charged DNA. Acetylated histones also serve as binding sites for bromodomain-containing proteins, which typically contain HAT activity and further enhance transcription.

Signaling by CaMKIV, as well as CaMKI, releases MEF2 from the repressive influence of HDACs by phosphorylation of two conserved serine residues in the N-terminal regions of

HDACs -4, -5, -7 and -9 (Figure 15). Phosphorylation of these residues creates docking sites for the intracellular chaperone protein 14-3-3, which disrupts HDAC–MEF2 complexes upon association with phospho-HDACs. Binding of 14-3-3 also masks the HDAC nuclear localization signal (NLS) [and induces a conformational change in HDACs that unmasks a nuclear export sequence (NES) near the C terminus. As a result, HDACs are exported to the cytoplasm and the released MEF2 is then able to associate with the p300 coactivator and stimulate MEF2-dependent genes. Similar to HDACs, p300 associates with the MADS/MEF2 domains of MEF2 factors, suggesting that interactions between MEF2 and HATs or HDACs are mutually exclusive. MITR is also subject to 14-3-3-dependent release from MEF2. However, MITR lacks an NES and thus remains in the nucleus after dissociation from MEF2. Interestingly, 14-3-3 was also recently shown to stimulate MEF2D activity by directly associating with the MADS/MEF2 domains. The mechanism by which 14-3-3 activates MEF2D remains unclear, but has been proposed to involve the disruption of MEF2–HDAC complexes.

Signal-dependent release of class II HDACs from MEF2 appears to play a role in skeletal muscle differentiation. In proliferating, undifferentiated myoblasts, MEF2 is expressed at a low level and HDACs -5 and -7 are localized to the nucleus, where they are available to repress MEF2-dependent genes. When myoblasts are triggered to differentiate upon removal of growth factors from the medium, HDACs -5 and -7 translocate to the cytoplasm, presumably through the action of CaMK or another kinase with similar substrate specificity. Nuclear export of these HDACs results in enhanced activity of MEF2, which activates downstream muscle structural genes and also positively autoregulates its own expression, resulting in a dramatic increase in MEF2 activity. MITR is also subject to phosphorylation-dependent release from MEF2 during muscle differentiation, but remains in the nucleus because it lacks an NES.

It is important to make the distinction between nuclear export of HDACs and release of HDACs from MEF2 in the control of muscle differentiation. Serine-to-alanine mutants of HDAC-4, HDAC-5 and MITR, which cannot be phosphorylated by CaMK, associate irreversibly with MEF2 and potently block skeletal myogenesis. However, mutants that contain intact CaMK phosphorylation sites but remain in the nucleus because of the action of a *cis*-acting SV40 NLS are less efficient inhibitors of muscle differentiation than the serine-to-alanine mutants. Thus, release from MEF2 appears to be a crucial requirement for muscle differentiation, and nuclear export of HDACs probably serves as a reinforcing mechanism to ensure maximal activation of MEF2 target genes.

Paradoxically, HDAC-4 is expressed in the cytoplasm of myoblasts and partially translocates into the nuclei of myotubes. Whether or not this nuclear pool of HDAC-4 has the capacity to associate with MEF2 and repress gene transcription remains unknown. Despite this uncertainty, these results suggest that nuclear export of class II HDACs is required for the early stages of muscle differentiation, and that accumulation of these repressors in myotube nuclei might help establish the terminally differentiated phenotype.

Why are repressors of MEF2 activity expressed most abundantly in skeletal muscle, heart and brain – the same tissues in which MEF2 function appears to be most crucial? With regard to muscle, expression of class II HDACs in undifferentiated myoblasts could serve to prevent premature stimulation of MEF2-target genes, which would lead to precocious muscle differentiation. In post-mitotic muscle, MEF2 activity is significantly downregulated, but can be stimulated to high levels in response to stress, including electrical stimulation of skeletal muscle and hypertrophic signaling in the myocardium. Thus, in these tissues, HDACs probably function to prevent inappropriate activation of downstream stress-response programs by MEF2. In neurons, CaMK signaling plays a key role in the transduction of synaptic signals that mediate changes in gene expression. It is intriguing to speculate that such cues could control neural gene expression patterns by altering the phosphorylation status of HDACs, with resulting effects on MEF2 activity.

Calcineurin signaling to MEF2

Calcineurin is a serine/threonine phosphatase that is activated by the binding of calcium and calmodulin. In contrast to CaMK, which is preferentially activated by transient, high-amplitude calcium spikes, calcineurin responds to sustained, low-amplitude calcium transients. The best-known transcriptional targets for calcineurin are members of the NFAT family of transcription factors, which translocate to the nucleus in response to dephosphorylation by calcineurin. However, recent studies have demonstrated that MEF2 factors are also downstream targets of calcineurin signaling.

The first link between calcineurin and MEF2 was made in studies of the Epstein-Barr virus (EBV) lytic cycle. In B cells latently infected with EBV, immunoglobulin crosslinking led to calcium-dependent activation of MEF2 which, in turn, stimulated the expression of viral genes that promote EBV replication. Importantly, this MEF2-dependent response was blocked in cells treated with cyclosporin A, an immunosuppressant that specifically inhibits calcineurin activity.

One mechanism for calcineurin-dependent activation of MEF2 involves recruitment of NFAT proteins (Figure 16). Upon dephosphorylation by calcineurin, NFATc2, also referred to as NFATp/NFAT1, translocates to the nucleus where it directly associates with MEF2A and -D. NFATc2 stimulates MEF2-dependent transcription by facilitating recruitment of the p300 coactivator to MEF2-response elements. This mechanism for MEF2 activation does not require an NFAT-binding site and is mediated by the direct association of NFAT with MEF2 bound to DNA. Other NFAT family members do not appear to associate with MEF2, which provides a basis for transcriptional specificity, depending on the spectrum of NFAT isoforms expressed by a particular cell. MEF2–NFAT interactions appear to play crucial roles in T-lymphocyte apoptosis (by regulating expression of the *Nur77* gene) and in the control of slow twitch fiber genes in skeletal muscle.

A unique mechanism for calcineurin-mediated activation of MEF2 was demonstrated in neurons (Figure 16), where MEF2 appears to play a key role in protection from calcium-induced apoptosis. Calcineurin was shown to dephosphorylate MEF2A in response to membrane depolarization. Hypo-phosphorylated MEF2A bound DNA with higher affinity than its hyperphosphorylated counterpart and, thus, served as a more efficient transcriptional activator. Recently, it was shown that, in the absence depolarizing potassium, MEF2D is phosphorylated and targeted for caspase-mediated cleavage. Caspases cleaved the C-terminal transactivation domain of phospho-MEF2D, leaving an intact DNA-binding domain that acted as a dominant negative inhibitor of MEF2 target genes. Thus, under conditions of depolarization and high intracellular calcium concentration, calcineurin is activated and dephosphorylates MEF2, protecting it from caspase-mediated cleavage and thereby stimulating anti-apoptotic MEF2 target genes.

Calcineurin-mediated dephosphorylation of MEF2A and 2D has also been demonstrated in cultured skeletal myoblasts treated with calcium ionophore, and in functionally overloaded and electrically stimulated mouse muscle *in vivo*]. However, it remains unclear which phospho-residues on MEF2A and D are targeted by calcineurin, and whether this mode of calcineurin-dependent control applies to other MEF2 family members and other cell types.

Does calcineurin-mediated activation of MEF2 involve derepression of MEF2-associated HDACs? Although calcineurin and CaMK synergistically activate MEF2, calcineurin signaling alone is sufficient to activate MEF2 in some cell types. This implies that calcineurin is capable of relieving MEF2 from the repressive effects of associated HDACs. However, we have found no evidence for calcineurin-mediated nuclear export of HDACs. Although a direct effect of calcineurin signaling on HDAC catalytic activity cannot be ruled out, it is more likely that

calcineurin indirectly antagonizes HDACs via cross-talk with other signaling molecules such as CaMK. An alternative possibility is that both HDAC-bound and HDAC-free pools of MEF2 exist within a given cell, and that only the HDAC-free pool of MEF2 is capable of being activated by calcineurin signaling.

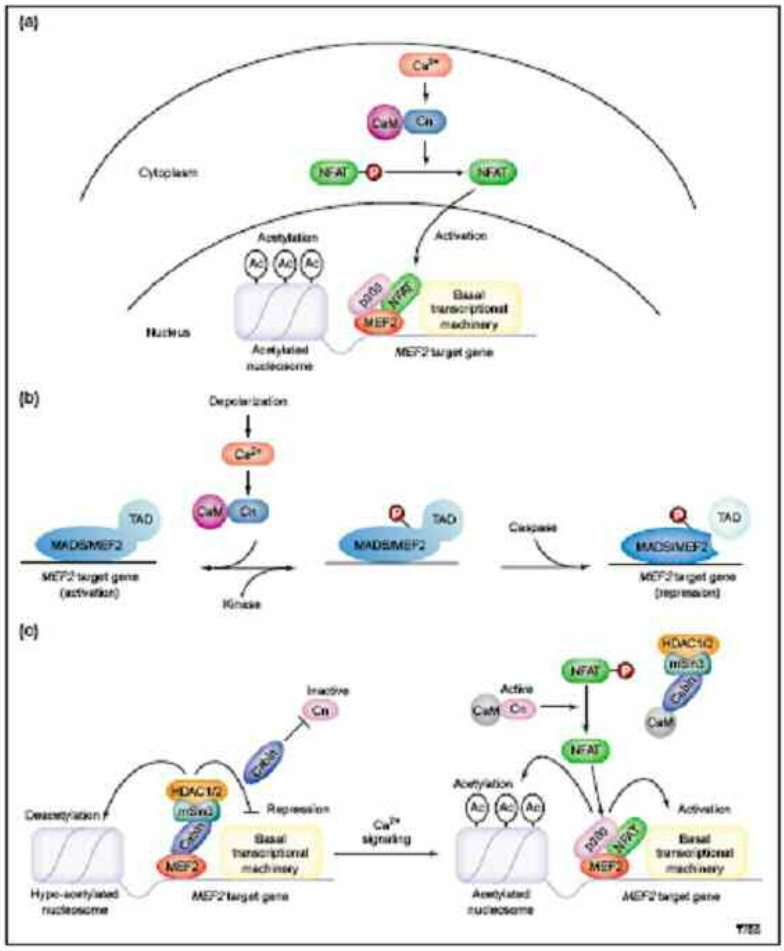


Figure 16. Three calcineurin-dependent mechanisms for regulation of MEF2 activity. (a) Recruitment of NFAT–p300 complexes to MEF2 target elements. (b) Stabilization of MEF2 protein. (c) Targeting MEF2–Cabin complexes.

Control of MEF2 by Cabin and calmodulin

Recent studies have also identified a more direct role for calmodulin in the control of MEF2 activity. In activated T lymphocytes, calcium-bound calmodulin disrupts interactions between MEF2D and a transcriptional repressor, termed Cabin1, which inhibits MEF2-dependent transcription by recruiting class I HDACs to MEF2 target genes via the mSin3 co-repressor (Figure). Calmodulin associates with the MEF2-binding site on Cabin . Thus, the competition between calmodulin and MEF2 for association with Cabin determines whether MEF2 target

genes are activated. Calcium-dependent release of MEF2 from Cabin frees the transcription factor to stimulate expression of the *Nur77* gene in association with p300 and NFATc2.

Interestingly, Cabin was originally identified as a protein that binds and inhibits the activity of calcineurin. The MEF2-binding region of Cabin is distinct from the region that interacts with calcineurin. In addition, Cabin was shown to block calcium-dependent association of MEF2 with ERK5, a mitogen-activated protein kinase (MAPK) that stimulates MEF2-dependent transcription (see below). Cabin therefore acts to inhibit MEF2 activity at three levels: (1) it recruits class I HDACs to MEF2 target genes; (2) it antagonizes the calcineurin phosphatase; and (3) it blocks association of MEF2 with ERK5.

Cabin is not expressed in muscle, whereas class II HDACs are abundant in these tissues. By contrast, both Cabin and class II HDACs are expressed in T cells. It remains unclear why these two disparate modes of repression evolved to regulate a single transcription factor family. One possibility is that distinct repressor complexes target different pools of MEF2 within a given cell and thus provide a means to differentially regulate subsets of MEF2-responsive genes. Such a mechanism could explain how MEF2 regulates genes that drive opposing cellular decisions such as differentiation and proliferation.

Calmodulin has also been shown to associate with HDAC-4 and disrupt MEF2–HDAC-4 complexes. In addition, we have demonstrated that CaMK signaling also disrupts these complexes. These distinct mechanisms probably function in concert to ensure maximal release of HDACs from MEF2 in response to calcium signaling.

MAPK signaling to MEF2

MAPKs couple MEF2 to multiple signaling pathways for cell growth and differentiation. MAPKs are calcium-responsive enzymes that are generally divided into three signaling cascades on the basis of the terminal effector kinase in the pathway: the extracellular signal-regulated protein kinase (ERK), c-jun N-terminal kinase (JNK) and p38 kinase pathways. The p38 and ERK pathways have been shown to stimulate MEF2 activity.

Using p38 as bait in a yeast two-hybrid screen, MEF2C was identified as a p38-binding protein]. There are four p38 isoforms (α, β, γ, δ) and each has now been shown to phosphorylate residues in the transcriptional activation domain of MEF2, resulting in enhanced MEF2 activity. Phosphorylation of MEF2 by p38 has been linked to activation of the hypertrophic program in cardiomyocytes, stimulation of skeletal muscle differentiation, expression of pro-inflammatory cytokines during a host response to bacterial pathogens, and protection of neurons from apoptosis.

With regard to the role of p38–MEF2 interactions in skeletal myogenesis, p38 activation can partially overcome the block to muscle differentiation imposed by HDAC-4 and -5. Interestingly, unlike CaMK, which inactivates HDAC-4 and -5 by stimulating their nuclear export, p38 has no effect on the subcellular localization of these HDACs. Possible alternative mechanisms by which p38 overrides HDAC action are that it stimulates MEF2 transcriptional activity to a level that effectively negates the repressive activity of HDACs on transcription, or that it directly inhibits HDAC catalytic activity. Resolution of these issues will provide insight into the regulation of MEF2-dependent gene programs in skeletal muscle, heart and brain – the tissues in which class II HDACs are most abundant.

MEF2 activity is also regulated by ERK5. ERK5 associates with MEF2 but, in contrast to p38, this kinase binds the MADS domain of MEF2 factors. In T cells, ERK5–MEF2 interactions are stimulated by increases in intracellular calcium and inhibited by Cabin. Two distinct modes of ERK5-mediated regulation of MEF2 have been described. ERK5 has been shown to phosphorylate the transactivation domains of MEF2A, -C and -D, resulting in increased transcriptional activity. ERK5-mediated phosphorylation of the MEF2C transactivation domain has been implicated in the regulation of cell-cycle progression through its stimulatory effects on *c-jun* expression. Surprisingly, ERK5 has also been shown to possess a transcriptional activation domain, supporting the notion that ERK5 stimulates MEF2-dependent transcription by functioning as a coactivator that facilitates recruitment of the basal transcription machinery. It seems likely that both mechanisms will apply to the regulation of MEF2 activity by ERK5.

Other signaling systems regulating MEF2

The number of signaling pathways that have been shown to impinge on MEF2 is rapidly growing. For example, SMADs, transcriptional regulators that are activated by transforming growth factor-β signaling, have recently been shown to associate with, and stimulate the activity of, the MEF2 transactivation domain. In addition, phosphoinositide-3-kinase (PI3-K) signaling has recently been implicated in the regulation of MEF2. PI3-K is essential for muscle differentiation and is a key mediator of the pro-myogenic action of insulin-like growth factor (IGF). Activated forms of PI3-K and its downstream effector AKT stimulate MEF2-dependent transcription, and inhibition of PI3-K activity with pharmacological agents and dominant-negative constructs blocks the induction of MEF2 activity in muscle cells. We showed that IGF signaling could rescue MEF2 from the inhibitory action of class II HDACs during muscle differentiation. However, to date, we have been unable to demonstrate IGF-induced nuclear export of HDACs. It is possible that IGF antagonizes HDAC action by inhibiting its catalytic

activity or, more likely, that IGF stimulates MEF2 transcriptional activity to a level that overrides the inhibitory effects of HDACs on MEF2.

Functional characterization of wild-type and Δ7aa MEF2A protein

The deleted residues in the familial mutant of MEF2A, Gln-Pro-Pro-Gln-Pro-Gln-Pro, are conserved in MEF2A proteins from other species and in other MEF2 factors. They are contained in the region of the protein required for nuclear localization (56;65). Not surprisingly, this mutant MEF2A protein is sequestered in the cytoplasm of transfected cells and acts as a dominant negative mutant, presumably by forming heterodimers with wild-type MEF2A monomers or cofactors such as GATA factors. (66).

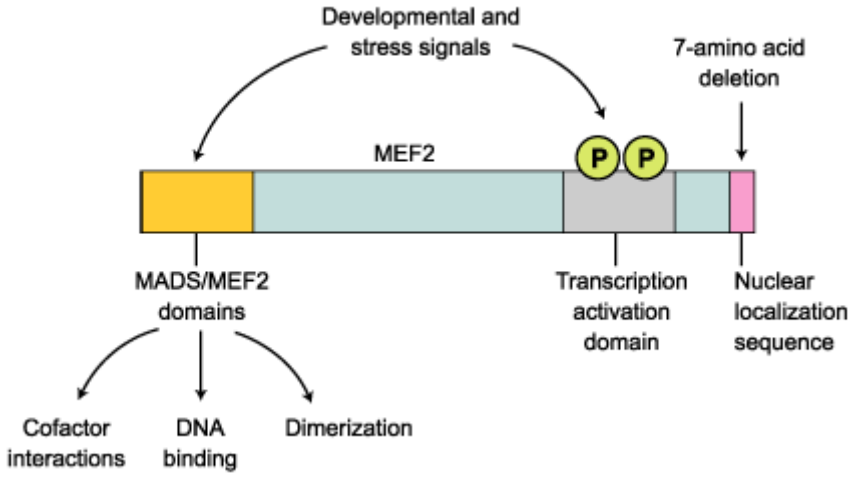


Figure 17. . Schematic diagram of MEF2 and its functional domains.

Because *MEF2A* was viewed as a potential candidate for causing CAD/MI susceptibility, Wang et al. undertook a systematic mutational screening of the entire *MEF2A* gene using direct DNA sequence analysis. A 21–base pair (bp) deletion was identified in exon 11 in all ten living affected members in the family (figure 17), resulting in a deletion of seven amino acids of MEF2A (Δ, Gln-Pro-Pro-Gln-Pro-Gln-Pro or Δ7aa). These seven amino acids are conserved among MEF2A proteins in the human, the mouse (GlnProProGlnProGlnPro), the pig (proglnProGlnProGln), and the Chamek spider monkey (GlnProglnGlnProGlnPro). Δ7aa is located in the conserved C-terminal region between MEF2A and MEF2C (a MEF2A homolog), a location that has been demonstrated to be important for nuclear localization of these two proteins (65). Δ7aa was not identified in the family members with normal phenotypes and was

absent in 119 individuals with normal angiograms, strongly suggesting that the deletion was responsible for CAD and MI in this large family.

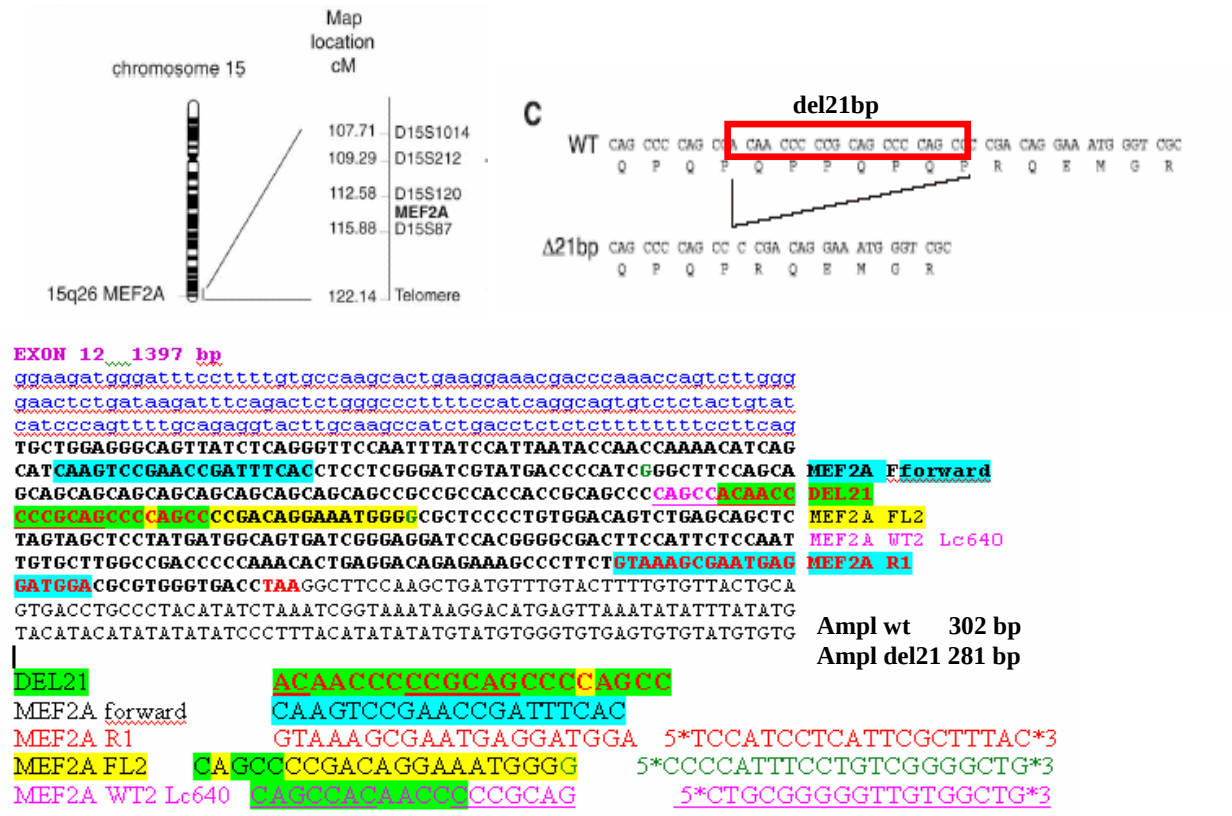


Figure 18 *MEF2A* intragenic deletion cosegregates with CAD in kindred QW1576. (**A**) The pedigree of kindred QW1576, showing genetic status: + indicates the presence of the 21-bp deletion of *MEF2A* (heterozygous); – indicates the absence of the deletion. (**B**) DNA sequence analysis of the wild-type (WT) allele and the 21-bp deletion allele (Δ21bp) of *MEF2A*. Sequence analysis of exon 11 of *MEF2A* in the proband (II.1) revealed the presence of a deletion. The wild-type and deletion alleles were separated by a 3% agarose gel and a single strand conformation polymorphism gel, purified and sequenced directly. The location of Δ 21bp is indicated. (**C**) Δ21bp results in a deletion of seven amino acids of MEF2A (Δ $Q_{440}P_{441}P_{442}Q_{443}P_{444}Q_{445}P_{446}$

The authors hypothesized that Δ7aa may cause a conformational change of the MEF2A protein that might result in protein trafficking defects that could prevent its function as a transcription factor. To test this hypothesis, they examined the cellular localization of mutant MEF2A protein by immunofluorescence staining. As expected, wild-type MEF2A localized to the nucleus (Figure 19). However, Δ7aa caused a marked defect in MEF2A trafficking, with a block of MEF2A entry into the nucleus (Figure 19). The mechanism by which the MEF2A deletion mutant is retained in the cytoplasm is not clear, although the corresponding region of MEF2C has been found to play an important role in its nuclear localization(65).

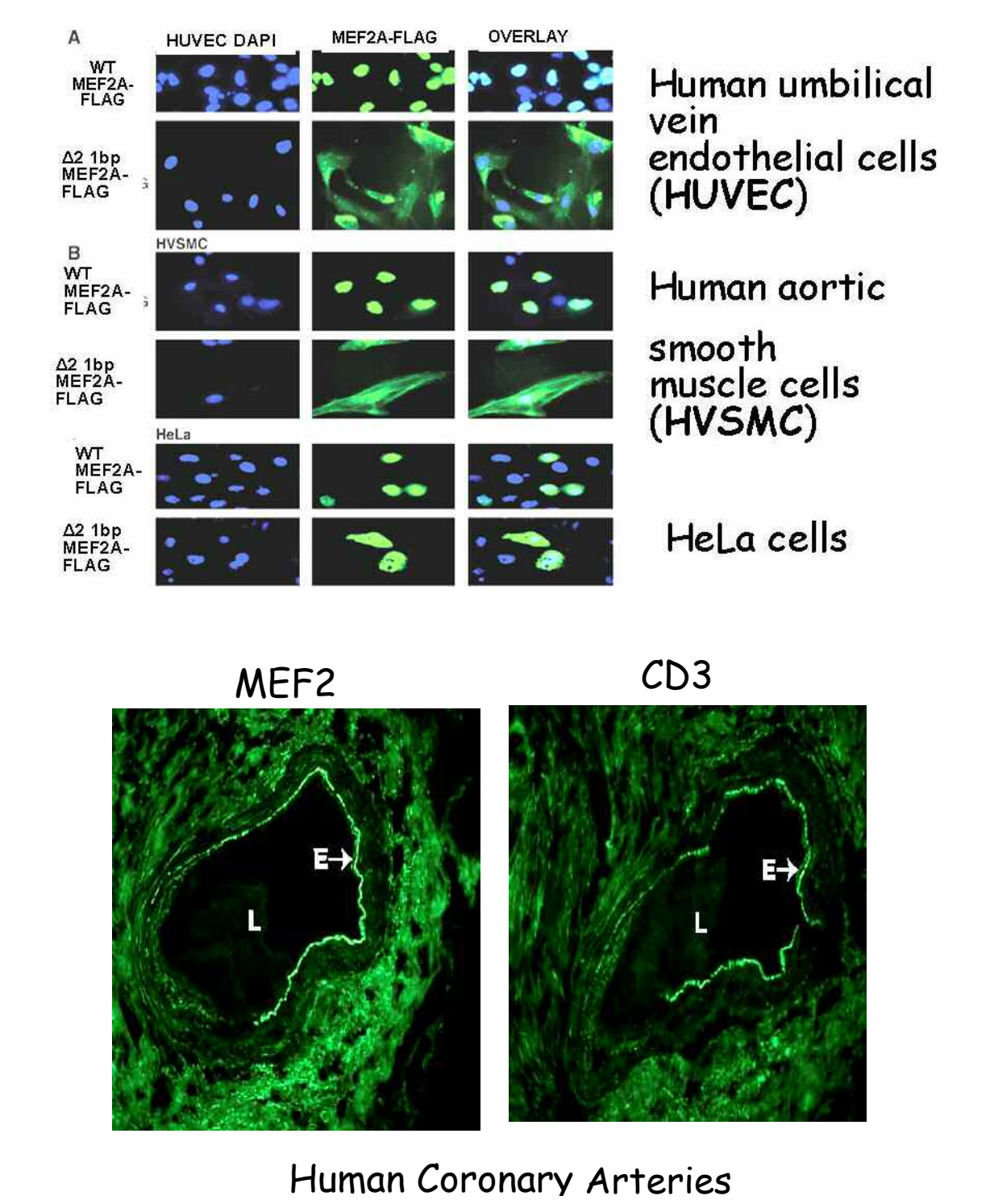


Figure 19. Functional characterization of wild-type and Δ7aa MEF2A proteins by immunofluorescence. (**A** to **C**) *MEF2A* deletion Δ7aa causes a defect in nuclear localization of the MEF2A protein in three cell types: (A) human umbilical vascular endothelial cells (HUVEC); (B) human aortic smooth muscle cells (HVSMC); and (C) HeLa cells. Cells were transfected with expression constructs for wild-type and mutant MEF2A proteins tagged with a FLAG epitope. Green, MEF2A signal; blue, nucleus. DAPI, 4',6-diamidino-2-phenylindole. (**D**) Colocalization of MEF2A and CD31 (PECAM, an endothelial cell–specific marker) in the endothelium of human coronary arteries. Cryosections (6 μm thick) of human coronary arteries were immunostained with the rabbit polyclonal antiserum to MEF2A. The adjacent sections were used for immunostaining with a monoclonal antibody to CD31. L, lumen; E, endothelium.

The functional consequence of the MEF2A deletion was also explored by transcription activation assays. The atrial natriuretic factor promoter (ANF_{-700}) can be activated by cooperation between MEF2A and GATA-1, a member of the GATA family of zinc-finger transcription factors (67). Mutant MEF2A with Δ7aa has only a third of wild-type MEF2A transcription activity (Figure 20), indicating that the deletion is a functional mutation that reduces transcription activation by MEF2A. Coexpression of the mutant and wild-type MEF2A showed transcription activity similar to that of mutant MEF2A alone (Figure 20). Cotransfection of MEF2A and GATA-1 showed synergistic activation of the ANF_{-700} promoter as reported previously (67). The synergistic activation of transcription by MEF2A and GATA-1 was abolished by coexpression of the mutant Δ7aa MEF2A with normal wild-type MEF2A. Together, these data suggest that Δ7aa acts by a dominant-negative mechanism. The dominant-negative effect of the mutant Δ7aa MEF2A can be explained by the findings that MEF2A can function as a dimer or in a complex with GATA factors.

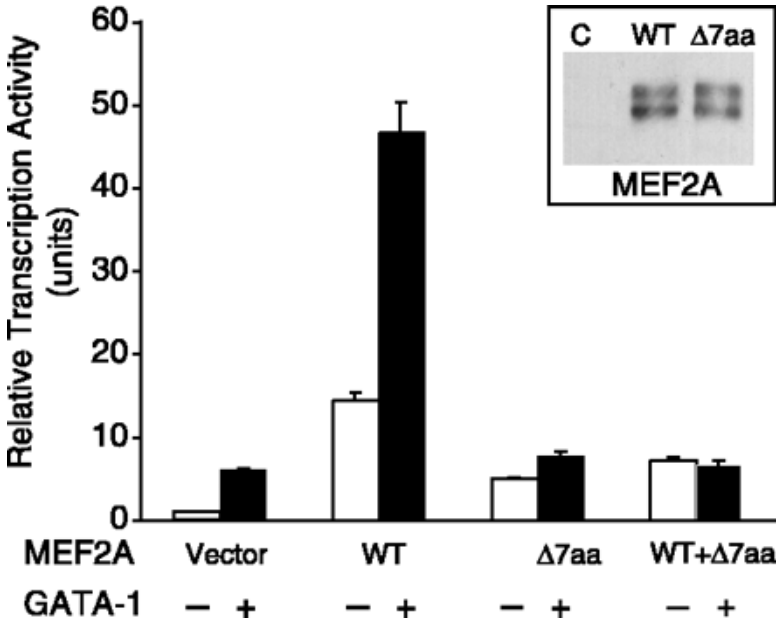


Figure 20. Functional characterization of wild-type and Δ7aa MEF2A proteins by transcriptional activation assays.

Immunostaining for MEF2A revealed strong MEF2A protein signal within the endothelial cell layer of coronary arteries (Figure 21). This pattern of expression is similar to that of CD31 [also known as platelet endothelial cell adhesion molecule (PECAM)], an endothelial cell–specific marker (Figure 21). Immunostaining and reverse-transcription polymerase chain reaction also detected *MEF2A* expression in human umbilical vascular endothelial cells. Collectively, Wang's data and previous work (55) implicate an important role for MEF2A in endothelial cell

development and function. A genetic defect in MEF2A may lead to a defective or abnormal vascular endothelium, which may promote the diapedesis of monocytes and expose the subendothelial matrix to the genesis of atherosclerotic plaque or thrombosis. M*EF2A* mRNA was also detected in cultured proliferating rat smooth muscle cells (SMCs) (63). MEF2A protein expression was detected in proliferating SMCs but not in differentiated SMCs in the rat model of arterial injury and clinical restenosis. Wang et al. have also detected expression of MEF2A protein in the nuclei of proliferating human SMCs (figure 21). Increased SMC proliferation was found to be associated with accelerated atherosclerosis. Therefore, the Δ7aa deletion in MEF2A may also affect the activity of proliferating SMCs, influencing the process of atherogenesis.

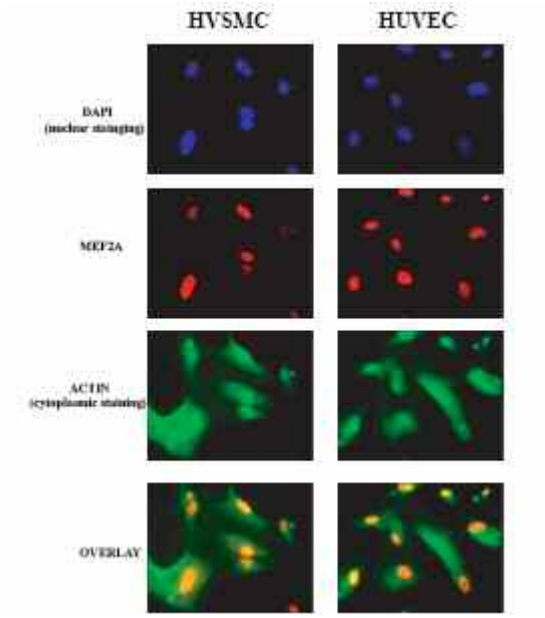


Figure 21. Expression of MEF2A protein in proliferating human vascular smooth muscle cells (HVSMC) and human umbilical vascular endothelial cells (HUVEC).

Results of Studies on Association of MEF2A gene variant in the CAD

The research on the role of genetic variants within the *MEF2A* gene for the pathogenesis of MI/CAD has been stimulated by Wang et al (52), who described a 21-bp deletion as the first disease-causing gene mutation for familial MI/CAD, regardless of classic risk factors (Figure 22 and 23). In a large kindred with 13 affected family members, genome-wide linkage analysis revealed a positive linkage signal with a logarithm of the odds score of 4.19 at chromosome 15q26. In the *MEF2A* gene, located within this critical region, a 21-bp deletion was identified in all living affected family members. This variant was absent in 119 controls with normal coronary angiograms.

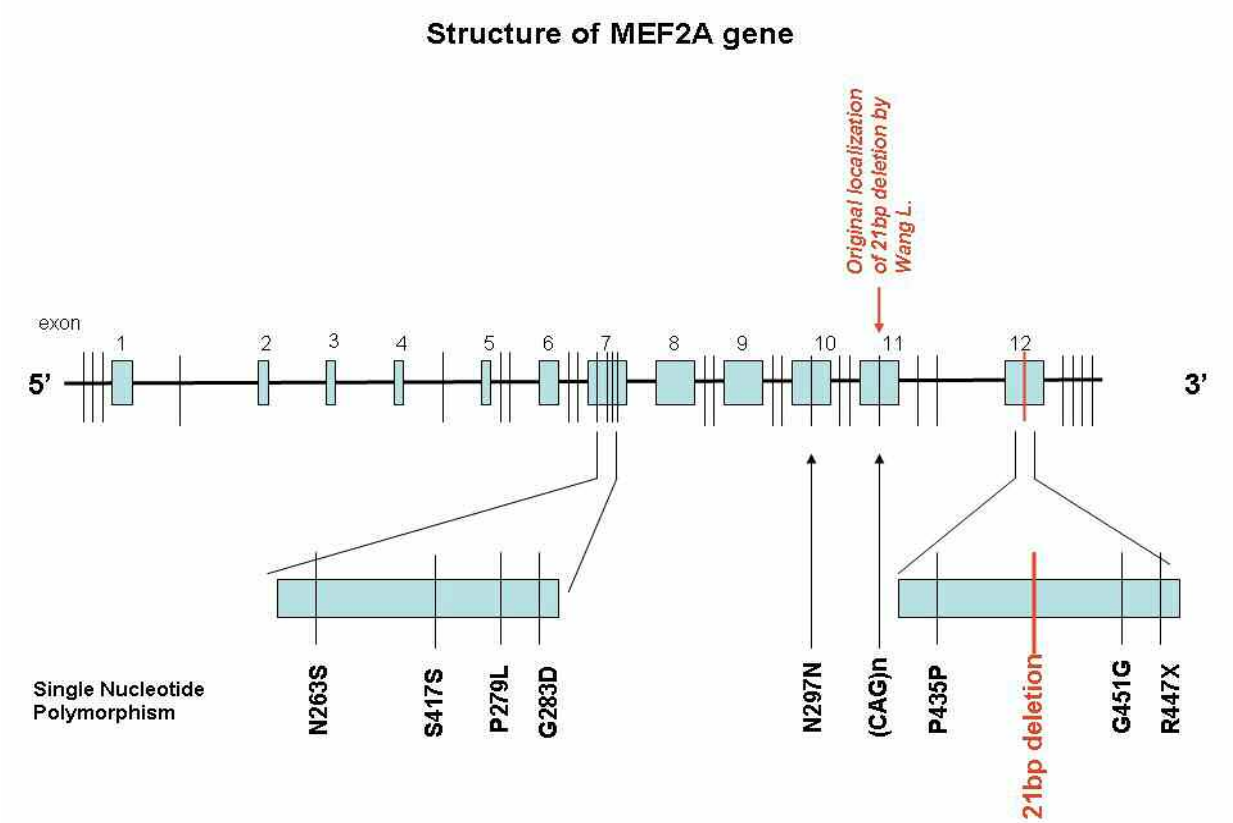


Figure 22. Scheme of the MEF2A gene. Promoter region and all of the 12 exon regions are shown (grey boxes). In the polymorphism found in exons, mutations of the amino acid sequence are also indicated.

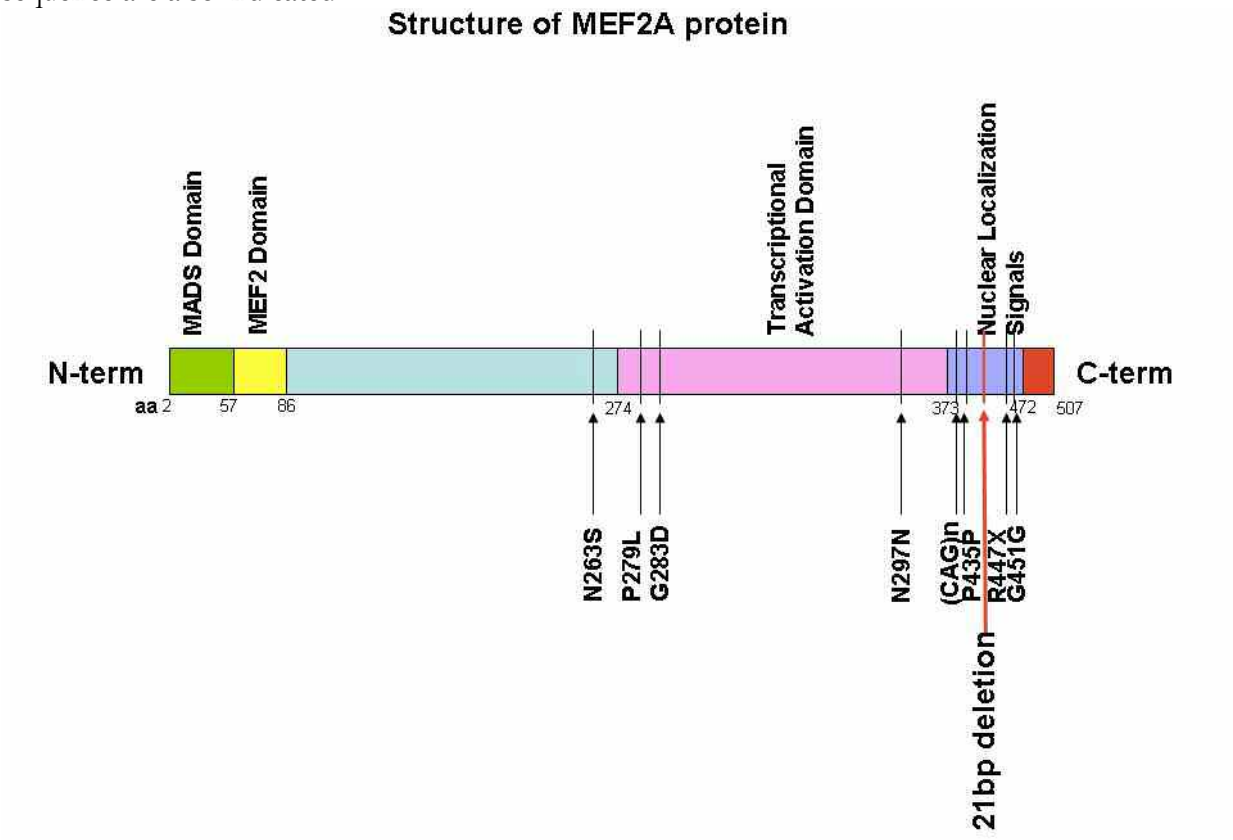


Figure 23 .Structure of MEF2A protein with CAD/MI-associated mutations indicated.

Subsequently, 3 genetic variants in the *MEF2A* gene (N263S, P279L, and G283D) were found in 4 of 207 independent patients (1.9%) with MI/CAD (in part without a positive family history for CAD), suggesting that the *MEF2A* gene could even play a significant role in the pathogenesis of MI/CAD in nonfamilial (sporadic) cases (50) (see Table 4 and 5). In accordance with these results, the P279L variant also was associated with the prevalence of MI in a Spanish case-control study (483 cases, 1189 controls (68). In the study of Lieb et al. (69), by contrast, no association of this genetic variant with MI was detected in 2 large samples of patients with sporadic and familial MI, respectively. A (CAG)n repeat in exon 11 displayed some evidence for association with CAD in a small Chinese case-control study, the (CAG)(9)-allele being overrepresented in the cases (70).However, this variant was not associated with MI in Lieb's much lager sample of patients with nonfamilial MI and displayed no association with MI in the above-mentioned Spanish MI population(68).

The findings of Lieb et al. (69) are in line with the results obtained by Weng et al (71) and Horan et al (72). Weng and colleagues found no causative MI mutation in 300 CAD cases, and Horan and associates failed to detect the 21-bp deletion described by Wang et al (52)in 1481 individuals with a positive family history for ischemic heart disease. *MEF2A* mutations are responsible for only a relatively small proportion of familial MI cases. By sequencing the *MEF2A* gene it was identified several genetic variants within the *MEF2A* gene; however, these variants do not seem to be pathogenic because of their intronic localization or because they are not leading to a change in the amino acid sequence.

Comparable results were obtained by Kajimoto and colleagues (73), who found several genetic variants but no clearly pathogenic mutation within the *MEF2A* gene in Japanese MI patients by sequencing the gene in 379 MI patients.

Table 4 Genetic variations in the MEF2A gene

Gene variation	Reference
Single nucleotide peptide(SNP)	
N263S	(50)
P279L	(50;68;69)
G283D	(50)
S417S	(68)
G451G	(68), (73)
(CAG)n polymorphisms	(68)., (74)., (70); (69).
R447X	(73), (71), (72)
P435P	(73)
N297N	(73)
Deletion	
21 bp in exon 11	(52;73) (71)

The data from these studies suggest that CAD/MI is genetically heterogeneous and the hypothesis that MEF2A is a disease-causing gene needs further validation with more molecular biologic and genetic studies.

Table 5 Results of studies on association of MEF2A gene variable in the CAD

Gene variation	CAD phenotype	Type of study	References
-21 bp deletion in exon 11 of the C-terminus of MEF2A gene on chromosome 15q26	MI, angina	linkage	(52)
Single nucleotide protein polymorphisms in exon 7: -N263S -P279L -G283D	 IHD, angina IHD IHD, angina	association	Bhagavatula MRK, Human Molecular Genetics 2004; 24:3181
-Deletion of 21 bp -(CAG)n repeat polymorphisms -(CAG) 9 allele	- - -	association	(71)
Single nucleotide protein polymorphisms: -P279L -S417S -G451G -(CAG)n repeat polymorphisms	 MI - - -	association	(68)
Single nucleotide protein polymorphisms: -N297N -(CAG)n repeat polymorphisms -P435P -G451G Nonsense mutation downstream of the 21 bp deletion site: -R447X -Deletion of 21 bp in exon 12	 - - - - - -	association	(73)
-(CAG)n repeat polymorphisms in exon 11	MI before 60 ys	association	(74)
-(CAG)n repeat polymorphisms -(CAG) 9 allele	- Extent of CAD	association	(70)
-(CAG)n repeat polymorphisms -(CAG) 9 allele	IHD IHD		(72)
Single nucleotide protein polymorphisms: -P279L in exon 7 -(CAG)n repeat polymorphisms in exon 11 -Polymorphisms in intron 1, 8, 9, 11 -Polymorphism in 3' untransleted region -1 synonymous point mutation in exon 10	 MI MI - - -	Association, linkage analysis	(69)

CAD= coronary artery disease; IHD= ischemic heart disease; MI= myocardial infarction, SD= sudden death.

METHODS

THE GENICA STUDY (39;75-77)

Patient selection

a) Consecutive Caucasian patients of both genders consecutively referred to the Division of Cardiology of the Cittadella General Hospital for coronary angiography for investigation of chest pain and/or suspected CAD were enrolled between 1999 and 2001. The Medical Ethics Committee of our university approved the study protocol, and a written consent after explanation of the aims and details of the study was obtained from each participant. The refusal to participate in this study was the only exclusion criterion. Two groups served as controls: group 1 entailed patients in whom significant (e.g., stenosis ≥50%) CAD was eventually ruled out by coronary angiography; group 2 comprised 119 consecutive healthy normotensive blood donors enrolled at the local blood bank during the same period (see table 6). In these latter subjects, it was unethical to perform coronary angiography to rule out the presence of asymptomatic CAD. Therefore, the following inclusion criteria were used: negative family history of CAD, MI, and stroke; nonsmoking status; absence of hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, all defined as specified in the following text. Based on available data from epidemiologic and family studies, a cohort fulfilling these criteria is expected to have a very low prevalence of asymptomatic CAD.

b) As some of the hypertensive patients (PH) and normotensive (NT) subjects had been used for previous studies, the criteria for their enrolment have been detailed previously (77). Briefly, 131 PH and 51 healthy NT subjects consented to participate in this study, which was approved by our ethics committee. Exclusion criteria were hypercholesterolemia, diabetes mellitus, cardiac and/or cerebral ischemic vascular disease, impaired renal function and other major diseases. Subjects were defined as NT if they had no history of hypertension and blood pressure (BP) values consistently < 140/90 mmHg. The vast majority (>80%) of PH patients had never been treated; the rest reported a history of discontinued (>=6 months) or ineffective pharmacological treatment. Pharmacological treatment was withdrawn at least 2 weeks before performing the study. At the time of the study the patients were asked to have a normal sodium intake and underwent routine diagnostic procedures to exclude secondary forms of hypertension.

Demographic and clinical measurements

A standard questionnaire was used to carefully ascertain medical history in all participants (transient ischemic attack, stroke, angina, MI, coronary artery bypass, percutaneous transluminal coronary angioplasty, renal failure, peripheral artery disease, and history of

vascular surgical interventions), smoking habits, presence/absence of hypertension, diabetes, hypercholesterolemia, hypertriglyceridemia, and current medications. Body mass index was calculated as weight/height^2 (kg/m^2). Patients were classified into three groups: current smokers, nonsmokers, and ex-smokers (who had stopped smoking for at least one year). Diabetes mellitus (type I or II) was defined as a previous diagnosis of the disease, history of antidiabetic medications, or plasma fasting levels of glucose ≥126 mg/dl (7.0 mmol/l) on at least two occasions. Impaired glucose tolerance was defined as plasma fasting levels of glucose ranging between 110 to 126 mg/dl (6.1 to 6.9 mmol/l) (78). Hypercholesterolemia was defined as a low-density lipoprotein (LDL) cholesterol ≥100 mg/dl according to the National Cholesterol Education Program guidelines for patients with CAD ; hypertriglyceridemia was defined as plasma fasting levels ≥134 mg/dl, that is, higher than the 95th percentile value of our group 1 control subjects. Blood pressure was measured by mercury sphygmomanometer using Korotkoff phase V for diastolic, according to the World Health Organization guidelines. Hypertension was defined as systolic pressure ≥140 mm Hg, and/or diastolic pressure ≥90 mm Hg , or use of any antihypertensive agents.

Coronary angiography

Angiography was carried out and evaluated by experienced cardiologists who were blinded to patients' genotype. The severity of CAD was determined by the number of significantly stenosed coronary arteries Patients were classified as follows: code 1 = normal vessels; code 2 = <50% stenosis; code 3, 4, and 5 = stenosis ≥50% in one, two, or three major coronary arteries, respectively.

Laboratory measurements

Each patient was studied between 8:30 and 12:00. Before coronary angiography, blood samples were taken from the femoral artery and were immediately put on ice and centrifuged at 3,000× *g* (at 4°C for 10 min). Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glycemia, sodium, potassium, blood urea nitrogen, and creatinine levels were measured with conventional methods.

Demographic characteristics

Of the 1,271 patients originally recruited in the GENICA study who had complete coronary angiography data, 17% (n = 217) were found to have normal coronary arteries, and 14% (n = 178) stenosis <50%, 23% (n = 290), 24% (n = 305), and 22% (n = 281) had significant (≥50%) stenosis in one, two, or three major epicardial vessels, respectively. To maximize contrast at the phenotype level, it was decided to combine two- and three-vessel CAD patients, as CAD group, and normal vessel and <50% stenosis as control group (group 1); thus, the CAD group

and group 1 comprised 749 and 330 patients, respectively. Of the latter, 21% were healthy individuals, 39% had valvular heart disease, 15% had cardiomyopathy (13.7% dilated, 1.3% hypertrophic), 7% had hypertensive heart disease, and 18% had ischemic cardiomyopathy. The latter diagnosis was based on resting and stress electrocardiogram and on results of stress myocardial scintigraphy that showed inducible ischemia.

The demographic and clinical characteristics of the CAD patients and of group 1 and 2 (119 subjects) controls are shown in Table 6.

The CAD patients were older, had higher plasma glucose and triglycerides, and lower HDL cholesterol than control subjects. There were more males in CAD patients than in group 1 ($p < 0.001$), but not group 2 control subjects. Compared with both control groups, more CAD patients had diabetes mellitus, hypercholesterolemia (both $p < 0.001$), hypertriglyceridemia ($p < 0.001$), and were smokers or ex-smokers ($p < 0.0001$). Compared with controls, the CAD patients more commonly reported a history of myocardial infarction ($p < 0.0001$), coronary artery bypass surgery ($p < 0.0001$), percutaneous transluminal coronary angioplasty ($p < 0.0001$), peripheral artery disease ($p < 0.001$), vascular surgery ($p < 0.001$), but not of transient ischemic attack or stroke.

Table 6. The demographic and clinical characteristics of the GENICA Study

Group Variables	CAD (n = 749)	p vs. Group 1	Controls			
			Group 1 (n = 330)	p vs. Group 2	Group 2 (n = 119)	p vs. CAD
Age, yrs	64,3 ± 9,4	0,001	62,0 ± 10,8	< 0,001	32,8 ± 8,0	< 0,001
Gender M/F (%)	611 (82%)/138 (18%)	< 0,001	191 (58%)/139 (42%)	< 0,001	103 (87%)/16 (13%)	NS
BMI (kg/m^2)	26,9 ± 3,8	NS	26,8 ± 4,4	< 0,001	23,9 ± 3,6	< 0,001
Systolic BP (mm Hg)	135 ± 18	NS	134 ± 17	< 0,001	119 ± 8	< 0,001
Diastolic BP (mm Hg)	78 ± 10	NS	79 ± 10	< 0,001	70 ± 7	< 0,001
Mean BP (mm Hg)	97 ± 11	NS	97 ± 11	< 0,001	86 ± 7	< 0,001
Heart rate (beats/min)	66 ± 10	NS	69 ± 10	NS	NA	NS
Total cholesterol (mg/dl)	206 ± 44	NS	202 ± 41	< 0,001	175 ± 22	< 0,001
HDL cholesterol (mg/dl)	45 ± 11	< 0,001	48 ± 13	–	NA	–
LDL cholesterol (mg/dl)	131 ± 36	NS	129 ± 34	–	NA	–
Triglycerides (mg/dl)	147 ± 98	< 0,001	125 ± 71	< 0,001	73 ± 35	< 0,001
Glycemia (mg/dl)	115 ± 40	< 0,001	108 ± 25	< 0,001	88 ± 8	< 0,001
Left ventricular ejection fraction (%)	60 ± 14	NS	60 ± 16	–	NA	–
Diabetes mellitus (%)	19,0	0,004	11,5	< 0,001	0	< 0,001
Nonsmoker/smokers/ex-smokers (%)	35,0/14,2/50,8	< 0,001	59,1/14,3/26,5	< 0,001	100/0/0	< 0,001
Hypertensives (%)	61,4	NS	56,6	< 0,001	0	< 0,001
Hypercholesterolemia (%)	59,4	< 0,001	46,1	< 0,001	0	< 0,001
Hypertriglyceridemia (%)	22,7	< 0,001	13,3	< 0,001	0	< 0,001
Cholesterol-lowering drugs (%)	38,6	< 0,001	18,8	< 0,001	0	< 0,001
History of						
Transient ischemic attack (%)	3,5	NS	4,6	0,009	0	0,026
Ischemic stroke (%)	1,7	NS	1,2	NS	0	NS
Acute myocardial infarction (%)	45,6	< 0,001	11,7	< 0,001	0	< 0,001
Coronary artery bypass surgery (%)	12,1	< 0,001	0	NS	0	< 0,001
Percutaneous coronary angioplasty (%)	9,3	< 0,001	0,3	NS	0	< 0,001
Peripheral arterial disease (%)	17,5	0,008	11,7	< 0,001	0	< 0,001
Vascular surgery (%)	5,4	0,036	2,2	0,051	0	0,002
Chronic renal failure (%)	6,7	0,042	3,7	0,027	0	< 0,001

Extraction of deoxyribonucleic acid

The blood was stored at −20°C until deoxyribonucleic acid was extracted using a commercially available kit (DNA Blood Extraction Fast KIT, Analitica Srl., Padova, Italy). Details of the methodology used were reported (79).

Extraction of deoxyribonucleic acid (DNA) and deletion genotyping

In consecutive white patients of the GENICA (Genetic and Environmental Factors in Coronary Atherosclerosis) study, we determined a deletion of 21 nucleotides in the gene encoding for the transcription factor MEF2A by a novel Real Time Polymerase Chain Reaction. This technology utilizes melting curve analysis of amplicons from allele-specific fluorescence resonance energy transfer (FRET) probes. The blood was collected in ethylenediamine-tetraacetic acid and stored at -20°C until DNA was extracted from buffy coat using commercially available kits, and quantified by spectrophotometer. The DNA was also extracted from saliva according to Oragene DNA purification Kit.

For genotyping we designed two FRET probes specific for two adjacent sequences in target DNA. These probes contiguously hybridize to an intenal sequence of the amplified fragment during the annealing phase of polymerase chain reaction. When this occurs, the close proximity of the two fluophores allows the energy transfer from one (donor) to the other (acceptor) probe, resulting in a fluorescence signal that is detected. The acceptor probe was labeled at 5'-end with a red fluorophore (LCRed640), while the donor probe at the 3'-end with fluorescein (X). Signal fluorescence allows to find the deletion by analysis of fluorescence variation compared to increase of temperature (analysis of melting curve profiles). Hence, distinction of wild type and mutant genotype can be easily accomplished by differences in their respective melting temperature (Tm).

	Sequence
Primer forward MEF2A	CAAGTCCGAACCGATTTCAC
Primer reverse MEF2A	TCCATCCTCATTCGCTTTAC
MEF2A FL2	CCCCATTTCCTGTCGGGGCTG - FL
MEF2A WT2	LC Red 640 – CTGCGGGGGTTGTGGCTG -PH

PH= phosphate, FL= fluorescein, LC Red640= fluoroforo Roche

Table7. Specific primers.

Genotyping was performed with LightCycler 1.5 (Roche, Milan, Italy) and PCR reaction mixture consisted of LightCycler DNA Master Hybridization Probes 10X (Roche Diagnostics, Milan, Italy) to which primers and probed were added. The cycling program entailed a

denuration step (95°C for 2 min) followed by 50 cycles (95°C, 5 s ; 58°C, 15 s ; 72°C, 12 s). For the analysis of the melting curves at the end of PCR, temperature was raised to 95°C, lowered to 45°C, and then slowly raised to 85°C to allow monitoring of the decline of fluorescence. Melting curves were automatically converted to fluorescence peaks, thus allowing distinction of genotypes.

Experimental Design of HRMC (high resolution melting curve)

An Gene Scanning Platform (Light Cycler 480, Roche) will be used to identify deletion end mutation in DNA of 1142 patients. Total DNA will be extracted from tissues of ACC using a standardized method, concentration and quality will be valuated with spectrophotometry. High-Resolution Melting Analysis (Light Cycler 480, Roche), will be used specific primers (see table 7) and particular SyberGreen for detection of deletion in exon 12 (21 bp) of MEF2A.

The sample DNA is first amplified via real-time PCR in the presence of a proprietary saturating DNA dye contained in the LightCycler 480 High Resolution Melting Master. A melting curve is then produced using high data acquisition rates, and data are finally analyzed using the LightCycler 480 Gene Scanning Software Module for identify the deletion.

Protocol: Denaturation: 95°C x 10'; Amplification: 95°C x 15", 8°C x 10", 72°C x 15"; High Resolution Melting: 95°C x 1', 40°C x 1'; then from 65°C x 1" to 95°C we obtain 25 acquisitions per °C. At the end cooling at 40°C x 15".

DNA sequence analysis

DNA sequence analysis of the wild type (WT) allele and the 21 bp delete allele (Del21) of MEF2A gene was performed by BMR-Genomics Service (www.bmr-genomics.it).

Endothelial-Dependent Flow-Mediated Vasodilatation of brachial artery For the subject preparation, the equipment, the image acquisition and the endothelium-dependent flow mediated dilation technique we refer to the Guidelines (80).

Aim of the Study.

Available evidences suggest that MEF2A plays a role in vascular ontogeny and shows its predominant expression in the coronary artery endothelium. Considering the pivotal role played by the latter in atherogenesis, we investigated:

5. the prevalence of MEF2A deleted gene in a large case-control study (GENICA Study)
6. if the deletion might be associated with coronary artery structural and functional abnormalities;
7. if it might be associated with widespread endothelial dysfunction;
8. if either one or the other or both alterations might eventually result into clinically relevant coronary artery disease.

RESULTS

Accuracy of the methodology developed for genotyping

We applied the methodology that was developed to the positive control that was kindly provided from Dr. Wang L. of the Center for cardiovascular Genetics of the Cleveland Clinic Foundation, Cleveland, Ohio.

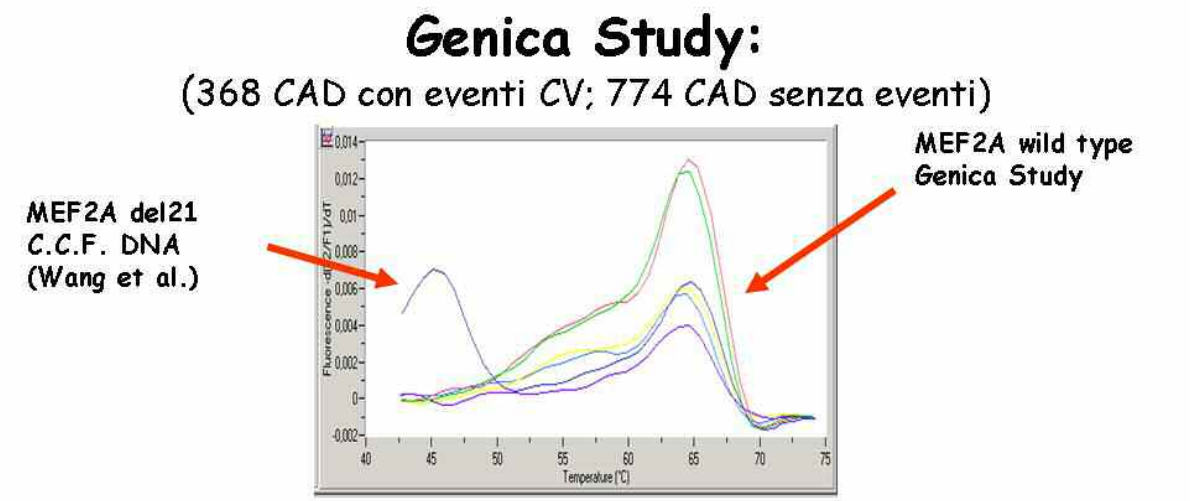


Figure 24. Results of the genotyping of MEF2A

We first sequenced the DNA and found that the deletion was located in exon 12.

We had then applied our melting curve analysis fluorescence resonance energy transfer (FRET) technologies for the assessment of MEF2A deletion and found that our methodology was allow to unequivocally identify the deletion.

As showed in figure 24 the curves assume these aspect because the deleted DNA showed a fluorescence peak at a hybrid melting temperature of 45°C, which was obviously different from that (65°C) of individuals carrying the wild type gene.

Moreover, no false positive results were obtained in several negative controls DNA.

We next tested the hypothesis that the deletion was identifiable in a more simple and cost effective way by using the HRMA (high resolution melting amplicon) analysis methodology. This methodology was developed by Idaho Research (Idaho Technology, Salt Lake City, Utah) and was found to provide accurate genotyping without use of fluorescent probes when applied to investigation of deletion and single point mutation in the EGF receptor gene (81;82). We found that also in this case it was possible to unequivocally identify the deleted DNA with 100% accuracy (data shown in figure 27).

Prevalence of MEF2A deletion

The prevalence of MEF2A gene mutation was studied in 3 different cohorts of patients:

- 1a. healthy normotensive blood donors (n=119) (76)
- 1b. healthy normotensive volunteers (n=51) (77)
- 2. primary uncomplicated hypertensive patients (n=131) (77)
- 3. consecutive patients undergoing coronary angiography for suspected CAD of the GENICA study cohort (n=1142) (76)

The prevalence of MEF2A deletion in the three cohorts is shown in figure 25.

Based on these findings our further research work was devoted to an in depth investigation of the pedigree of index case n=730 of the GENICA study.

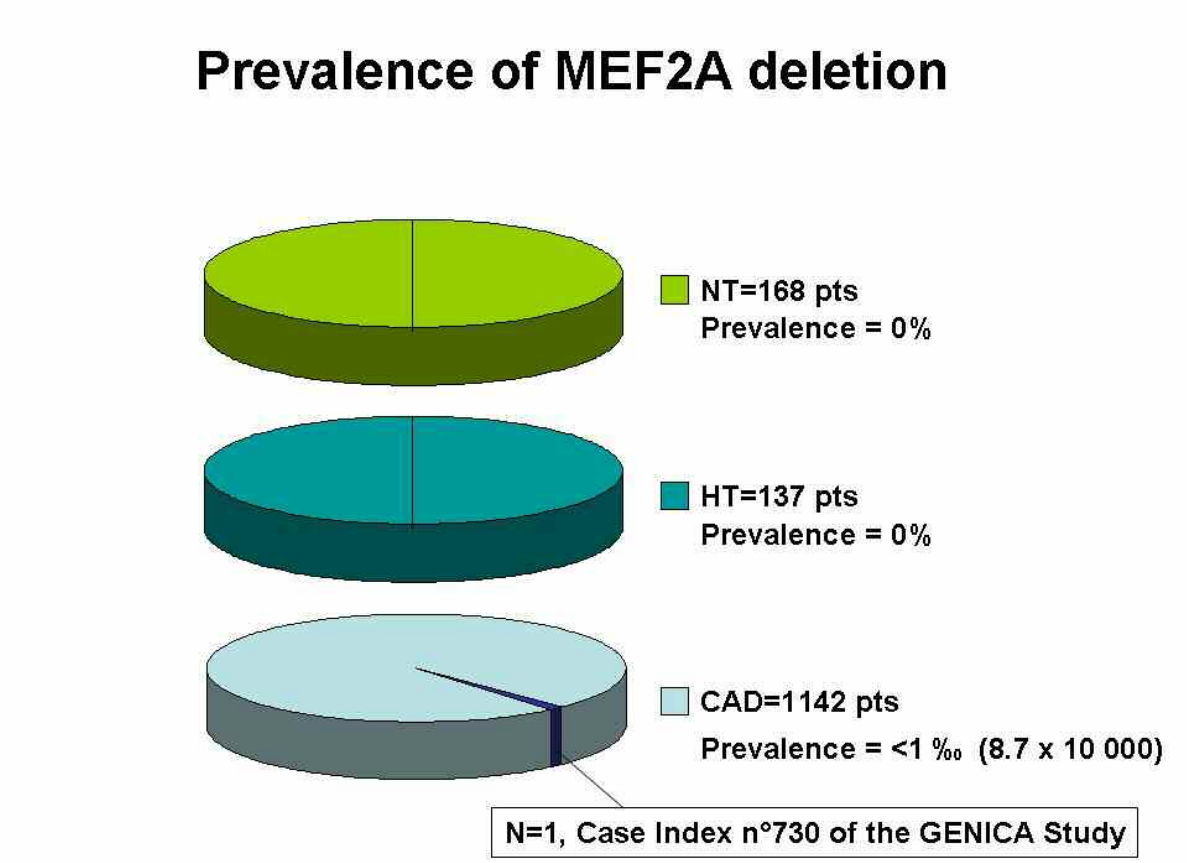


Figure 25.

Phenotypic and Genotypic Analysis of Pedigree of Case Index n° 730

Among the 1142 patients of the GENICA study, we found the deletion of 21 nucleotide of the gene encoding for the transcription factor MEF2A in one patient (case index n°730).

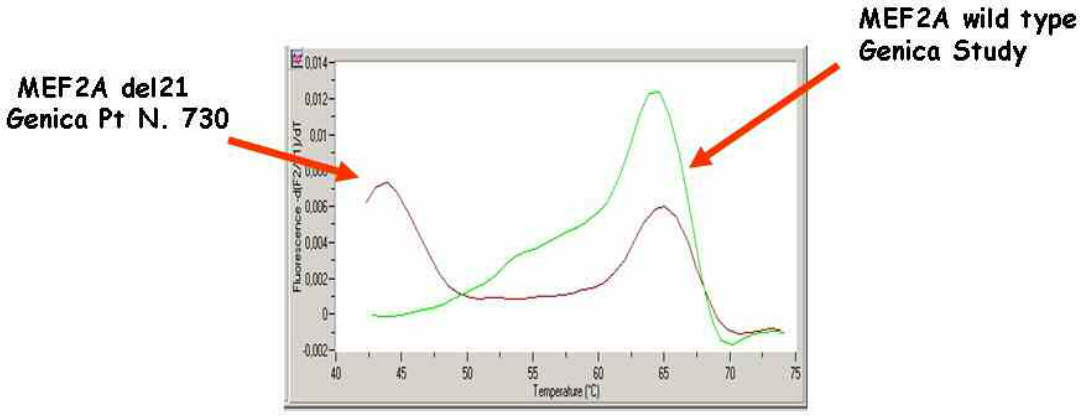


Figure 26.

The HMRA also allowed the identifying of the delete region with no false positive results.

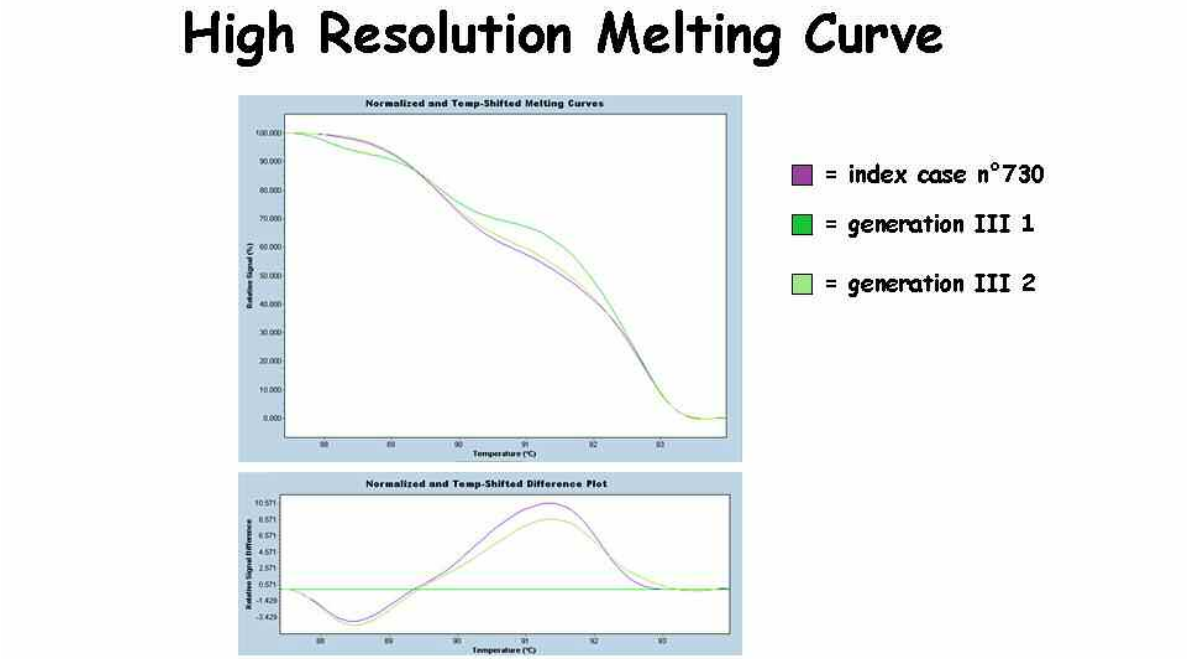


Figure 27.

He has a family history with high incidence of cardiovascular events (see figure 28).

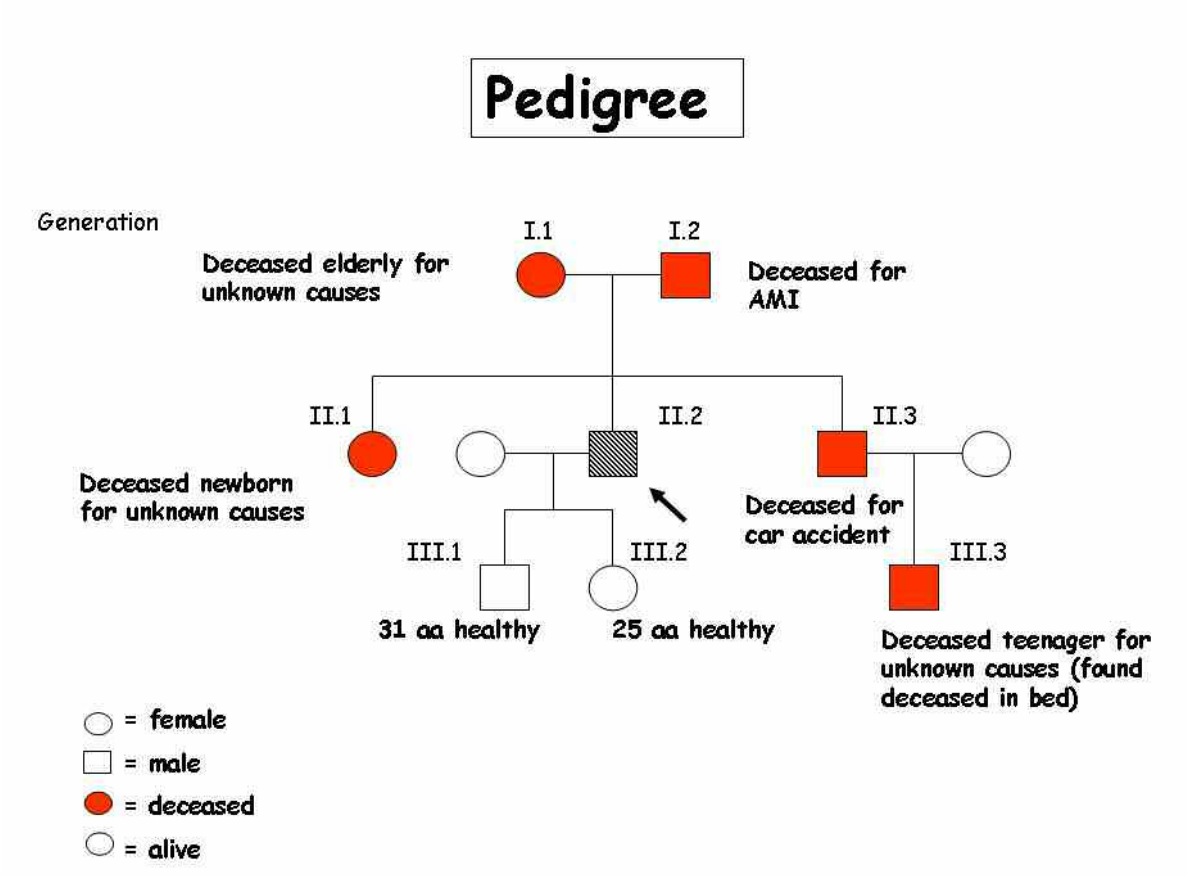


Figure 28.

His grandfather died for acute myocardial infarction, while his grandmother deceased for unknown causes. He had a sister and a brother; the former died one newborn in crib for unknown causes, the latter for car accident at age 30. His brother had a son, who died at age 15 for unknown causes (sudden death?). Unfortunately no further information is available about concomitant disease.

The two sons are healthy.

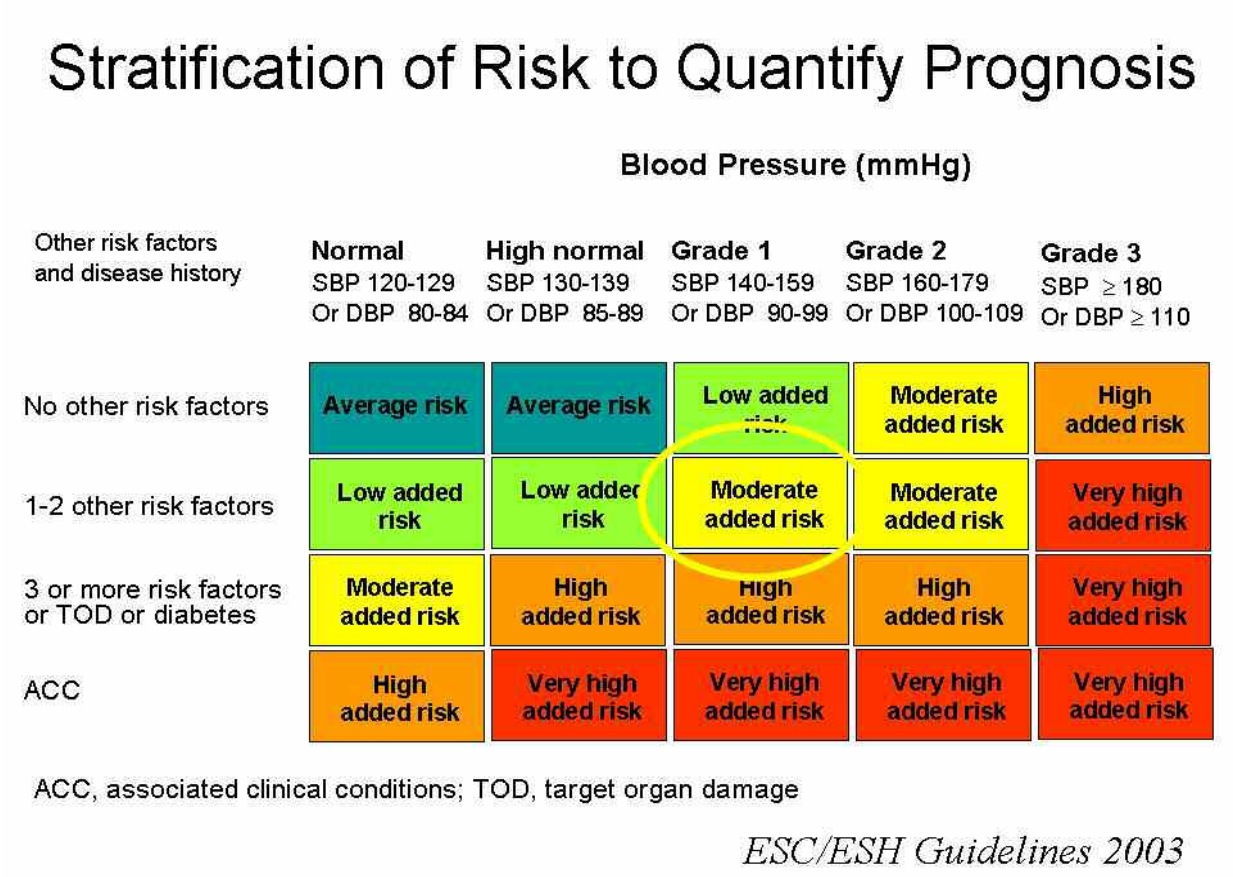
Risk stratification by conventional approach

Index case n°730 was a 52 years old man who reported a history of hypertension and cigarette smoking, nor dyslipidemia neither diabetes mellitus.

According to the PROCAM risk score (34)and the ESC/ESH guidelines (35) he could be considered at moderate added risk. The PROCAM risk score was 46, with an estimated risk of acute coronary events in 10 years of 10.00-19.99 %.

PROCAM Score	Estimated Risk of Acute Coronary Events in 10 y (in %)	Mean Estimated Risk of Acute Coronary Events in 10 y (in %)	Observed Incidence of Acute Coronary Events in 10 y (in %)	Prevalence of This PROCAM Score Category (in %)
0–20	<1.00%	0.7	0.5	16.8
21–28	1.00–1.99	1.6	1.5	16.7
29–37	2.00 –4.99	3.3	2.3	25.7
38–44	5.00–9.99	6.8	6.6	18.2
45–53	10.00–19.99	13.5	14.8	15.0
54–61	20.00–39.99	26.3	28.1	5.5
>61	≥40.00	49.9	43.2	2.0
Total		6.8	6.7	100

Table 8 . Observed Incidence of Acute Coronary Events Occurring Within 10 Years of Follow-Up in the PROCAM Study



Physical examination

The patient was referred to the Cardiology Department of the Cittadella Hospital on26th June 2000 for dispnea and chest pain arose from a month for low-moderate physical activity. Physical examination, blood testing, echocardiography, ECG, standard interview were performed, and resting blood pressure (data not shown) was measured with the resulting diagnosis of post-ischemic dilatative cardiomyopathy. He thereafter underwent coronary angiography that showed three-vessel disease. Two months later he underwent coronary artery bypass graft.

During the follow up, the patient had no more symptoms and referred a good physical activity.

Then we extended the analysis to the other family members, the son and daughter, (Generation III of the pedigree) who are the only still alive members of the family. DNA was extracted or from buffy coat or saliva as described. This latter strategy was the only feasible because of the two cases in generation III, one is living in Sicily and the other in Moscow, Russia.

The physical examination, blood testing, echocardiography, ECG, standard interview, and resting blood pressure (data not shown) of the subject III1 resulted in the normal range. He performed echocardiography and physical stress test that were normal.

Genotypic Analysis of Generation III

Then we applied our melting curve analysis fluorescence resonance energy transfer (FRET) technologies for the assessment of MEF2A deletion in generation III and found that the deletion has been transmitted to the subject III2 (Figure 29), while the subject III1 had wild type gene allele.

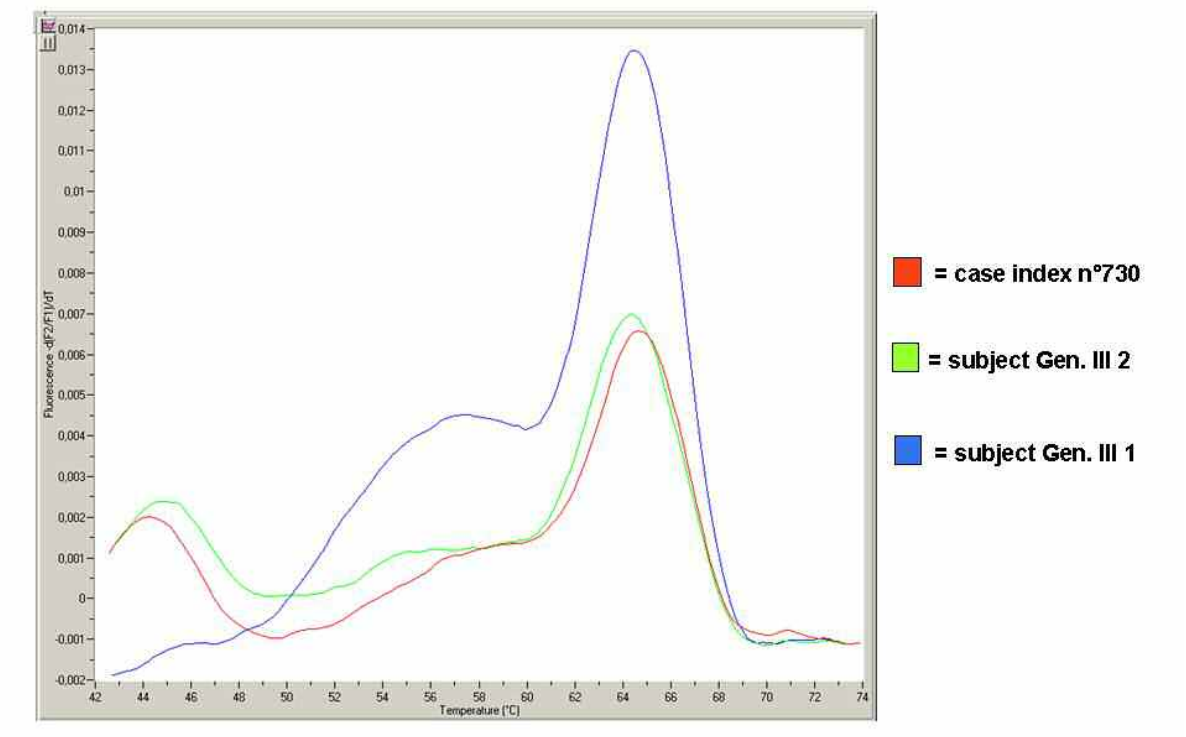


Figure 29.

These results were confirmed by performing High-Resolution Melting Analysis and by sequencing the DNA, which confirmed that the deletion was located in exon 12 (figure 30).

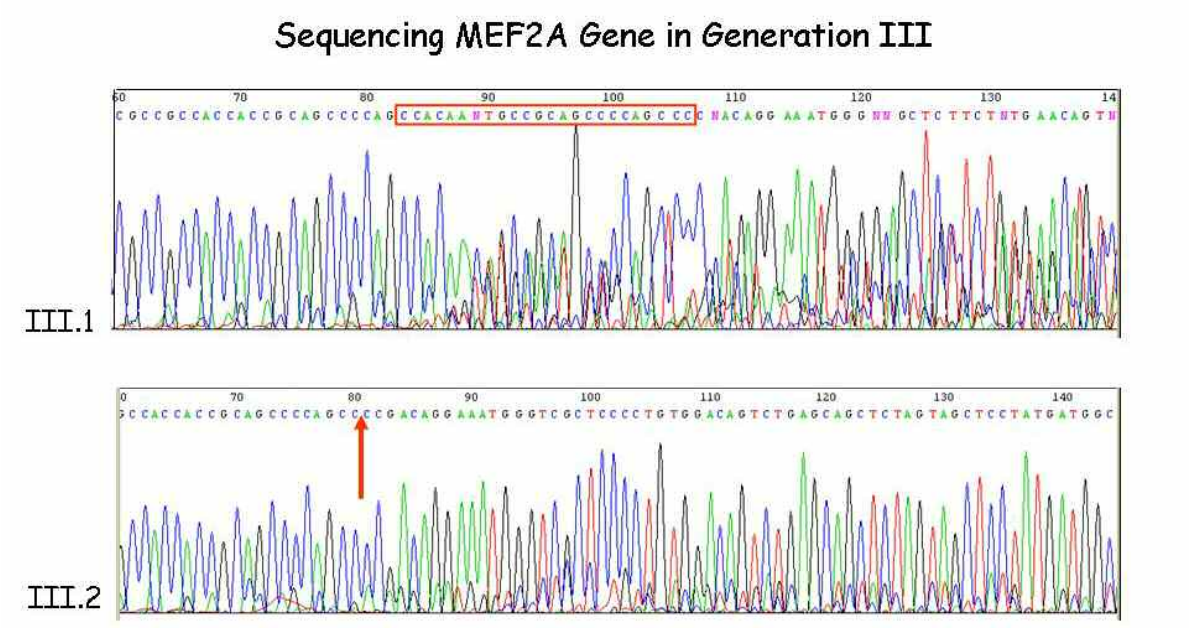


Figure 30.

Study of Endothelial Dysfunction in the Pedigree of index case n°730

As available evidences suggest that MEF2A plays a role in vascular ontogeny and since the immunofluorescence study by Wang L. (52) showed the predominant expression of MEF2A in the coronary artery endothelium, considering the pivotal role played by the latter in atherogenesis, we hypothesised:

9. That the deletion might be associated with coronary artery structural and functional abnormalities;
10. That it might be associated with widespread endothelial dysfunction;
11. Either one or the other or both alterations might eventually result into clinically relevant coronary artery disease.

To test the hypothesis that the risk for premature myocardial infarction is due to an endothelial dysfunction, multiple tests were performed by the 2 subjects available.

Laboratory test

In addition to the normal lab measurements routinely, we have also determined a number of factors that could be involved in endothelial dysfunction (see table 9).

Table 9. Laboratory Measurement.

Case	Index case n°730	Generation III1	Generation III2	Normal value
MEF2A deletion	+	-	+	-
PCR	1.3	3.36	N.A.	0 - 6.0 mg/dL
Total Cholest.	3.01	4.97	N.A.	<5.18 mmol/L
HDL Cholest.	1.17	1.70	N.A.	>1 mmol/L
Insulin	11.8	< 2.0	N.A.	6 - 29.1 mU/L
s-TNF	9.4 *	5.9	N.A.	0 - 8.1 ng/L
Homocysteine	11.6	16.2*	N.A.	5 – 15 μmol/L

PCR=protein C reactive; TNF= tumor necrosis factor

Only the s-TNF value was over the normal range, but at the moment it remains of uncertain interpretation. The most widely documented cytokines and chemokines implicated in the progression of heart failure are TNF-α, ILs, IFNs, and other. These mediators are produced by endothelial cells, myocardial cells and macrophages. These pro-inflammatory cytokines have been shown to contribute to cardiac dysfunction under various pathophysiological conditions associated with heart failure, including ischemia/reperfusion injury, myocardial infarction. And atherosclerosis (83-85). So, its role seems very interesting and remains to be investigated.

Endothelial-Dependent Flow-Mediated Vasodilatation of brachial artery was performed and the results are shown in Table 10 .

Case	%FMD	PSV basal (cm/sec)	PSV PI (cm/sec)
Index case n°730	4.5%	66	98
Generation III 1	7.9%	90	158
Generation III 2	N.A.	N.A.	N.A.

FMD= flow mediated dilation; N.A.=not available; PSV= peak systolic velocity; PI= post ischemic.

Index case n°730 shows a clear endothelial impairment as a flow mediated dilation from the baseline (only 4.5%, with a normal value of 8%) and a weak peak systolic velocity, that not improve after post-ischemic stimulus. Generation III1 subject had normal values.

Magnetic Resonance

We also performed cardiac Magnetic Resonance to the microvasculature study. Index case n° 730 shows a first passage hypoperfusion in the postero-lateral wall (Figure 31A) with a late enhancement as a post-ischemic fibrotic tissue (Figure 31B). He has an ejection fraction of 40%. Generation III1 subject had no signal alteration. Magnetic Resonance of Generation III 2 subject is still not available.

Probably the result we will get from the study of the microcirculation (including multi slice angio-CT) could a detect if the carrier of MEF2A gene deletion have an early endothelial dysfunction. Indeed in the index case No. 730 the results are affected by the 3 major vessels disease.

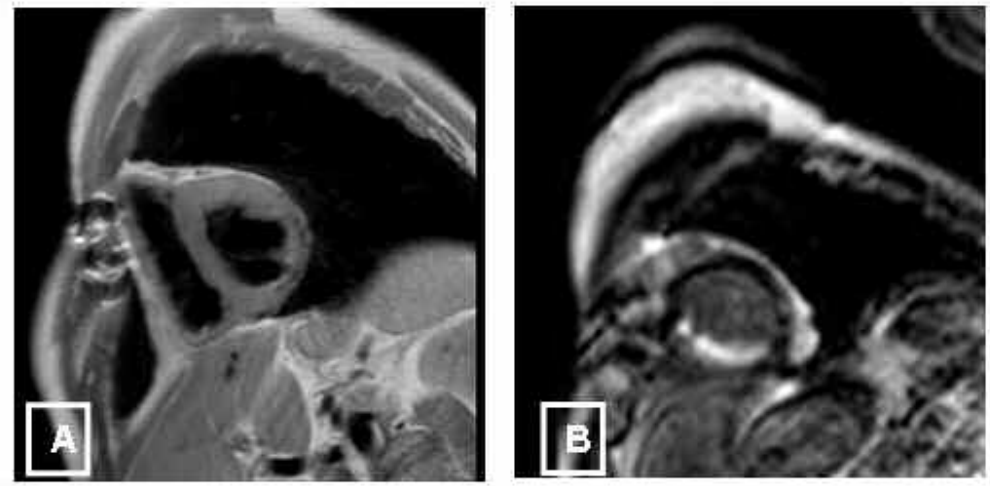


Figure 30. Cardiac Magnetic Resonance. **(A)**: first passage ipoperfusion of the postero-lateral wall. **(B)**: late-enhancement.

COMMENT

In 2003 the Cleveland Clinic Group reported the first gene that directly related to myocardial infarction (MI) and coronary artery disease. MEF2A. Subsequently, different groups had been stimulated to research in this field but there is still controversy as to whether MEF2A can be unequivocally considered as a disease causing gene for MI.

In the present study, the prevalence rate of the MEF2A deletion resulted to be very low as we could find no deleted individuals in healthy subjects of 2 cohorts (n= 170 pts); in primary hypertensive patients (n=131 pts) and in the vast majority of the GENICA Study (n=1141 pts) cohort. In this study we found the MEF2A deletion in only one of 1142 consecutive patients referred for coronary artery angiography. Therefore, the prevalence in the latter was 8.7 per 10.000 (<1 ‰) patients.

This result is consistent with those of a recent screening in Germany (69) in which no deleted patients could be found among 1181 from 23 families selected because they had at least 3 living siblings that had suffered myocardial infarction before 60 years of age, and at least 2 additional second- or third-degree affected relatives.

They are also consistent with the results of a study conducted in Ireland (72) where no deletion was identified among 1494 patients from 580 families selected because of at least one family member affected with proven premature ischemic heart disease (onset <60 years of age).

Of interest, to our knowledge, our pedigree is the first to be identified in Europe. It has moreover to be pointed out that the pedigree discovered by Wang L. and Topol E., was that of a family originally immigrated to Iowa from Scandinavia.

If the further genetic studies will confirm that the MEF2A mutation is rare, a general screening is not necessary. But the significance of identification of *MEF2A* as the first disease-causing gene for CAD and MI makes genetic testing possible for many individuals with a very high risk for CAD and MI. and wise to extend screening to pedigree of subjects with acute myocardial infarct or acute coronary syndrome and few or no risk factors.

By diagnosing the genetic susceptibility at an early age, efforts can be initiated far sooner in the individual's lifetime with respect to healthy lifestyle, including more rigorous attention to diet and exercise, along with more aggressive risk factor control. Eventually it should be possible to reclassify their cardiovascular risk as in our experience with case index n°730, otherwise low-moderate.

This could even extent to much earlier pharmacologic intervention with medications such as statins or aspirin in appropriate individuals deemed at high risk as the genomic level.

Ultimately, in the years ahead, patients who are interested in the information will able to undergo a panel of MI genes for determination of their risk and a genetic consult for tailored prevention, especially if genotyping would be inexpensive as allowed by our HMR technology (about 50 €/assay).

The recent discovery of high expression in the endothelium of MEF2A, lead us to hypothesize that an early trigger for the pathogenesis of CAD and MI may be dysfunction or abnormal development of the endothelium, which increases susceptibility of the coronary arteries to inflammation, leading to the development of CAD and MI.

It is important to note that the last view remains a hypothesis that needs further validation with more molecular biologic and genetic studies.

In the future, advances in genomic medicine will pave the way for much improved prevention of MI through genetic screening of individuals deemed at increased risk. Such insight should greatly catapult us forward in our efforts to triumph over this exceptionally important complex trait.

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