Cerebrovascular Research and Disorders

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ATHEROSCLEROSIS

RISK FACTORS, PREVENTION AND TREATMENT

Etsuo Murakami
Hayato Sakamoto
Editors

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ETSUO MURAKAMI
AND
HAYATO SAKAMOTO
EDITORS

Nova Science Publishers, Inc.
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PREFACE

Atherosclerosis is a chronic disease affecting the entire arterial tree, representing an inflammatory response in the vessels. The pathogenesis of atherosclerosis involves complex processes leading to the formation of atherosclerotic plaque. In this book, the authors present current research in the study of the risk factors, prevention and treatment of atherosclerosis. Topics include questioning the cholesterol-atherosclerosis hypothesis; diabetes-associated atherosclerosis and new therapies; the pathogenesis and oxidative mechanisms in atherosclerosis; peripheral arterial vasodilation for primary atherosclerosis prevention; and coronary artery atherosclerosis measured by a hybrid SPECT/CT camera.

Chapter I - The hypothesis that total cholesterol or LDL cholesterol is a risk factor or causative factor for coronary atherosclerosis was advanced without supporting evidence but rather was based on what appeared to be an association between serum cholesterol and acute coronary events. This observation was then extrapolated backwards since it was assumed that atherosclerosis was a prerequisite precursor of myocardial infarction or unstable angina. But this ignored several autopsy studies that showed no correlation between coronary atherosclerosis and circulating cholesterol in healthy accident victims, studies that were prior to or contemporary with the early evolution of the cholesterol-atherosclerosis hypothesis . Atherosclerosis frequently starts at a relatively young age and progresses to eventually generate plaque, atheromas and stenosis with an impact on coronary circulation, and this pathology increases dramatically with age. To study the cholesterol-atherosclerosis hypothesis, it seems reasonable that one must actually observe the associated arterial manifestations and progression and how they relate to cholesterol levels, rather than focus on acute coronary events or one or two selected atheromas. However, for a considerable period, this was not or could not be done, but the cholesterol-atherosclerosis hypothesis still evolved to become a dogma as did the cholesterol-heart disease hypothesis.

Chapter II - Over the past 150 years, there have been numerous efforts to explain the complex events leading atherosclerosis. In this endeavor, several hypotheses and the risk factors have emerged that currently are under active investigations. However, these hypotheses are not mutually exclusive, but rather emphasize different concepts as the necessary and sufficient events to support the development of atherosclerotic lesions. In this review, the combination concept of ‘‘response-to-injury’’ and ‘‘oxidative modifications’’ for the initiation of atherosclerosis, rather than the progression of atherosclerosis associated with risk factors, is mainly discussed for the prevention and treatment.

Chapter III - Atherosclerosis is a disease of the medium to large size systemic arteries, and is the leading cause of death world-wide. Although they do not mimic the human disease

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exactly, a cause of much debate, animal models of atherosclerosis are crucial to the authors' understanding of the risk factors and mechanisms behind the disease's initiation and progression. Various approaches have been taken, be it treatment with drugs or manipulation of the diet, to give us an insight into possible approaches to the prevention and treatment of the disease. The fat-fed apolipoprotein E knockout mouse has proven itself as a valid and extremely valuable model of atherosclerosis, and in particular as a model of atherosclerotic plaque rupture, the cause of the majority of severe clinical cardiovascular-related consequences. It is this model, and in particular its history, development, key findings, and current use in research, that shall be discussed further in this chapter.

Chapter IV - Atherosclerotic lesions initiate at bifurcations of arteries, regions characterized by low shear stress and reduced activity of endothelial atheroprotective molecules such as nitric oxide and grow towards high shear stress areas. Extensive epidemiological research has established that major, traditional risk factors, such as gender, age, cigarette smoking, family history, diabetes, hyperlipidemia, and hypertension, significantly contribute to the development of atherosclerosis as independent risk factors However, these so called classical risk factors not always explain the initiation and the progression of atherosclerosis. Therefore, novel risk factors have been proposed such as inflammatory biomarkers, lipoprotein (a), infections, triglyceride-rich remnants, homocysteine, thrombotic and haemostatic factors. As regards the treatment of these novel risk factors several drug categories have been proposed, such as angiotensin converting enzyme inhibitors/angiotensin receptors blockers, statins, along with a variety of other agents.

Chapter V - It has long been recognised that diabetic patients have accelerated atherosclerotic disease, particularly due to the underlying inflammatory response associated with the diabetic milieu resulting in atherosclerosis. Accelerated atherosclerosis contributes to the high rates of myocardial infarction and stroke observed in diabetic patients. Whilst current treatment regimes do slow the progression of diabetes-associated atherosclerosis they do not prevent it. This lack of effective treatment, coupled with the increasing prevalence of diabetes worldwide makes identification of novel therapeutic targets imperative. This review will outline recent work from other laboratories and the authors' own with respect to novel therapeutic targets including peptide hormones, such as urotensin II and endothelin I. Furthermore the authors will outline strategies which target generation of reactive oxygen species (ROS) directly by inhibition of the main enzymatic source of ROS, NADPH oxidase. It is now considered that the innate and adaptive immune system also play an important role in diabetes-associated atherosclerosis. This review will therefore also outline potential novel therapeutic targets within the immune response which contribute to the progression of diabetes-associated atherosclerosis. Ultimately identification of new therapies will lead to a reduction in the burden of cardiovascular disease in diabetes.

Chapter VI - Atherosclerosis, also known as atherosclerotic vascular disease (ASVD), is a prime cause of premature death and long-term disability throughout the world. Various theories like injury hypothesis, retention hypothesis and oxidative mechanism hypothesis were put forward over the years to explain the pathogenic mechanisms involved in ASVD. With so much already known about the etiology and pathology of this disease, the primary focus of investigation in the last two decades has shifted to prevention. One such area of attention has been the role of oxidative mechanisms in the development of ASVD, and the potential benefit of antioxidants in preventing oxidative vascular damage. Despite the presence of compelling evidence that oxidation mechanism plays a major role in

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atherosclerosis, the precise molecular mechanisms which lead to oxidative changes are still being studied. Although there is an accumulating body of evidence that does suggest that the oxidative mechanisms do play a role in ASVD, several large trials with antioxidants have failed to demonstrate any reduction in mortality from ASVD. In this chapter the authors discuss the evidence for and against antioxidants having a role in preventing atherosclerotic disease, with special attention to Alpha Tocopheral (vitamin E) and Ascorbic Acid (vitamin C). The authors also review the literature regarding the role of Uric Acid and Bilirubin in the pathogenesis of ASVD. Finally, they will discuss potential implications of current research regarding the prediction and prognosis of atherosclerotic disease.

Chapter VII - It is unlikely that cholesterol initiates atherosclerosis, because most of the risk factors (smoking/nicotine, low physical activity, high blood pressure, stress and apnea) or protective factors (high physical activity, vitamin D and alcohol consumption) are minimally linked with serum cholesterol levels. Furthermore, the site of the initial development of atherosclerosis cannot be explained on the basis of cholesterol hypothesis. The authors propose an alternative hypothesis called "vasa vasorum constriction/hypoxia", which logically covers all previous hypotheses and is in good agreement with risk and protective factors. They postulate that a small constriction of peripheral arteries (including external vasa vasorum) will lead to a progressive hypoxia in the branching areas of these end arteries deep in the smooth muscle layer. This leads to a prolonged contraction and increasing oxygen consumption. Hypoxia will develop to a severe anoxia and capillary damage. Macromolecules (HDL-, LDL-cholesterol= "cholesterol hypothesis", microbes= "microbe hypothesis", matrix vesicles = "microvesicle hypothesis" etc) leak into the wall of the artery (extravasation). An inflammation begins ("inflammation hypothesis"). Hypoxia/anoxia will also cause neoangiogenesis and regeneration.

According to the authors' hypothesis, a high physical activity prevents atherosclerosis, because it causes a peripheral vasodilatation. On the other hand, nicotine is known as peripheral vasocontrictor and therefore it is a risk factor. The beneficial effects of statins might be due to their vasodilatatory properties in addition to their anti-inflammatory action. The hypothesis suggests that peripheral arterial vasodilatation could be more suitable for primary prevention of atherosclerosis than statins, which are not as successful in the primary prevention as in the secondary prevention.

Chapter VIII - Detection of coronary calcification is a highly sensitive marker of underlining coronary atherosclerosis. A hybrid SPECT/CT made it possible to detect coronary atherosclerosis and myocardial perfusion simultaneously. The authors discuss coronary atherosclerosis, including calcification and coronary endothelial function, to determine whether the combined approach may serve as an important method for monitoring the treatment and making decision in further invasive investigations.

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Chapter I

DOES CHOLESTEROL DRIVE CORONARY ATHEROSCLEROSIS?

William R. Ware*
Emeritus Professor, Faculty of Science, University of Western Ontario,
Ontario, Canada

INTRODUCTION

The hypothesis that total cholesterol or LDL cholesterol is a risk factor or causative factor for coronary atherosclerosis was advanced without supporting evidence but rather was based on what appeared to be an association between serum cholesterol and acute coronary events. This observation was then extrapolated backwards since it was assumed that atherosclerosis was a prerequisite precursor of myocardial infarction or unstable angina. But this ignored several autopsy studies that showed no correlation between coronary atherosclerosis and circulating cholesterol in healthy accident victims, studies that were prior to or contemporary with the early evolution of the cholesterol-atherosclerosis hypothesis [1-3]. Atherosclerosis frequently starts at a relatively young age and progresses to eventually generate plaque, atheromas and stenosis with an impact on coronary circulation, and this pathology increases dramatically with age. To study the cholesterol-atherosclerosis hypothesis, it seems reasonable that one must actually observe the associated arterial manifestations and progression and how they relate to cholesterol levels, rather than focus on acute coronary events or one or two selected atheromas. However, for a considerable period, this was not or could not be done, but the cholesterol-atherosclerosis hypothesis still evolved to become a dogma as did the cholesterol-heart disease hypothesis.

In a review published in 2008, Paul Rosch describes and documents the early history of the cholesterol-heart disease hypothesis which was originally also called the heart-diet hypothesis [4]. In 1856 Virchow found cholesterol in human atheroma and thought it arose from an inflammatory process that injured the intimal lining of the arteries. This notion was reinforced in a studies where rabbits were fed large amounts of meat, eggs and milk. In 1909

* 14 Metamora Cres. London, Ontario, Canada N6G 1R3. E-mail: warewr@rogers.com. Tel: 519-472-2359.

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Ignatowski reported this produced lesions similar to those seen in human atherosclerosis and Windaus subsequently found that such intimal atheromatous deposits contained enhanced cholesterol compared to normal arterial walls. These observations were quickly followed by experiments where rabbits were fed purified cholesterol and this also produced lipid-laden lesions. However, evidence of inflammation characteristic of obstructive plaque in humans was absent in rabbits. It is also well known that rabbits do not normally consume meat, eggs or cholesterol, and similar experiments on carnivores were a failure.

Rosch also points out that few physicians in the U.S. were aware of this early research and there was little interest in coronary heart disease. It was not a major problem since it caused less than 10% of all deaths in the 1920s. The dormant period lasted until the 1950s when Ancel Keys became interested in the increasing rate of heart disease in the US which was not seen in Italy. His approach involved ecological studies in a limited number of countries which produced remarkable correlations between mortality from heart disease and either fat intake or serum cholesterol levels. However, when others examined the data from 22 countries actually available at the time Keys published his early work involving 6 countries, the correlation was poor [5]. The cholesterol-heart disease hypothesis was nevertheless successfully launched and Keys' early research labelled "landmark." However, twenty years after the Keys' studies, Rosch quotes him as admitting on the basis of his feeding experiments that cholesterol in the diet does not matter unless you are a chicken or rabbit [4]. A recent review of the literature confirmed this, finding that the risk CVD associated with dietary cholesterol is at best very low and that dietary cholesterol has negligible clinical implications [6]. It is thus interesting that limiting dietary cholesterol is still a pillar of modern guidelines.

The cholesterol-heart disease hypothesis was then examined in the famous and still ongoing Framingham study, based on a long follow-up of the population of a small city in Massachusetts. Framingham is widely viewed as providing solid evidence that high cholesterol levels are associated with elevated risk of heart attack. Cholesterol was described as a powerful predictor in spite of the observation that half of heart attacks occurred in individuals with normal or low cholesterol [7]. Framingham also found that those with a decline in serum cholesterol over a 30-year period had a *greater* risk of heart-disease related mortality. With each 1% drop in cholesterol resulted in an 11% increase in coronary mortality [8]. In 1992 in a paper on Framingham results, William Castelli, the director of the study at the time, stated that "the more saturated fat one ate, the more cholesterol one ate, the more calories one ate, the lower the person's serum cholesterol" [9].

The next significant study was the *Multiple Risk Factor Intervention Trial* (MRFIT). The results of one part of the MRFIT trial which involved following 300,000 so-called screenees for 6 years were presented as definitive by pointing to the result that the risk of dying from a heart attack (MI) with total cholesterol (TC) above 265 mg/dL was an impressive 4.3 times higher than if it was below 170 mg/dL [10]. However, the absolute percentage difference between the number of deaths from heart attack when these two groups were compared was only 1% (1.3% vs. 0.3%). Thus as a risk factor, elevated cholesterol did not seem to make much difference to almost all of the subjects studied. This is an example where relative risk can be very misleading, but the practice of emphasizing relative risk continues to this day. Nevertheless, cholesterol was accepted as a major risk factor for heart disease and even cardiovascular disease in general, and dietary cholesterol, while having a significant influence

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on serum levels in only a very small fraction of individuals, was demonized and this became a significant food-marketing tool.

The large number of lipid lowering trials that followed after the development of statin drugs provided the icing on the cake. Very small absolute differences in benefit were found for true primary prevention and somewhat larger but still small benefits observed for secondary prevention, but the large relative risk reductions of 20-30% were extremely persuasive. Only the critics kept harping on absolute risk reduction. The decrease in events associated with statin therapy was believed to be due to the lowering of LDL cholesterol, a fallacy in formal logic commonly termed *post hoc ergo propter hoc*. Attempts to establish a causative relationship have been plagued by the ever increasing number of pleiotropic effects (non-lipid lowering effects) associated with statins [11-13]. Furthermore, a series of recent negative lipid lowering trials have unsettled some who held the cholesterol-heart disease hypothesis as proven [14].

Over the years since the cholesterol-heart disease hypothesis was put forward and accepted, there have been numerous studies which have exposed inconsistencies and contradictions. For example, a further examination of the Framingham data revealed that once men reached 47, it did not matter if cholesterol was high or low if survival was the issue [8]. For sudden cardiac death, which is the case in about 50% of acute cardiac event-related deaths, the risk has been found in all studies to be completely independent of cholesterol levels [15]. Many other examples could be cited, but in the overall picture that emerged, it became evident that there were many factors at play including age, gender, country of residence and its culture, diet, exercise, smoking, hypertension, lipid sub-fractions such as lipoprotein(a) and apolipoproteins A and B, C-reactive protein, diabetes, obesity, the dyslipidemia of the metabolic syndrome, poor dental hygiene, homocysteine and psychological stress [16, 17]. In the context examining hypotheses concerning coronary atherosclerosis and the primary prevention of coronary heart disease, the formation, prevalence and progression of coronary plaque should now be primary endpoints in studies that evaluate the significance of this collection of risk factors, especially since plaque can be quantified by non-invasive CT scans. To some extent, this is ongoing, but it may take decades for a real evidence base to be developed and observational studies will remain essential. As will be discussed and documented below, non-invasive CT scans have provided a serious obstacle to the cholesterol-atherosclerosis hypothesis by failing to find any association between cholesterol levels and the prevalence or progression of calcified coronary plaque, which incidentally is in agreement with the early autopsy studies.

Since the cholesterol-heart disease hypothesis was advanced, critics wrote books, commentary in journals and letters-to-the-editor of journals detailing and documenting the problems with the hypothesis in an attempt to provide perspective, but these were ignored or ridiculed or simply not published. The authors of three of the books summarizing the critics' evidence are medical doctors [18-20]. These books, while written for a mixed audience, are a good source of citations for studies in the earlier peer reviewed literature that over the years have raised concerns. A very comprehensive and thoroughly documented book dealing with all the essential aspects of the heart-diet-cholesterol subject also recently appeared [21].

Unrecognized heart disease and in particular silent atherosclerosis is very common. When an "inside view" from a CT scan is lacking, clinicians attempt to non-invasively quantify the risk of developing symptomatic coronary or cardiovascular disease and clinically relevant events by using factors such as blood pressure, total and HDL cholesterol, smoking, age,

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gender and the presence or absence of diabetes, the latter being generally regarded as a risk equivalent to established heart disease. The end result is a score such as the Framingham Risk Score from which is derived an estimated 10-year risk of an acute coronary event and patient stratification into low, intermediate and high risk categories [22]. Such an approach is common but not without its critics or its defects, especially since age plays a central role. A man can be 74 with no risk factors and still be at intermediate risk. The Framingham Risk Score also has variable validity outside of the North America. The results of risk assessment influence medication decisions and treatment targets. So called "novel" or "emerging" risk factors such as homocysteine, triglycerides, C-reactive protein, apopolyprotein B, lipoprotein (a) and fibrinogen are also on occasion considered in treatment decisions since they can be quantified [23, 24], but evidence of the actual benefits of intervention to influence these emerging risk factors is sparse, inconsistent or non-existent.

Intermediate risk according to the Framingham Risk Score (10-20%) involves a huge grey area. Thus one school of thought believes that a more effective approach should employ the non-invasive measurement of coronary plaque by CT scanning in order to confirm the assignment of intermediate risk of acute events. When this approach has been tested, it is always found that some individuals change risk categories, both up and down, and such changes have a significant impact on treatment recommendations [25]. However, the use of CT screening for large populations has encountered considerable opposition from those concerned with overdiagnosis and overtreatment, and also from those who believe the cancer risk of the radiation exposure is significant. Incidentally, the magnitude of the risk associated with low-level radiation is considerably more debatable than generally recognized, and the field of so-called radiation hormesis is attracting increased attention [26-29]. Given the discovery of significant coronary plaque, there remains the question of an appropriate and in particular an effective intervention and, as will be discussed below, the typical response to a significant plaque burden often entails aggressive management of all traditional risk factors.

It is not the purpose in this review to examine in detail the all the issues outlined above. Rather, the focus is on subclinical atherosclerosis in both non-diabetic and diabetic individuals and its association with circulating cholesterol. Related aspects include primary prevention intervention based on cholesterol levels in populations free of symptomatic cardiovascular disease at baseline. Coronary atherosclerosis is, for almost all individuals, a prerequisite precursor of acute coronary events. Evaluating coronary atherosclerosis advanced rapidly after the introduction of electron beam computed tomography (EBCT) and the Agatston method of generating a convenient measure (calcium score) of the extent of calcified coronary artery plaque. The coronary artery calcium score provides significant improvement in risk assessment of coronary atherosclerosis in asymptomatic individuals, both non-diabetics [25] diabetics [30], as compared to using traditional risk factors. EBCT also represents an improvement over ultrasonic carotid artery assessment extrapolated to coronary atherosclerosis since there is very poor correlation between carotid and coronary atherosclerotic burden [31]. EBCT or variations of this non-invasive technique also offer an attractive adjunct to studies using acute coronary events as endpoints and allows examination of risk factor correlations that are relevant to the arterial process that preceded these events.

In the past 15 years a number of studies have examined the prevalence or progression of coronary plaque for both non-diabetic and non-diabetic individuals viewed as free of symptomatic coronary heart disease or cardiovascular disease. In these studies there was also extensive risk factor information collected, including blood lipid levels. In addition, the

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results of pharmaceutical intervention to halt or reverse progression of coronary atherosclerosis have been reported in a number of studies. Thus the information needed for a critical evaluation of the cholesterol-atherosclerosis hypothesis now exists. The focus in this review will be on the association between serum cholesterol and silent or so-called subclinical atherosclerosis, as manifest by calcified plaque.

INDIVIDUALS FREE OF CORONARY HEART DISEASE AND DIABETES

Cholesterol and in particular LDL has been called *the driving force of atherosclerosis* [32]. But this widely held view is merely a hypothesis based almost entirely on studies with cardiac event endpoints rather than direct measurement of coronary plaque burden and progression. But as briefly discussed above, cholesterol and LDL are rather weak predictors of CHD events and statin drugs produce very small absolute risk reduction in the context of primary prevention.

As will be discussed at length below, extensive data continues to accumulate indicating that serum total cholesterol (TC) and LDL cholesterol in individuals asymptomatic of CHD are not associated with either the extent or progression of coronary plaque [33]. The vast majority of the data relevant to the issues addressed in this review are based on EBCT scans. Most radiologists use the Agatston Score protocol to quantify plaque burden. This score ranges from zero to several thousand and provides the basis for inter-study comparisons and the development of generalized risk-score relationships. This non-invasive imaging can prevent unnecessary concern or lead to therapy [34]. A zero calcium score is particularly significant for the individual involved. Eight studies involving over 27,000 asymptomatic patients found that those with zero calcium score had an extremely low average annual coronary event rate (6.6 per 10,000), even though individuals with a zero calcium score may still have non-calcified plaque or stenosis [35]. Furthermore, the risk of adverse coronary events strongly increases with calcified plaque burden [34].

Some Inconvenient Questions

Those who believe the conventional wisdom that cholesterol in general and LDL in particular drives coronary atherosclerosis must address the following inconvenient questions which are based on the measurement mostly of calcified but also non-calcified coronary plaque as surrogates for coronary atherosclerosis. It is important to note that this approach avoids the use of carotid artery intima thickness as a surrogate for coronary atherosclerosis, a popular approach which suffers from being indirect and, as mentioned above, from a very poor correlation. It is also important to note that the correlation between carotid artery intima-media thickness and coronary atherosclerosis, the earlier traditional method of judging atherosclerosis, is modest, especially in asymptomatic individuals or those merely suspected of CHD, where correlation coefficients range mostly between 0.2 and 0.3 [31]. Results based on carotid intima-media studies, still a popular approach especially in studies of progression-regression, should be extrapolated to coronary artery atherosclerosis only with reservations.

If the hypothesis is true, then:

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- Why have autopsy studies of the correlation between the extent of coronary atherosclerosis and serum cholesterol yielded null results [1-3]? The objection that the blood samples, mostly from accident or suicide victims, were obtained too long after death has been discredited [3]. These studies, incidentally, looked all types of plaque.
- Why was it found by Hecht *et al* [36] that total cholesterol (TC) and LDL cholesterol did not correlate with either the extent or prematurely of coronary artery calcium (CAC) burden in 1105 consecutive, asymptomatic individuals self-referred for EBCT? Why did these same researchers [37] fail to find a correlation between LDL or TC and the CAC percentile (correlation coefficient 0.06 with a scatter plot showing no visible correlation) for 304 asymptomatic women?
- Why did Johnson *et al* [38] find when 1653 men and women without a history of CHD were subjected to coronary CT angiography using contrast media that there was there no correlation between total plaque burden (calcified, mixed and non-calcified) and total serum cholesterol with Spearman's rho = -0.04?
- Why in a study of the impact of psychosocial factors on coronary calcification in 780 male and female asymptomatic individuals, did Kop *et al* find that there was no correlation between TC or LDL and CAC score with Spearman correlation coefficients near zero? Multivariate analysis gave an odds ratio of 1.005 for LDL [39].
- Why in the St. Francis Heart Study [40] was no correlation found between LDL levels and CAC scores in 4,903 asymptomatic individuals?
- Why for adults with familial hypercholesterolemia, were age-adjusted CAC scores not associated with cholesterol levels, according to a study by Jensen *et al* [41]?
- Why in a study by Kornmal *et al* [42] of approximately 2900 individuals was the relative risk of incident CAC associated with LDL only 1.03 per 10 mg/dL and barely reached statistical significance (lower CI 1.01)?
- Why did Sung *et* al [43] in a recent study of CAC and coronary risk find negligible correlation between LDL or TC and calcium scores in 1653 asymptomatic individuals judged free of CHD (Spearman's coefficients = 0.07 and 0.08 respectively)?
- Why in a study by Ramadan *et al* [44] of 177 asymptomatic patients of intermediate risk of CHD was a null result found for the odds of a positive association between CAC and LDL in a multivariate model (OR = 1.022, p = 0.361)?
- Why when 100 asymptomatic individuals underwent EBCT were Takamiya *et al* [45] unable to find any association whatsoever between LDL and CAC when using three multiple logistic regression models?
- Why did a study by Lee *et al* [46] find that while insulin resistance positively correlated with CAC progression in a community based group of about 900 healthy adults, they failed to find in multivariate regression any correlation with TC or LDL.
- Why in a study by Budoff *et* al [47] of CAC progression and all-cause mortality, was a strong association found for subjects with calcium score (CACS) $\geq$ 30, and this relationship was not changed by adjustment for hypercholesterolemia?

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- Why in a study by Park *et al* [48] of 5239 asymptomatic patients free from known coronary artery disease did multivariate analysis of factors significant for a CACS > 100 not include TC or LDL?
- Why did a recent study from Korea by Lee *et al* [49] of the relationship between CAC and bone mineral density fail to find in either univariate of multivariate analysis any correlation between the CACS and LDL?
- Why in a study by Jeon *et al* [50] of the association between estradiol and CAC in postmenopausal women did a univariate analysis of factors associated with a calcium score ≥ 100 fail to find LDL significant (p = 0.49). Age, time since menopause and hypertension were the strongest risk factors.
- Why in a study by Rondanelli *et al* [51] of progression in a group initially having CACS of zero, was it found in univariate analysis that dyslipidemia was insignificant and thus omitted from multivariate analysis? Diabetes, hypertension and age > 40 remained significant.
- Why in a study by Coylewright *et al* [52] was neither TC or LDL significant in a multivariate analysis when one compared the risk of having high (400-999) or very high (≥ 1000) calcium scores. This also found that while high coronary calcium was associated with the risk of CHD death and myocardial infarct, very high values were more predictive of angina.
- Finally and perhaps the most important question, why is there no association between TC or LDL and the progression of CAC? At least 10 studies in addition to those mentioned above have directly addressed this issue and all cited involved EBCT [42,53-61].

One study Turner *et al* [62] recently appeared which appears to contradict the above picture. As part of the Multi-Ethnic Study of Atherosclerosis (MESA), the association of CAC with a combination of lipid parameters with was examined. For CAC, the question was zero or > zero. The lipid parameters were hypercholesterolemia (LDL ≥ 160 mg/dL, TGs < 150mg/dL) and no restriction on HDL. Combined hyperlipidemia changed the TGs to ≥ 150 mg/dL. The analysis treated the variables as dichotomous and found a small but statistically significant risk of CAC > 0 when hypercholesterolemia or hyperlipidemia was present. The association with LDL became progressively weaker with increasing age. While elevated TGs have been implicated in some studies, the finding for just hypercholesterolemia is surprising and may reflect the design of the study since the result is contrary to all of the above cited studies as well as those discussed below that concern diabetics.

A noteworthy exception to the above picture was reported in 2010. The results were part of the ongoing Coronary Artery Risk Development in Young Adults study (CARDIA) and indicated that non-optimal levels of LDL and HDL during the early years of adulthood were independently associated with coronary atherosclerosis two decades later, but at this later time, the plaque burden was no longer dependent on lipid levels [63]. The investigators calculated time-averaged cumulative exposures to blood lipids between ages 20 and 35 and then examined CAC burden later in life at mean age of 45. This implies LDL as a factor in the initiation of atherosclerosis at a young age, but as has been discussed above and confirmed in this study, in older persons the correlation disappears.

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One explanation for this apparent inconsistency is psychological stress which elevates LDL cholesterol in this age group [64-66]. The cited studies apply to the age group studied in CARDIA, the stress was mostly of academic origin, and the LDL elevations similar when compared to the CARDIA data. In this view, LDL is simply a marker for psychological stress. When the stress was continuous, as it was in one study, the elevation of LDL levels persisted [64]. Psychological stress might then operate to produce atherosclerosis through a blood pressure mechanism, and this has been documented in another CARDIA study of young adults where blood pressure reactivity to psychological stress was a positive factor in predicting CAC 13 years later [67].

Hypotheses are not proven but exist until falsified (to paraphrase Karl Popper). One is reminded of the famous black swan example. Here we seem to have a significant number of black swans! The obvious answer to the questions raised is that the hypothesis is false. Twenty-eight studies, all clearly relevant, contradict the hypothesis that TC or LDL drives atherosclerosis, either when plaque burden or progression is at issue, and this observation does not include twelve studies cited below that focus entirely or type 2 diabetic populations and reach the same conclusion. These questions directly address coronary plaque and thus do not involve arguments based on studies concerning other vascular beds. The results are consistent over a wide range of age and nationality for both genders. In many of the studies presented as evidence, the lack of an association was based on multivariate analysis. It seems justified to regard this as compelling evidence.

It might be argued that total plaque rather than calcified plaque should be the basis for judging the hypothesis, but in general, non-calcified plaque represents a small fraction of the total plaque burden. One study cited in the above set of questions [38] did indeed look at total plaque including non-calcified, calcified and mixed and found no correlation with TC. In addition, another study [68] found progression of non-calcified plaque volume in coronary arteries to be independent of whether patients had high or low LDL. Also, the autopsy studies looked at total plaque. The issue of different plaque types will be discussed in more detail below.

IMPLICATIONS AND RELATED CHOLESTEROL ISSUES

LDL as a Surrogate Endpoint for CHD Risk

The above results do not support the widely held view that a diet high in saturated fat is atherogenic and increases the risk of CHD because it modestly raises LDL which in turn, so it is argued, stimulates atherosclerosis. This role of saturated fat in CHD was already conclusively challenged two decades ago [69]. Since then three studies and a meta-analysis all reached the same negative conclusion [70-73]. Also, increased saturated fat intake leads to a beneficial *decrease* in small dense LDL, and greater intake in saturated fat was found to *reduce* progression of coronary arthrosclerosis [74]. Nevertheless, reduction of saturated fat intake has for a long time been central to preventive and treatment guidelines. A general discussion of the use surrogates in the context of CHD has recently appeared [75].

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Determinants of Plaque Progression

In the ten coronary plaque progression studies cited above, the prior existence of calcified plaque and hypertension were the most frequently found statistically significant positive risk factors, followed by diabetes, lipoprotein(a), triglycerides, smoking, the Framingham risk score, and HDL (negative association), but these latter factors were not consistently identified. In terms of modifiable factors, the only strong association consistently found was with hypertension. This association was also found in a study of individuals having a zero calcium score at baseline [57]. However, insulin resistance also appears very important not only in progression but also in plaque burden [46, 76]. Diet and exercise can influence insulin resistance, HDL and triglyceride levels, and patients are presumably always advised to stop smoking.

Plaques and Lipid Lowering

The null results from the large number of trials cited above suggest that lowering LDL should have no impact on the prevalence or progression of coronary plaque and calls into question the proposed approach which targets LDL for asymptomatic persons of intermediate traditional risk of CHD who exhibit elevated coronary calcium [77, 78]. Several randomized clinical trials employing statins and enrolling asymptomatic individuals support this inference. Randomized, placebo controlled studies found that statin therapy had no effect on the progression of coronary calcification as measured by the calcium score [79-81].

In trials comparing doses or different statins, atherosclerosis progression as measured by calcified plaque showed no relationship with on-treatment LDL levels and intensive therapy was unable to attenuate coronary artery calcium progression [80, 82, 83]. A recent randomized trial also failed to support the hypothesis that baseline CRP levels modify the vascular benefits of statin therapy [84]. However, one study found statin therapy reduced plaque burden for non-calcified plaque but not CAC score or total plaque burden [85]. This is inconsistent with the studies discussed above and only 11% of the patients had non-calcified plaque.

Lipid-Lowering Trials in Primary Prevention

The failure of statins to influence prevalence or progression of atherosclerosis prompts questions regarding statins for primary prevention of CHD. Michel de Lorgeril has discussed nine CHD-related negative cholesterol lowering trials done since the Vioxx affair of 2005 resulted in tightening of rules and regulations concerning conduct and reporting of trials [14]. This paper extends earlier related observations from de Lorgeril and colleagues [86, 87]. These recent results challenge the cholesterol hypothesis as did the observation that half of a large serial cohort admitted to a hospital for heart attack (MI) had low or very low LDL [88]. In addition, a recent meta-analysis that recalculated primary prevention trial results after excluding all subjects with CHD found no impact on all-cause mortality [89]. It is interesting in view of the recent JUPITER lipid and CRP lowering trial [90], that three recent studies directly examined the correlation between the coronary calcium score and high-sensitivity

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CRP levels and found that there was no association [54, 91, 92]. This was confirmed in the JUPITER cohort where obesity was the important factor associated with atherosclerosis [93]. JUPITER has also been criticized as flawed [94]. It may also have been confounded by the fact that rosuvastatin appears unique among statins in that it strongly elevates vitamin D levels and it is well known that low vitamin D status is a risk factor for CHD and also for coronary atherosclerosis [95].

A recent study reported a meta-analyses of statin intervention RTCs restricted to pure primary prevention (11 studies) and selected studies after eliminating 4 trials because of suspected high risk of bias according to the Cochrane Risk of Bias Tool. It was found for the restricted set that there was no statistically significant risk associated with overall mortality, an absolute risk reduction of 1.3% in major CHD events with an a number needed NNT to treat over the period of the study (typically 4-5 years) of 77 to prevent one event. For 11 studies the absolute risk reduction for major CHD events was 1% with a resultant NNT of 100 [96]. Of the seven RTCs, AFCAPS, ALLHAT-LLT, ASPEN, PROSPER, WOSCOP carried most of the weight with HYRIM, and PREVEND-IT contributing less than 2%.

Cholesterol as a Risk Factor for CHD

This association is based mostly on studies of the risk of CHD events and not on directly observed coronary atherosclerosis identified by calcified plaque. A recent review [97] summarizes six disturbing facts including the evidence that for women, men over 47 and in general the elderly, hypercholesteremia is a very weak risk factor for CHD, or in most cases, not a risk factor at all. Studies of familial hypercholesteremia (FH) also fail to support the hypothesis if one considers that in young people, where most of the excess mortality is seen, FH includes well known abnormalities in the coagulation system which constitute a strong CHD risk factor. In young men, the observed association may be confounded by the connection between cholesterol levels and stress-induced exaggerated blood pressure response [98]. The major studies frequently cited to support the hypothesis that cholesterol causes coronary heart disease continue to come under attack [99]. If one uses the prevalence and progression of coronary atherosclerosis as criteria instead of events, the association disappears completely.

DIABETIC INDIVIDUALS FREE OF CORONARY HEART DISEASE

Individuals with diabetes represent a special case since they are at enhanced risk of cardiovascular mortality and morbidity. Current estimates indicate that 1 in 3 individuals in the US will develop diabetes in their lifetime with concomitant lifespan reduction of 10--15 years. Furthermore, more than half of diabetics will die as the result of cardiovascular disease [100]. In the INTERHEART Study, a large case-control study of incident acute MI, diabetes was among the strongest risk factors identified. The combination of diabetes and hypertension was found to be particularly dangerous [16].

This review will focus on cholesterol and atherosclerosis in type 2 diabetics since they represent the vast majority of those with this disorder. While type 2 diabetes is generally

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characterized by much later onset, current trends in obesity and the metabolic syndrome among young people will no doubt alter this natural history dramatically over the next few decades with the associated comorbidities occurring much earlier.

In 2011, the American Diabetes Association (ADA) published their view of the standard of medical care for diabetes [101]. Under prevention and management of complications, they discuss hypertension, blood lipids, antiplatelet therapy and smoking cessation. Central to the lipid management recommendation is the focus on LDL cholesterol and the recommendation of statin use by many diabetics. Thus, it is of interest to examine the evidence for the central role given LDL and the magnitude of the benefits associated with statin use in diabetic patients, especially those without cardiovascular or coronary heart disease but with type 2 diabetes.

There are a number of studies including randomized, placebo controlled trials and observational studies published over the past 8 years that are of interest. In addition, it is clearly important to examine the factors associated with the prevalence and progression of subclinical coronary atherosclerosis in diabetics using non-invasive imaging.

CORONARY ARTERY CALCIFICATION PREVALENCE STUDIES IN DIABETICS

A number of studies have been published recently that find LDL is not a factor in the prevalence of coronary artery calcium (CAC) in diabetics as measured by electron beam computed tomography (EBCT) or coronary computed tomography angiography.

- Mazzone *et al*. (CHICAGO study) examined the prevalence of CAC in type 2 diabetics [102]. They excluded type 1 diabetics and anyone with coronary artery disease (CAD), cerebrovascular or peripheral vascular disease, heart failure, very high triglycerides (TGs) or BMI >45. Lipoprotein determinants of the CACS were HDL, TGs, ApoB, and cholesterol components called triglyceride rich lipoproteins (TRLs). Significant factors on multivariate analysis were age, systolic blood pressure, gender, race, and TRLs. Neither LDL nor statin use (50%) was significant in the multivariate analysis.
- Anand *et al* examined factors associated with CHD event-free survival over about 2 years and the prevalence of CAC in Type 2 diabetics [103]. They excluded subjects with documented CAD, atypical angina, abnormal ECGs, cerebrovascular or peripheral artery disease, or renal impairment. Multivariate analysis predictors of CAC were age, male gender, ethnicity, hypertension, duration of diabetes, statin use (positive) and UKPDS risk score (weak association). The input data included hyperlipidemia which was not significant in multivariate analysis. This study also examined cardiovascular event-free survival over about 2 years. LDL was not a significant predictor and statin use did not enter into the analysis.
- Elkeles *et al* studied the association between CAC and conventional risk factors in individuals with type 2 diabetes [104]. Subjects had no known coronary heart disease (CHD). Multivariate analysis yielded waist-hip ratio, systolic blood pressure, male gender and statin use as positive risk factors. Factors not appearing as significant in

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multivariate analysis included non-HDL cholesterol, LDL, the cholesterol ratio TC/HDL, TGs and HbA1c.

- Wolfe *et al* examined the association between CAC and traditional risk factors [105]. They found in a study of 71 diabetics with no evidence of CHD that the extent of CAC was independent of LDL levels in an adjusted model. Age, male gender and BMI were the only significant factors.
- A study by Martin *et al* (Penn Diabetes Heart Study) examined factors associated with CAC in type 2 diabetics with no clinical evidence of CHD [106]. Only white individuals were enrolled. Analysis found only ApoB as a significant factor and in particular, LDL was not significantly associated with CAC after adjustment for age, sex, hypertension, smoking, alcohol, exercise, family history of premature cardiovascular disease (CVD), C-reactive protein, medications (including statins) and the metabolic syndrome. However, ApoB and LDL are correlated. Also, ApoB is a measure of LDL particle number and total the particle number viewed as atherogenic. In this study, there was a large difference between statin use in diabetics (57%) and non-diabetics (14%), but ApoB predicted CAC even after controlling for differences in statin use and in subgroup analysis of non-statin users. This study also found a weak but positive association with LDL and CAC in non-diabetics, an association which, as discussed above, is almost universally absent in numerous other studies.
- The PREDICT study reported in 2006 on the results of a study involving 573 subject with type 2 diabetes [107]. Excluded were those with past or present heart disease and uncontrolled hypertension. It was found in a multivariate analysis that age was the major factor influencing the CACS with weaker contributions from waist-to-hip ratio and duration of diabetes. Other traditional or novel risk factors were found to have insignificant positive effect, including TC, HDL,TGs and the use of statins (34-45% of subjects).
- A Japanese study examined the association of the prevalence and extent of CAC with insulin resistance in chronic kidney disease patients free of symptomatic CVD or any history of previous MI [108]. It was found that CAC becomes more prevalent and severe with an increase in the severity of kidney disease. In a univariate analysis, all the classical lipid parameters as well as statin use failed to achieve significant association with the CACS, with LDL the weakest ($p = 0.59$). In a multivariate analysis for factors associated with the CACS, only the common measure of insulin resistance (HOMA-IR) exhibited a significant association. Of the 111 subjects, 38 (34%) had type 2 diabetes although none used insulin.

In all but the study by Wolfe *et al*, a significant percentage of subjects were taking statin drugs (range 34% to 51% with most studies around 40%). Thus the mean LDL levels were, with the exception of Wolfe *et al*, about 108mg/dL, but there was in general a large enough range to make the LDL level a meaningful variable to examine. When the association with statin use was examined, two studies found the prevalence of CAC increased with statin use, while for remainder there was either no association or in Wolfe *et al* it was not noted in the baseline data. Based on the conventional wisdom, one might have expected an inverse correlation with statin use, i.e. statin use yields lower LDL which lowers the prevalence of

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CAC. This was not observed even though there were significant statin users, but rather, the opposite was found in two of the studies. This anomalous positive association with statin use persists in studies of progression of CAC, and a potential explanation will be discussed below.

CORONARY ARTERY CALCIFICATION PROGRESSION STUDIES IN DIABETICS

What factors impact the progression of CAC in diabetics with no symptoms of heart disease? There are several relevant studies.

- A substudy of the Veterans Affairs Diabetes Trial examined the progression of CAC in 189 type 2 diabetics [109]. Individuals were excluded who had experienced stroke, MI, or revascularization in the past 6 months or who had congestive heart failure or significant angina. CAC progression was found in > 75% of subjects but was not influenced by standard risk factors. Univariable predictors of CAC progression were ethnicity, lipoprotein-associated phospholipase (Lp-PLA2) and the albumin to creatinine ratio. Lipids were not significant. The albumin to creatinine ratio and LP-PLA2 predicted progression independent of adjustment for age or other traditional risk factors. Treatment assignment to standard or aggressive glycemic control did not influence progression, irrespective of baseline CACS. LP-PLA2 has also been associated with the progression of subclinical atherosclerosis in a study involving individuals either with or without type 1 diabetes [110].
- Anand *et al* have reported the results of a study of the determinants of progression of CAC in type 2 diabetics without prior CHD or peripheral vascular disease, intermittent claudication, stroke, transient ischemic attack or renal impairment [111]. In the final multivariate analysis, odds ratios for independent factors for progression of CAC were baseline CAC > 400 mm^3 (OR = 6.38), HbA1c > 7 (OR =1.95), and statin use (OR = 2.27), but not hyperlipidemia or smoking status. It is noteworthy that the researchers found that statin therapy failed to inhibit progression of CAC but rather accelerated it.
- Progression of atherosclerosis measured by CAC in type 2 diabetics without previous clinical CVD was also addressed as part of the PREDICT study [112]. CAC was measured at baseline and after 4 years follow-up. The rate of change was strongly related to baseline CACS and also correlated positively with waist to hip ratio, male gender, the use of antihypertensive drugs or statins and the albumin to creatinine ratio. The majority of subjects showed progression. There was no relationship with traditional lipid risk factors. Independent of baseline CACS, blood pressure, central adiposity and microalbuminuria were found significant in the context of progression and the authors suggest these as areas for risk factor modification especially relevant to type 2 diabetics.
- It is well know that depression is a comorbid condition associated with diabetes [113]. The nature and details of the interaction are not clear. This is relevant to the present discussion since depressive symptoms are a risk factor for CHD and it has

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now been shown that depressive symptoms are a risk factor for the progression of coronary calcification. A recent report documented this in midlife women in the SWAN Heart Study, where depressive symptoms were independently associated with progression as measured by CAC [114]. In a multivariate analysis, the only other predictors of significance for progression of CAC were systolic blood pressure, and a low level of education. Lipids did not qualify statistically for inclusion in the analysis, and statin use, which was included, turned out to be insignificant.

- In a CAC progression study based on the Kaiser Permanente of Northern California database, Lee *et al* [46] examined a sample of about 900 healthy adults age 60-72 initially free of clinical CAD. They included measures of insulin resistance, noted the presence of diabetes in the group, and then examined associations via univariate and multivariate analysis. In the univariate analysis, TC, LDL, HDL and TG were not even close to being significantly associated with progression of CAC over 2 years. However, dyslipidemia was a factor, but the authors fail to provide their working definition. In the context of the study this probably means the dyslipidemia associated with prediabetes and diabetes which involves elevated TGs and low HDL. In multivariate analysis, progression was associated with age, female gender, African American, diabetes, fasting insulin, dyslipidemia, hypertension, diastolic BP and pulse pressure but not lipid lowering medication.

In some of these studies, 30-50% of the subjects were on statin therapy at baseline. It is thus curious that statin use turns up in multivariate analysis in some of these studies to be a positive risk factor. Elkeles in his 2010 review of coronary calcium and CVD in diabetics suggests that the positive associations may have been caused by some study cohorts including individuals who had been given statins because they were perceived to be at the greatest risk [115].

Not all of the studies of prevalence or progression examined the same set of baseline factors but all included the traditional blood lipids and blood pressure. For prevalence, systolic blood pressure and the waist to hip ratio were most frequently found on multivariate analysis. For progression, the albumin to creatinine ratio, blood pressure and statin use were the most common positive factors. The strength in these studies appears to be the consistent finding of no association between total cholesterol or LDL cholesterol and the extent or progression of subclinical coronary atherosclerosis, a result consistent with studies on non-diabetic cohorts discussed above. Nevertheless, statins are recommended for most diabetics. This raises the question of the comparative effectiveness of lipid lowering in the context of acute events in diabetics as compared to non-diabetics.

PRIMARY AND SECONDARY PREVENTION OF DIABETIC CHD/CVD EVENTS WITH STATINS

Individuals with diabetes have enhanced risk of coronary heart disease and vascular disease in general. As discussed above, in the context of true primary prevention in non-diabetics, statins provide only a small (approximately 1%) absolute benefit in terms of acute coronary events and no impact on mortality. Thus it is of interest to examine this therapy in

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diabetics. If one examines the 2011 ADA standard of care [101], three primary prevention lipid lowering studies are cited where it is possible to stratify both by diabetes and the presence or absence of baseline CHD, thus obtaining information on the role of statins in risk reduction in the context of primary prevention of acute CHD events in type 2 diabetes. Their Table 11 indicates endpoint restriction to fatal CHD and non-fatal heart attack, but it is extrapolated to 10-year risk. It is thus of interest to look at the individual primary prevention trials. The results were as follows for absolute percent risk reduction, (ARR) the numbers needed to treat to prevent one event (NNT), the relative risk reduction (RRR) and the untreated event rate (UTER) for the endpoint of fatal and non-fatal heart attack over 4-5 years (calculated from reported events over the study period).

The HPS (MRC/BHF) trial [119], which the ADA included, is omitted because for the endpoint in question, the study included in the diabetic group individuals with arterial disease, including 33% with previous heart attack or other CHD and 18% with other occlusive artery disease. HPS only stratified diabetics by prior CHD when the endpoint includes coronary events, all strokes, and coronary and non-coronary revascularization, which inflates the benefit. Its inclusion with the data presented in the ADA table is inconsistent with the other primary prevention studies cited.

The very small absolute risk reduction (average 1.1%) with large NNT seen in the above table was also found in studies of cohorts where diabetes is present in only in a small fraction or absent. In this essentially non-diabetic population, when meta-analyses were rigorously restricted to primary prevention statin trials [96], absolute risk reduction for major CHD events was 1.0% (11 studies) and the NNT 100. Thus it is interesting that in the context of primary prevention, there is little difference between diabetics and non-diabetics. In addition, the above results call into question the use of statin therapy for diabetics free of CHD/CVD.

The largest meta-analysis of statin trials where both diabetes and primary vs. secondary prevention were stratified is the Cholesterol Treatment Trialists' Collaborators (CCT) study. The results are summarized below for the composite endpoint of MI, coronary deaths, stroke, or coronary revascularization over 4-5 years, calculated from reported event numbers [120]. Most participants in these trials were at elevated risk of adverse CVD events.

These results illustrate the greater impact of statins in secondary prevention. The result for diabetics with no vascular disease is somewhat higher than in CARDS, ASPEN and ASCOT discussed above, presumably because of the expanded endpoint which increases event rates. The results for no diabetes and no vascular disease are slightly higher than those reported above [96], again probably for the same reason. The recent Cochrane meta-analysis [121] of primary prevention trials which included up to 10% subjects with heart disease and significant numbers with diabetes and hypertension presented data that yielded a 1.9% AAR (27% RRR) for CHD [75], which, given the cohorts, compares well with the CCT value of 1.6%.

Table 1. Primary prevention trials of statins among patients with diabetes

Trial	ARR	NNT	RRR	UTER
ASCOT [116]	0.6%	166	16%	1.4%
ASPEN [117]	0.7%	142	27%	3.6%
CARDS [118]	1.9%	53	36%	3.6%

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Table 2. Summary of outcomes from the Cholesterol Treatment Trialists' Collaborators study

Category	ARR	NNT	RRR	UTER
Diabetes and no vascular disease	2.6%	39	27%	11.8%
Diabetes with vascular disease	5.3%	19	20%	31.6%
No diabetes and no vascular disease	1.6%	63	22%	8.3%
No diabetes with vascular disease	5.0%	20	21%	23.5%

The CCT results tabulated above which include the NNT should be viewed with some caution since the combined studies average over somewhat different populations. The NNT, as it is being used to illustrate clinical utility, can only be rigorously applied for comparisons when the baseline absolute risks are similar. Also, the NNT decreases dramatically with the duration of studies [122-124]. In the CCT results, duration probably does not confuse the issue. Nevertheless, for primary prevention when 60-80 individuals without diabetes and about 40 with diabetes must be treated to prevent one acute event, the treatment appears to be without benefit for too many individuals receiving the drug to make it attractive, given that there are potentially serious side effects. These results, instead of justifying the use of the therapy, should prompt a search for something better.

There is growing suspicion that a significant and perhaps major part of the action of statin drugs involves non-lipid lowering (pleiotropic) effects, of which there is a long and impressive list containing actions that can influence acute events, but have nothing to do with lower circulating cholesterol [11-13]. This may explain the higher absolute risk reduction in secondary prevention trials seen in Table 2. Pleiotropic effects significantly impact the use of LDL lowering trials to prove that LDL is a causative factor. Some might argue that for secondary prevention it does not matter what the mechanism is as long as there is a modest (5%) absolute benefit.

However, there are the statin adverse effects. For liver dysfunction, cataracts and myopathy, the number needed to treat to harm one patient with statin therapy has been estimated for men at 142, 52 and 81 and for women 136, 33 and 259, respectively [125]. It is doubtful that cataracts disappear if statin therapy is terminated. It is also strongly suspected that adverse events are significantly under-reported, downplayed or prevalence data suppressed. The point at which the ARR is large enough and the adverse side effects small enough to justify treatment is of course debatable, and the attitudes of both patients and physicians highly variable. Conservative clinicians might conclude that the treatment is not justified in general for very small ARRs, especially in the absence of symptomatic heart disease [96].

A side effect of statin treatment which is receiving increasing attention involves the enhanced risk of incident diabetes. In fact, at the 2011 annual meeting of the European Association for the Study of Diabetes held in Lisbon, Portugal in September, a special symposium was devoted to this topic. Diabetes incidence has been examined as a side effect in studies that were controlled by a placebo and studies where two doses of statin were compared.

Sattar *et al* [126] have performed a meta-analysis on a large number of randomized statin trials. When diabetes incidence was not in the published report, they obtained the data from the authors. A total of 13 trials were used involving over 91,000 participants. Statin therapy

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was associated with a statistically significant 9% relative risk increase. In absolute terms, this represented one new case per 255 statin users or an absolute risk enhancement of about 0.4% over 4 years of therapy.

Waters *et al* [127] has reviewed three studies not included in the above analysis that employed atorvastatin. Only the SPARCL study compared the drug with a placebo. This study involved 80 mg /day, a dose considered high. A 37% increase in the risk of new-onset diabetes was found (2.7% absolute increase). The diabetes risk factors were fasting glucose ≥ 220 mg/dL, triglycerides ≥ 150 mg/dL, BMI ≥ 30 and history of hypertension (yes/no). Individuals with none of these risk factors had a hazard ratio (HR) or 1.37 whereas when all for factors were present, the HR jumped to 2.44. The other two trials reviewed involved comparison of 80 mg and with 10 mg (TNT trial) or 20 mg (IDEAL trial) of atorvastatin. For the TNT trial, the HR for new-onset diabetes for all statin users was 1.10 but not significant. For the IDEAL trial, the HR was 1.19, also not significant. While the SPARCL result indicated higher risk, the upper confidence limit overlapped that in the meta-analysis of Sattar *et al* discussed above.

The general conclusion seems to be that there is a small absolute enhanced risk of diabetes associated with statin treatment which becomes stronger with the presence of all the identified risk factors, but that the absolute risk increase may be smaller than the absolute risk reduction for incident CVD. These studies involved mostly cohorts at high risk of CVD or were CVD secondary prevention trials.

FIBRATE THERAPY

A class of drug called fibrates has also been tested for reducing CVD/CHD events in diabetics. Fibrates are interesting in the context of diabetes because diabetic dyslipidemia is characterized by elevated TGs and low HDL and fibrates are very effective in lowering TGs (30-50%). When combined with a statin, they dramatically reduce TGs and modestly elevate HDL. The most extensive testing has occurred with fenofibrate. In the FIELD randomized controlled study [128] which reported in 2005, fenofibrate did not significantly reduce the risk of CHD death or non-fatal MI, but there was a significant (about 14%) relative risk reduction in total cardiovascular events, driven in part by revascularizations which represent a soft endpoint due to prevalence influenced by physician recommendations (and persuasive ability??). Additional analysis of the outcomes in the FIELD study has revealed beneficial effects on the need for laser therapy for patients with or without known baseline diabetic retinopathy and fenofibrate treatment was also associated with slower progression of kidney disease and reduced risk of diabetes-related amputation [129].

The FIELD trial was followed by the ACCORD-DIAB-FENO trial [130] which compared type 2 diabetics taking simvastatin to those where fenofibrate was added for a combined therapy. This combined therapy did not reduce the rate of fatal cardiovascular events, non-fatal MIs, or non-fatal stroke (the combined primary endpoint) as compared to the statin alone. However, there was evidence of benefit in a reduction in the rate for the primary endpoint in individuals with significantly elevated TGs (≥ 204 mg/dL) and low HDL (≤ 34 mg/dL) where approximately a 30% decrease in relative rate was found, but the result was only close to statistical significance.

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In 2011,the FDA has issued a warning regarding fenofibric acid, stating that the drug may not lower the risk of major cardiovascular events, which is the rationale for its use. This was prompted by the ACCORD trial. Furthermore, women receiving the combination may have an increase in risk of a major cardiac event compared to the statin treatment alone. Fenofibrate was approved in 2008 to be used with a statin to reduce triglycerides and increase HDL in diabetics with dyslipidemia and coronary heart disease or at risk of coronary heart disease who were already at their "official" LDL target.

A PARADOX?

The above studies, viewed together, suggest a paradox. Acute coronary events, aside from those associated with "electrical problems," almost always require atherosclerosis. The more CAC the more coronary plaque and the higher risk of acute events with very low risk associated with zero plaque [131]. The prevalence and progression of coronary plaque as measured by EBCT is independent of serum LDL and TC levels over a very wide range of calcium score, age, gender, ethnic background and the presence of diabetes. But it is almost universally believed that the risk of CHD as manifest by acute ischemic events is strongly and continuously associated with LDL levels and thus LDL lowering targets form one of the pillars of modern preventive medicine in asymptomatic individuals with and without diabetes and as well in secondary prevention. This has the earmarks of a paradox.

The belief that the risk of coronary heart disease (CHD) events is strongly and continuously associated with LDL levels has resulted in LDL lowering targets which can generally only be attained with drugs. These targets are strongly dependent on meta-analyses of intervention studies which involve mostly secondary prevention. Cholesterol, we are also told, is related in observational studies to the risk of acute CHD evens which mostly require atherosclerosis, and at the same time, the evidence is compelling that neither TC nor LDL is driving atherosclerosis in men or women.

This apparent paradox can be resolved merely by assuming that the pathophysiology of acute events is different from that involved in the natural history of atherosclerosis. This gives LDL the role of an event trigger. However, this seems inconsistent with the apparent absence of a dose relationship since as pointed out above, it has been observed that half of a large cohort presenting with MI had low or very low LDL. Nevertheless, this explanation may have some merit.

There is an alternative. A way to resolve this paradox is to take the view that the association between TC and LDL and acute coronary events is in fact very weak, clinically insignificant, and for some segments of the population, nonexistent, a view that goes strongly against the conventional wisdom and guidelines from a number of professional organization. This has already been briefly discussed above. The literature supporting this contrary view is extensive, spans almost three decades, is in general ignored and has had negligible impact on those who believe in the cholesterol-heart disease hypothesis.

Put another way, the critical question is simply, does elevated TC and LDL increase the risk of heart disease related acute coronary events to an extent which is clinically significant? The conventional wisdom is that this question is already answered, and that the science clear with vast and robust evidence, even if the mechanism is far from clear or at least far from

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proven. If the notion that cholesterol increases the risk of symptomatic heart disease is viewed as a hypothesis, then careful consideration must be given to evidence that falsifies it, since as mentioned above, hypotheses are not proven, they survive until falsified.

The several meta-analyses cited above involving primary prevention of healthy individuals and yielding large numbers needed to treat with lipid lowering to prevent one acute CHD event would appear to support the notion of only a very weak association between cholesterol and CHD even after significant LDL lowering and up to 4-5 years follow-up.

A dispassionate examination of this contrarian evidence is thus important and It turns out to be informative to examine this question with stratification by age and gender.

Women

A pooled analysis involving 15 observational studies and about 125,000 women examined the question of total cholesterol and all-cause, cardiovascular, and coronary heart disease mortality [132]. Only events that occurred ≥ 5 years after the study baseline were considered and the reference was 160-199 mg/dL. Hazard ratios adjusted for age, diastolic blood pressure, BMI, and alcohol and smoking were derived. For total cardiovascular mortality, there was no significant association at any cholesterol level from < 160 to ≥ 240 mg/dL. For coronary heart disease mortality, the association became significant at levels ≥ 240 mg/dL but this may have included individuals with familial hypercholesterolemia which confuses the issue because of the non-lipid related triggers for acute coronary events associated with this disorder.

In 2004, Walsh and Pignone reported on pooled analysis of 6 lipid lowering trials involving women with endpoints of CHD and total mortality and CHD events [133]. Follow-up was from 3 to 5 years and 5 of the 6 studies used statins. For women without cardiovascular disease, lipid lowering was not associated with total or CHD mortality. For CHD events, the data was insufficient to permit a conclusive result. A more recent meta-analysis found that for women without prior cardiovascular disease, statin therapy did not reduce CHD events nor the risk of total mortality [134]. A similar conclusion was reached in a study motivated by legal aspects of drug company claims of efficacy which was also based on meta-analysis [135]. It is unusual to see a study on this subject in a legal journal, although one of the two authors was a professor of medicine at Harvard.

The famous JUPITER trial was the first to find benefit of lipid lowering for women. This study enrolled subjects with relatively low LDL (<130 mg/dL, mean 108 mg/dL) with C-reactive protein levels > 2 mg/L [136]. While described as healthy, 42% of the cohort had the metabolic syndrome and most were overweight and some obese. The study was prematurely terminated at a median follow-up about 2 years with maximum follow-up of 5 years, even though the number of events was very small. Using the tabulated events during the study, the absolute risk reduction for any heart attack was 0.41% and for combined heart attack, stroke or death from cardiovascular causes, it was 0.83%. Since these results span a considerable range of follow-up times of up to 5 years, the small absolutes risk reductions appear more meaningful than approximate numbers needed to treat which one might calculate. Women and men had similar relative risks, and thus the absolute risk reductions for women were also very small, although the relative risk reductions were very large. What is generally not recognized about this trial is that Crestor is unique in its very strong influence on vitamin D

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levels. The increase in vitamin D levels could easily account for the small absolute cardiovascular benefits [95]. Until this potential confounding is taken into account, JUPITER should be viewed with reservations. Serious problems with JUPITER have also been discussed by de Logeril [137]. Put simply, some of the numbers do not make any sense clinically.

Thus from the above studies one may reasonably conclude that TC or LDL are only very weak or non-existent factors for acute coronary events for women. This of course accounts for the interest from the legal profession. It is important to recognize that women younger than 50 years have such low risk of CHD that studies of events are difficult due to the very large number of participants needed to obtain useful data. It is no doubt also the reason why, historically, women were under-represented in studies conducted to justify drug therapy.

The Elderly

There have been a number of studies addressing the association between cholesterol and CHD or CVD events and related mortality in the elderly.

- Framingham looked at an elderly subgroup and the data for those 60 to 74 years of age assessed statistically and discussed by Larson [138]. For women, the rate ratio for cardiovascular disease was J shaped and showed *enhanced* risk at levels below about 200 mg/dL and then no risk until there was a modest increase above 300 mg/dL, which is both very high and in the FH range. For men, the curve was U shaped, with small *enhanced* risk below about 160 mg/dL and a modest increase in risk with a threshold of about 250 mg/L.
- Nissinen *et al* [139] found no association between cholesterol and CVD or CHD in a group aged 65-75. Systolic blood pressure was the most significant factor, and smoking declined in importance with age.
- Siegel *et al* [140] found for men and women with a mean age of 72 that when the data was adjusted for covariables, cholesterol was not associated with the risk of first cardiovascular event.
- Krumholz *et al* [141] studied the association between cholesterol and CHD mortality and morbidity in persons older than 70. They state that their results failed to support the hypothesis that hypercholesterolemia was an important factor for CHD mortality, or hospitalization for MI or unstable angina.
- Simons *et al* [142] found cholesterol and other lipids predicted CHD in the elderly, but only in those below 70 years of age. However, for the 60-69 age group, a hazard ratio > 1.0 was found only for the 5^{th} quintile of TC which for women was 274 and men 294 mg/dl.
- The most frequently cited study in the context of primary prevention with a statin in an elderly population is the PROSPER trial which reported in 2002. This was a combined primary and secondary prevention trial which involved 5800 men and 3000 women aged 70-82 treated with a statin drug. For the primary prevention arm, no statistically significant treatment benefit was observed for CHD death, non-fatal

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MI or fatal and non-fatal stroke [143]. PROSPER is frequently cited supporting the opposite result, but this is incorrect in the context of primary prevention.

In a review published in 2011, Long *et al* attempt to convince readers that in fact statin intervention is important in the elderly for primary prevention [144]. If one ignores JUPITER because of issues discussed above, then the review is based on 5 primary prevention studies, AFCAPS, ASCOT, MEGA, PROSPER AND WOSCOP which included the elderly as a fraction or all of the study cohort. The researchers only cite stroke results for PROSPER. If one examines the data from these studies for the endpoint of major coronary events, the relative risk reductions range from 23% to 36%. The authors then claim that in studies where subgroup analysis was done, age did not influence the results. However, aside from PROSPER, the studies covered a range of age from 40 to 76, and thus did not provide an ideal design to examine the impact of old age. Absolute risk reductions were not mentioned. However, if one calculates the overall pooled absolute risk reduction it is only 1% yielding a NNT of 100 over the average duration of the studies. As discussed above, the PROSPER study, which was specifically targeted at the elderly, found no association with the incidence of major cardiac events from statin intervention. Thus for those who are impressed by relative risk reduction, the elderly definitely benefit from statins. However, if one considers the observational studies cited above and PROSPER which found no impact and the other intervention studies cited by Long *et al* which found 1% absolutes risk reduction from lipid lowering, then a different view of the significance of cholesterol in the context of the elderly emerges.

It therefore appears that there is no or a very weak statistically or clinically significant association between serum cholesterol levels and thus also LDL levels and CHD events in the elderly of either gender, although at very high levels there is only an inconsistent suggestion of the appearance of some risk.

Younger Men

There seems to be general agreement even among critics of the heart-cholesterol hypothesis that in this age group there is statistically significant association between the risk of CHD and serum cholesterol or LDL. The reason this age-gender group is different is unclear but one possibility is that psychological stress, its association with blood pressure and cholesterol levels, and in particular the cholesterol elevation associated with exaggerated blood pressure response to stress and aggravation may play a significant role [98]. This is the age group where men are subjected to high levels of stress associated with academic and career achievement, financial problems, domestic stress associated with the initial stages of raising children, and finally the severe stress associated with the breakup of a marriage or relationship. Stress is never considered quantitatively as a confounder in studies, but stress is recognized as a highly significant factor and trigger for acute coronary events and as well, coronary plaque progression [17]. Note that these same arguments were used and documented above in connection with the CARDIA study of LDL exposure and atherosclerosis in young individuals.

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Back to the Paradox

Thus an attractive resolution of the above described paradox is that the association between serum cholesterol and thus LDL and the acute coronary events is weak or non-existent for women of all ages and the elderly, and cholesterol may only be a marker for stress in younger men where the association does appear to exist. However, younger men have a very low risk of acute CHD events compared to older individuals, and for those who experience sudden cardiac death, there is no correlation with cholesterol at all [15]. It is noteworthy that many studies do not stratify endpoint results by both age and gender, and furthermore, meta-analyses, which have profound influence on practice and guidelines, are also not generally stratified both by age and gender.

The paradox as outlined does not include a detailed discussion of the relative importance of non-calcified plaque, mixed calcified and non-calcified plaque and calcified plaque. Only recently has research started to differentiate these subtypes.

For the hypothesis that cholesterol causes acute heart disease related events, there appear to be quite a few black swans and it can be argued that there is no paradox at all. The greater absolute risk reduction in secondary statin therapy, which has had a strong but unjustified impact on primary prevention strategies, may be due to pleiotropic effects, and this view is reinforced by the very rapid impact of statin therapy seen in some studies subsequent to a major coronary event with statin naïve patients.

ZERO CORONARY CALCIUM

When large groups are screened, there will always be some where the radiologist cannot detect any coronary calcification and describes the arteries as “normal” or assigns a CACS of zero. Hecht has briefly reviewed prognostic studies where the CACS was zero [145]. From a pooling of three prospective studies with a median follow-up of about 4 years, the event rate per year of any adverse coronary event was 0.17% with a range of 0.11 to 0.9%. Hecht summarizes meta-analyses of studies involving over 49,000 individuals with a zero calcium score at baseline. A yearly event rate of 0.1% over 4.2 to 5.6 years follow-up was found.

In a review, Gottlieb *et al* [146] discuss zero calcium scores in the context of diabetic and other high-risk patient populations. They cite one study showing that diabetic patients with zero coronary calcium had the same risk for events as non-diabetic patients after a mean follow-up of 5 years even though only 50% of the non-diabetic and 25% of the diabetic patients still had a zero calcium score. They cite two other studies consistent with these results.

In a recent study, Min *et al* [147] looked at the predictors of CAC in individuals with CAC = 0 progressing to a finite calcium score. A total of 25% of patients with CAC = 0 developed CAC during the 4-year follow-up. The mean age was 49 and the cohort was about equally divided between men and women. Progression from CAC = 0 was not linear but occurred at an increasing rate each year. Consistent with the studies reviewed above, cholesterol levels were not a significant factor in univariate analysis and thus omitted in multivariate analysis where only the presence of diabetes, smoking and age > 40 were significantly associated with the development of calcified plaque.

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A zero calcium score, while an excellent indication of very low risk of acute cardiac events, is nevertheless no guarantee of the absence of non-calcified plaques or a major or minor stenosis [148]. In fact, coronary calcification is only marginally related to the extent of coronary stenosis and both obstructive and non-obstructive coronary artery disease can occur in the absence of calcification. Coronary stenosis is frequently not accompanied by calcification, highly calcified plaques are frequently non-obstructive and the calcium score is not suitable for ruling out obstructive CAD nor is relying on unenhanced CT justified when making the decision regarding discharge home for individuals presenting in the emergency department with suspected acute heart disease [146].

NON-CALCIFIED AND MIXED PLAQUE

For 20 years, electron beam computerized tomography (EBCT) without contrast media has been used to identify calcified coronary plaque. This has been of great value even though the scans do not pick up non-calcified plaque. The amount of coronary calcium correlates linearly with total coronary plaque burden and the higher the atherosclerotic burden, the higher the probability of acute events. The ability of the calcium score to predict CVD events in asymptomatic individuals has been confirmed in many clinical trials and studies indicate superiority over traditional risk stratification tools including carotid intima–media thickness (IMT) [146]. In fact, in one cohort study even patients who were in the greater than 75th percentile of carotid IMT but zero coronary calcium score had less than 1% per year risk for CVD events [149].

Recently improved CT technology termed computed coronary tomography angiography (CCTA), also called multislice computed tomographic angiography (MSCAT) or multidetector computed tomography (MDCT), has been developed and used in studies. This CT technology is used both with and without contrast enhancement. This makes it possible with a non-invasive technique to identify calcified, mixed and non-calcified plaque as well as stenosis. The study of plaque subtypes as compared to total CAC has only recently become an area of interest, and although the accumulated data is sparse compared to that associated with calcified coronary plaques quantified by conventional electron beam computed tomography, the situation is changing rapidly. CCTA or other improved coronary CT techniques provide a much more complete assessment of coronary atherosclerosis which then will make possible a re-examination of questions such as prevalence, progression, risk factors and factors impacting prognosis for each subtype.

A recent study has demonstrated that contrast-enhanced CCTA can also be used to measure CAC and found an excellent correlation with unenhanced CAC measurements. This suggests that two scans are unnecessary order to obtain a risk assessment which includes both non-calcified plaque and stenosis as well as a calcium score [150].

This is now an active area of research. Furthermore, this more sophisticated non-invasive technique has opened new avenues of research into the characteristics of vulnerable plaques. CCTA with contrast has been used mostly to study small groups of subjects with heart disease or symptoms strongly suggestive of this disorder. Studies have involved a variety of goals and information is accumulating, but it appears too early to attempt any general conclusions concerning risk factors or the impact of interventions for each plaque subtype or the

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distribution or seriousness of stenosis. Calcified plaque and the calcium score remain the only widely used and extensively studied surrogates for the presence, progression, response to medication and event risk evaluation of silent coronary atherosclerosis.

PREVENTING, SLOWING OR REVERSING CORONARY PLAQUE PROGRESSION

The traditional approach to studying progression and regression of atherosclerosis focused on the carotid arteries since ultrasound measurements were simple and non-invasive. This is still popular today although the clinical significance of small changes in carotid artery intima thickness or atheroma volume has never been demonstrated. The same is true for invasive studies using intravascular ultrasonography where a recent study found statin treatment reduced progression by measuring the percent change in atheroma volume [151]. One can question the clinical significance of a 1% decrease. The clinical significance of decreases in the CACS is also unknown, and in addition, the number of studies addressing reversing of halting CAC progression is limited.

A recent study involved dietary advice and combined intensive lipid management (statin plus niacin) along with supplementation with omega-3 fatty acids and increased vitamin D status. The targets were triglycerides ≤ 60 mg/dL, HDL ≥60 mg/dL, and 25-hydroxy vitamin D levels ≥50 ng/mL. Out of 45 male and female subjects with calcium scores ≥50 Agatston units, after about 18 months 20 subjects experienced a 15% drop in calcium score (maximum 64%), and 22 had their progression arrested or slowed to 12%, whereas a 22% to 52% increase in score per year was expected [152]. In another study involving hypertensive patients, a calcium channel blocker was found to significantly slow the progression of coronary calcification when the control was a diuretic [153]. Also, lowering the triglyceride/HDL ratio with the drug pioglitazone was associated with a beneficial impact on the progression of coronary atherosclerosis in diabetic patients [154]. This study suggests the importance of examining the impact of addressing the dyslipidemia of the metabolic syndrome with aggressive dietary intervention with the goal of reversing coronary atherosclerosis. In a mouse study, high HDL was found to promote rapid atherosclerosis regression and to alter the inflammatory properties of monocyte-derived plaque [155]. Potential modifiable risks factors associated with the severity or progression of coronary calcification also include physiological stress [156], depression [114] and sleep apnea [157]. Unfortunately, little effort has gone into aggressively addressing the problem of interventions that significantly slow or reverse directly measured coronary plaque progression and as well the impact on events, and yet this in fact is the real challenge facing those interested in CHD primary prevention.

CONCLUSION

Coronary atherosclerosis, which typically begins at a rather young age and progresses throughout life, appears independent of total cholesterol or LDL levels, which calls into doubt classical etiological models and should encourage the development and testing of new

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hypotheses [97]. Lowering LDL with statins has only a very small absolute effect on the risk of acute events in asymptomatic individuals, has proved slightly more useful in secondary prevention, but there remain numerous serious questions as to the importance of pleiotropic effects vs. lipid lowering, and the pleiotropic actions may be better achieved by other drugs or interventions. However, the risk of sudden cardiac death is independent of cholesterol levels, over 50% of all cardiac deaths are sudden and as discussed above and statins also do not appear to influence mortality [15]. Primary prevention lipid lowering drug trials remain controversial with criticism that has continued over several decades concerning issues such as age, gender, subject selection, cohort contamination by individuals with CHD, very high-risk subjects, very low absolute benefits, high numbers needed to treat to prevent one adverse event, no impact on overall mortality and indications of the prevalence of conflicts of interest and bias [96].

Diabetics represent a special case since there is little doubt this disease strongly enhances the risk of acute coronary events and in general, cardiovascular and peripheral vascular problems. Thus the obvious question. Is targeting LDL in diabetics indicated when it rarely appears as significant when one examines correlations with the prevalence or progression of silent coronary atherosclerosis or coronary event free survival, or when one takes into account the very small absolute benefits, similar to that found in non-diabetics, seen in true primary prevention statin intervention trials when the endpoint is fatal or non-fatal MI. It is interesting that in commentaries and reviews, ASCOT and ASPEN are frequently ignored in favour of HPS and CARDS, evidence of cherry picking for higher but still small absolute risk reductions. In a review concerning treatment of type 2 diabetics, the question addressed for lipids was “How low should we go?” Only CARDS and HPS were cited as justification for statin therapy, and only relative risk reductions were discussed. The authors did, however, comment on the limited data concerning taking cholesterol levels to very low values and suggest that this is an area for future trials [158]. But using relative risks, viewing HPS as a primary prevention trial without discussing the expanded endpoint, and omitting ASCOT and ASPEN inflated the suggested benefit.

In one recent comprehensive review on cardiovascular outcomes in type 2 diabetes which emphasized preventive therapies [159], the principal citation in the lipid section was the Cholesterol Treatment Trialists’ Collaboration Study [160] which was viewed as robust evidence of the merits of statin intervention, pointing to the 18,000 subjects with diabetes out of a total of over 90,00 total participants. But in this meta-analysis of 14 studies, only one study involved true primary prevention (CARDS) and for a another trial (HPS), as discussed above there were problems separating primary from secondary prevention which were endpoint dependent. The analysis failed to include ASCOT and ASPEN. In fact, the use of large meta-analyses which involve mostly secondary prevention studies as justification for statin use in primary prevention is commonly encountered in review articles. Furthermore, while LDL targets are an integral part of guidelines and standard practice, there are no studies where the main objective was establishing the optimum LDL targets to go with the recommendations in the guidelines, especially for diabetics [158]. A recent review even suggested that type 2 diabetes should in fact not be considered a true risk equivalent for CHD [161].

LDL is universally regarded as the “bad” cholesterol. The issues discussed above suggest a different view. Why is it consistently absent as a factor in studies of the risk of prevalence and progression of silent atherosclerosis in both diabetics and non-diabetics? Furthermore,

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why does low LDL characterize half of hospital admissions for MI? In addition, non-statin drugs that lower LDL do not reduce event risk? Why are the numbers needed to treat so high in true primary prevention? If the NNT found in intervention studies is 50 or 100, or even 25, there are 49, 99 or 24 who were treated, typically over 4 years, but did not benefit from lowering of circulating LDL. Then there are the statin adverse effects. It is widely recognized, post-introduction side effect monitoring is notoriously inefficient. In fact, the actual prevalence of side effects of statins is unknown and an area of active investigation.

The focus on relative rather than absolute risk reduction has resulted in widespread inflated perceptions of benefit, confused the risk/benefit analysis and produced a false sense of security among the millions of statin users. The extensive use of relative risk in this field has been the subject of criticism for several decades. A 20-30% relative risk reduction is impressive and strongly influences therapeutic decisions, but it can be associated with a 1%-2% or even smaller absolute event risk reduction which is not impressive [162, 163].

There seems to be no consensus regarding the magnitude of an absolute risk reduction that is clinically significant. This is understandable since many factors come into play. It can be argued that a low-cost, low-risk preventive measure for a potentially disabling or fatal disease can have a high threshold for NNT. However, if a treatment carries high risk and high cost, this can in the view of some clinicians reduce the limit of the NNT to quite a low number above which treatment is not justified [162]. It can be argued that patients need to be advised on the basis of absolute risk reductions, but not only do the above factors enter in, but the particular situation presented by the individual patient needs to be considered to ascertain if study-derived NNTs are applicable or if the probability of benefit may be much higher or lower [163]. Furthermore, underreporting of adverse effects makes a meaningful risk/benefit analysis impossible.

The notion is gaining support that widespread screening for the presence and extent of coronary calcium is indicated, especially for those of intermediate risk by the Framingham Risk Score. The results are intended to guide the degree of treatment aggressiveness. But there is almost no data regarding how to significantly arrest or reverse the progression of atherosclerosis nor the associated clinical benefit. The few glimmers of hope, amplified into suggestions of significant success, have been based on very small changes in targeted carotid or coronary artery atheromas or in carotid intima media thickness. The significance of these very small changes appear to be of questionable clinical significance and there is a poor correlation between atherosclerosis in these two vascular beds. Neither statins, most antihypertensive drugs or sevelamer in the case of those with chronic kidney disease have shown any impact on progression of CAC [164].

There do not appear to be studies in the context of arresting or reversing coronary atherosclerosis measured by coronary calcium which provide guidance regarding interventions such as cessation of smoking, exercise, a Mediterranean diet, moderate alcohol consumption, optimal vitamin D status, consumption of ample long-chain omega-3 fatty acid and reducing psychological stress, actions suggested by observational studies which are associated with reduced risk of acute events, especially when combined into an integrated program. One study discussed above did find substantial regression of CAC associated with triglyceride targets combined with omega-3 fatty acid and vitamin D supplementation, but much more research is obviously needed.

The difficulties individuals experience in loosing weight, adopting dietary strategies that might assist in weight loss and improve nutrition, avoiding the development or reversing the

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metabolic syndrome, diabetes and the associated characteristic dyslipidemia, and stopping smoking appear to be a serious impediment to progress in prevention. Even a strengthened evidence base for the merits of such actions based on intervention studies with both event and CAC endpoints may not help motivate patients. Professional advice and encouragement appear to have limited impact.

The literature cited and discussed above suggests that a new paradigm is urgently needed, especially in the context of primary prevention of CHD [17, 86]. This issues has been recently discussed and documented by Ravnskov and others [4, 18, 97, 165, 166], and a new hypothesis has been advanced by Ravnskov and McCully which focuses on action in the arterial media initiated mostly by agents entering via or blocking the vasa vasorum with only monocytes crossing the endothelial barrier to enter the media. The hypothesis addresses many of the ongoing issues [97]. Any new paradigm will also have to accommodate the information now accumulating regarding plaque subtypes and vulnerable vs. non-vulnerable plaques. It is noteworthy since the paper by Ravnskov and McCully was published in 2009, there is virtually no activity in the area of new hypotheses. It is suggested that this lack of progress is due to the still unquestioned primacy of the cholesterol-heart hypothesis, the associated cholesterol-atherosclerosis hypothesis, and contentment with the current model of atherosclerosis. Cholesterol appears to only be a distraction and, given the large body of evidence suggesting that it is irrelevant in the context of atherosclerosis and a weak or non-existent factor when acute coronary event risks are considered. It is unlikely that additional evidence will be brought forward that neutralizes this large body of significant evidence that calls into question the cholesterol-heart hypothesis.

Science becomes dysfunctional when dogma and vested interests trump critical analysis and constant questioning of the conventional wisdom. Yet critics believe that there is a culture today in medical science, which aggressively and successfully resists new ideas and challenges to widespread beliefs and the conventional wisdom, and it can be argued that this is obviously not in the best interests of patients and inhibits meaningful scientific progress. It is time for a change.

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Chapter II

ATHEROSCLEROSIS: RISK FACTORS, PREVENTION AND TREATMENT

Katsuyuki Nakajima[1,2] and Masaki Q. Fujita[2]
[1]Graduate School of Health Sciences, Gunma University,
Showa-machi, Maebashi, Gunma, Japan
[2]Department of Legal Medicine (Forensic Medicine),
Keio University School of Medicine, Shinano-machi,
Shinjuku-ku, Tokyo, Japan

1. INTRODUCTION

Over the past 150 years, there have been numerous efforts to explain the complex events leading atherosclerosis. In this endeavor, several hypotheses and the risk factors have emerged that currently are under active investigations. However, these hypotheses are not mutually exclusive, but rather emphasize different concepts as the necessary and sufficient events to support the development of atherosclerotic lesions. In this review, the combination concept of ‘‘response-to-injury’’ [1] and ‘‘oxidative modifications’’ [2] for the initiation of atherosclerosis, rather than the progression of atherosclerosis associated with risk factors, is mainly discussed for the prevention and treatment.

HMG-CoA reductase inhibitors (statins) are well known to decrease cellular cholesterol synthesis and consequently reduce the hepatic production of VLDL and increase expression of LDL receptor [3]. Clinical trials have shown that improvements in plasma LDL-C levels are associated with retardation of atherosclerosis and reduction in coronary artery morbidity and mortality [4, 5]. The major mechanism of this therapeutic effect has been recognized as the increase of LDL receptor expression in liver to remove elevated LDL-C in plasma. However besides LDL-C, remnant lipoproteins (RLP) have been increasingly implicated in progression of atherosclerosis, with elevated fasting RLP-Cholesterol (RLP-C) levels shown to predict clinical events independently in coronary artery disease patients [6]. Oxidized lipoproteins, notably oxidized low-density lipoproteins (Ox-LDL) and RLP-C in plasma emerged as the new risk factors after lowering LDL-C. Both risk factors are paid attention by the clinical laboratories as diagnostic tools for the cardiovascular disease with the progress of

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new technology. Accordingly, attempts are made to provide an insight into the atherogenecity of remnant lipoproteins and Ox-LDL including their contribution to endothelial cell dysfunction, for example through the lectin-like oxidized LDL receptor-1 (LOX-1 receptor) [7] which has been discovered as an Ox-LDL receptor. It was recognized that activation of LOX-1 receptor by remnant lipoproteins plays a key role for the initiation of endothelial cell dysfunction [8] and may present a major factor in atherogenesis which is independent from plasma LDL concentration. The new concept on prevention and therapeutic target and its associated risk factors in cardiovascular disease are described in this review recognized from our previous sudden cardiac death research performed during last two decades.

2. TWO MAJOR RISK FACTORS OF ATHEROSCLEROSIS

2.1. The Oxidized LDL Hypothesis Associated with High Plasma LDL-C

In 1989, Steinberg et al. [2] put forward the original oxidative modification hypothesis based on the notion that oxidation represents a biologic modification analogous to chemical modification discovered by Goldstein et al. [9] that gives rise to foam cells. Since then, numerous studies have supported the Ox-LDL hypothesis which says Ox-LDL can promote foam cell formation through the so-called ''scavenger receptor'' pathways [9, 10]. Scavenger receptor, SRA, in macrophage was first characterized in 1988 by Kodama et al. in Krieger's laboratory [11], but it should be noted that macrophages express more than one scavenger receptor.

Several new receptors for Ox-LDL in macrophage such as CD36 [12], LOX-1[7] and SR-PSOX [13], etc., have been discovered after Steinberg proposed Ox-LDL hypothesis. Sawamura et al. [7] noticed the absence of scavenger receptors for Ox-LDL in endothelial cells which may cause the endothelial dysfunction to initiate atherosclerosis in elevated LDL-C cases. They found a new receptor for Ox-LDL in endothelial cells and named LOX-1 receptor.

The Steinberg's hypothesis was proposed under the situation that Ox-LDL receptor (SRA) was the only one receptor found in macrophage associated with the formation of atherosclerotic lesions, but no other receptors ware yet found in endothelial cells. The Ox-LDL hypothesis was proposed mainly for the formation of foam cells from macrophages via scavenger receptor as the major cause of atherosclerosis, but not for the dysfunction of endothelial cells which most probably associated with the initiation of the formation of atherosclerotic lesions. However, the present concept is that atherosclerosis represents a state of heightened oxidative stress characterized by lipid and protein oxidation in the vascular wall generated by LDL penetrated through between the endothelial cells from plasma. The current oxidative modification or stress hypothesis of atherosclerosis predicts that LDL oxidation is an early, essential event in atherosclerosis and that Ox-LDL does contribute to both initiation and progression of atherosclerosis. But besides Ox-LDL, the possibility still exists that other lipoproteins which are oxidized in plasma with normal LDL concentration may cause to form the atherosclerotic lesions.

As shown in Figure 1, we have proposed the oxidative modification hypothesis of remnant lipoproteins on athegenesis.

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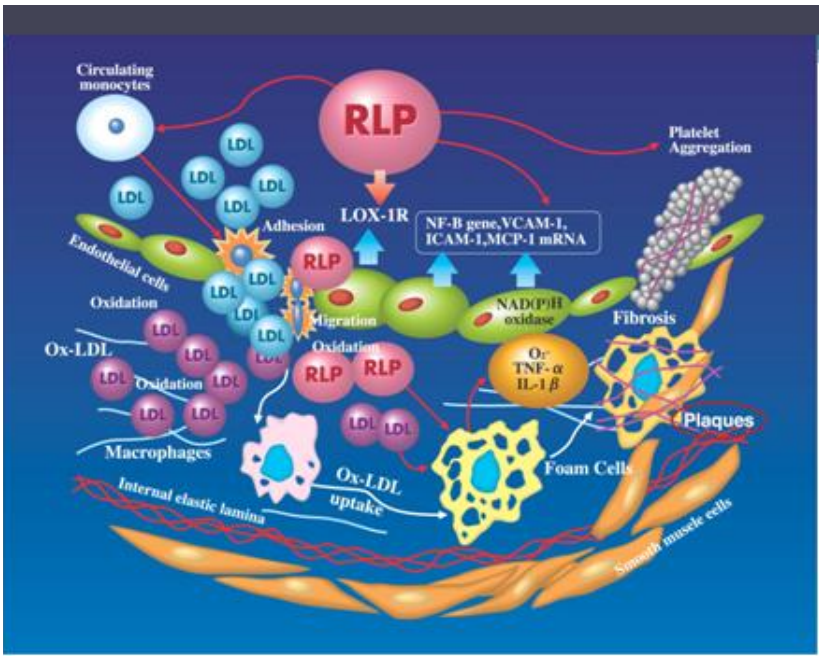

Figure 1. Effect of RLP and Ox-LDL on the formation of atherosclerotic plaque. The endothelial cell dysfunction is initiated by RLP in plasma (not oxidized LDL) followed by the induction of LOX-1 receptor. The associated pathway of various cytokines and enzymes are activated and promote for the endothelial cell damage. After increased permeability of endothelial cell by damage, a large amount of LDL move into the subendothelial space and form Ox-LDL which are incorporated into macrophages and foam cells and promote the progression of atherosclerotic plaques in blood vessel wall.

The endothelial cell dysfunction is initiated by RLP in plasma followed by the induction of LOX-1 receptor and the associated pathway of various cytokines and enzymes. Ox-LDL promotes the progression of atherosclerosis in subendothelial space after a large efflux of LDL from plasma and form foam cells and atherosclerotic plaques. As small dense LDL-C is a part of LDL, or a kind of Ox-LDL and the similar target of cardiovascular disease by statins, we have not described in this review.

2.2. Characteristics of Ox-LDL and Remnant Lipoproteins on Atherogenesis

The oxidative modification hypothesis focuses on the concept that LDL in its native form is not atherogenic [14]. However, LDL modified chemically is readily internalized by macrophages through the so-called scavenger receptor pathway [9]. Exposure to vascular cells in medium that contains transition metals also results in modification of LDL such that it serves as a ligand for the scavenger receptor pathway [10]. Therefore, it is now clear that only one mechanism whereby cells in vitro render LDL a substrate for the scavenger receptor pathway is via oxidation of LDL which results in modification of apo B-100 as well [15]. These observations form the basis for the oxidative modification hypothesis of atherosclerosis in which LDL traverses the subendothelial space of lesion-prone arterial sites. During this process, LDL lipids are subject to oxidation and modifications of the lysine residues on apo B-100 leads to an increase of the net negative charge on the lipoprotein particles [16]. This

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modification of apo B-100 renders LDL susceptible to macrophage uptake via a number of scavenger receptor pathways producing cholesterol ester-laden foam cells [17]. The presence of Ox-LDL in atherosclerotic lesions has been studied using antibodies that recognize specific epitopes on Ox-LDL that are not present in its native, non-oxidized form. The oxidation of polyunsaturated fatty acids can lead to the formation of aldehydes that modify lysine residues in apo B-100 [18]. Adducts of lysine residues with malondialdehyde and 4-hydroxynonenal have been characterized extensively and antibodies raised against these species. These antibodies avidly stain atherosclerotic lesions in LDL receptor-deficient rabbits [19, 20], apo E deficient mice [21], and humans [22, 23] with no demonstrable staining in normal arteries. As the oxidative modification hypothesis would predict, these epitopes largely co-localize in macrophages, although one might argue that they are not specific for LDL and could represent modification of other proteins and phospholipids in the atherosclerotic lesion as well. Consistent with this assertion is a study showing that LDL isolated from atherosclerotic lesions possesses properties that resemble those of Ox-LDL formed in vitro [23], indicating that lesion LDL is oxidatively modified and accumulated in vascular walls. Aggregated LDL is much more rapidly taken up by macrophages than native LDL but again the uptake appears to occur via the LDL receptor [24].

Aggregation of LDL in the subendothelial space has been demonstrated [25] and this may be encouraged by the proteoglycans in the artery wall to which LDL binds avidly [26]. On the other hand, it is generally agreed that LDL accesses the artery wall, but VLDL, another apo B-100 carrying lipoproteins also can access the artery wall and directly contribute to atherogenesis. As reviewed below, there is overwhelming evidence for VLDL and especially for VLDL remnants being major atherogenic lipoproteins [27, 28].

Particles resembling VLDL remnants, RLP can be taken up by macrophages to produce foam cells without oxidative modifications [29, 30], stimulate endothelial cells to express a monocyte-specific chemotactic factor [8], increase monocyte adherence to the endothelium [31] and so forth. Further, varying amounts of labeled VLDL apo B-100 have been found in aorta from human and experimental animals after intravenous administration [32–35]. Other possibilities are reported by several investigators that a major part of apo B-100 in atherosclerotic plaques was not originated from LDL. Rapp et al. [36] isolated and characterized immunoreactive apoB-containing lipoprotein particles from human atherosclerotic plaques.

These apoB-100 species were present significant amount in VLDL+IDL fraction, as much as in the LDL fraction. From these observations, VLDL, VLDL remnants or both showed the possibility of entering human atheosclerotic plaques as the origin of apo B-100 in spite of being larger in size than LDL particles.

2.3. Circulating Ox-LDL in Plasma as a Risk for Coronary Artery Disease

LDL circulates in plasma, including a fraction which reenters the circulation from the subendothelial space [37, 38]. The plasma antioxidants provide effective protection against oxidation of LDL [39]. This means that major site of LDL oxidation is the subendothelial space. The transit of LDL across this space may yield a small amount of circulating LDL that is oxidized. Chemical analysis of circulating LDL has been reported to yield a minor fraction, termed LDL that has an increased amount of oxidized lipid [40].

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Consistent with these findings, human plasma shows immunoreactivity towards epitopes generated on Ox-LDL [41, 42]. However, the existence of oxidized LDL in the circulation remains controversial on the basis of artifacts that may occur during ex vivo handling of plasma and isolation of LDL. Although the aforementioned data do not address a causal relation between Ox-LDL and atherosclerosis, several studies have shown that epitopes on circulating Ox-LDL can be used to distinguish between patients with and without clinical evidence of atherosclerosis [43, 44].

Using immunologic methods that detect oxidized phosphatidylcholine and their protein adducts [45,46], but not native, acetylated or malondialdehyde-treated LDL, Toshima et al. [47] and Ehara et al. [48,49] reported that acute coronary syndromes are characterized by increased circulating levels of Ox-LDL. Most recently, Tsimikas et al. [50] reported that circulating levels of Ox-LDL are strongly associated with angiographically documented coronary artery disease, and indicated the high association with serum Lp(a) which binds oxidized phospholipids in LDL.

Together, these data indicate that are relatively small amount of LDL containing different types of oxidation specific epitopes can be detected in blood and may reflect atherosclerosis and its different manifestations. What is less clear at present is where these epitopes originate from and which, if any, of the different oxidation-specific epitopes directly relate to and/or are important for disease burden [51].

The concentration of Ox-LDL detected in plasma (less than 0.5% in total LDL) is too low for the induction of Ox- LDL proatherogenic and proinflammatory properties shown from many in vitro studies.

On the other hand, autoantibodies against malondialdehyde- modified lysine residues (anti-oxidized LDL antibodies) have been demonstrated in the serum of both rabbits and humans [22, 52]. Some studies have reported that the titer of these autoantibodies is associated with the burden of and may predict progression of atherosclerosis [53, 54] and myocardial infarction [55, 56]. Higher titers of autoantibodies have also been associated with coronary artery disease [57], peripheral atherosclerosis [58] and higher risk for restenosis following balloon angioplasty [59]. In addition, there is support for a role for anti-oxidized autoantibodies in animal atherosclerosis [60].

But it is worth noting that these results were not observed consistently, because of the co-existence of similar antigens with Ox-LDL in plasma. The presence of autoantibodies in plasma is well known to reflect the results of cellular damage, indicating the possibility that oxidized LDL may play the role for the progression of atherosclerosis in vascular wall macrophages and smooth muscle cells, rather than the role for the initiation of atherosclerosis in endothelial cells. Small, dense LDL has been reported to be more susceptible to be oxidation than large, buoyant LDL [61], but the size of LDL particles, not the susceptibility to oxidation in vitro, seems to be more associated with cardiovascular disease [62].

However, currently an adequate technique to isolate native small, dense LDL from large, buoyant LDL is not available yet. Therefore, it is still difficult to compare the differences in oxidation susceptibility directly between these two LDL sub-types. Small, dense LDL levels in plasma were reported to be highly correlated with the levels of RLP-C [63, 64] which has been speculated as precursor lipoproteins of small, dense LDL. Further, Ando et al. [65] reported that plasma Ox-LDL levels were strongly correlated with RLP-C levels in hemodialysis patients.

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2.4. Triglyceride-Rich Lipoprotein Remnants in Plasma as an Risk Factor for the Cardiovascular Diseases, Independent from TG in Plasma

Patients at increased risk of coronary artery disease (CAD) frequently have an atherogenic lipoprotein profile characterized by elevated plasma triglyceride-rich lipoproteins (TRL) levels, a predominance of small, dense LDL and reduced high density lipoprotein (HDL) cholesterol which are highly associated with the characteristics of metabolic syndrome. This profile is often seen in patients with type 2 diabetes mellitus with normal LDL concentration and it is associated with an approximately three-fold increase in risk of atherosclerotic disease [66, 67]. An elevated remnant lipoprotein concentration determined as remnant-like lipoprotein particle-cholesterol (RLP-C) is also a characteristic feature of patients with this atherogenic lipoprotein profile [68] and there is considerable evidence linking increased plasma RLP-C levels with CAD. In this connection, Nakajima et al. [69] reported that plasma RLP-C levels were abnormally high in Japanese patients with coronary heart disease. Similarly, Ikewaki et al. [70] showed that plasma RLP-C significantly increased in postprandial state in patients with coronary artery disease. Further, Leary et al. [71] subsequently found that CAD patients (n =151) from nine centers in the United States and one in Canada had significantly higher median RLP-C levels compared with 302 gender and age-matched control subjects. Devaraj et al. [72] also showed that RLP-C levels were markedly higher in CAD patients compared with healthy control subjects ($P < 0.01$). These results have been supported by other case-control studies reporting that RLP-C levels were significantly high in patients with CAD, patients with resterosis after percutaneous transluminal coronary angioplasty [73,74], in vasospastic angina [75,76], coronary artery stenosis [77], coronary artery endothelial dysfunction [78,79], sudden cardiac death [80,81], intermittent claudication [82], increased intima-media thickness of the carotid artery [83] and in CAD patients with normal cholesterol or triglyceride (TG) levels [84,85].

In a large study, NcNamara et al. [86] measured RLP-C and RLP-TG in fasting plasma samples from 1567 women in the Framingham Heart Study. Multiple logistic regression analysis adjusting for other major risk factors (like age, hypertension, smoking, diabetes, LDL-C, HDL-C beta-blocker use and replacement hormones) revealed that RLP-C was an independent risk factor for cardiovascular disease (CVD) in these women and independent from TG (Table 1). RLP-C has similarly been shown to be an independent risk factor for CAD in Korean patients with type 2 diabetes [87] and in Japanese patients more than 65 years of age [88].

More recently, prospective data have been presented supporting the prognostic value of RLP-C measurement. Three studies have been reported by Kugiyama et al. at Kumamoto University Hospital in Japan, in which CAD patients (men and women [89], postmenoposal women [90], type 2 diabetes patients [91] who had angiographically documented arterial stenosis were investigated. Their lipid and RLP-C levels were measured at baseline and then were followed for 2 to 3 years until the occurrence of a clinical event (recurrent or refractory angina pectoris requiring coronary revascularization, nonfatal myocardial infarction, or cardiac death). In all these three studies, higher RLP-C levels were associated with greater probability of a coronary event and were found to be independent risk factors (other than hitherto known risk factors like age, gender, smoking, hypertension, triglycerides, cholesterol, HDL-C) and were shown to be statistically significant predictor of future coronary events.

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Recently high plasma levels of RLP-C have been reported in the metabolic syndrome as a risk for endothelial dysfunction and coronary artery disease [92–95].

3. PREVENTION

3.1. What Is the Most Effective Risk Factor Targeted for the Prevention of Cardiovascular Disease?

Plasma total cholesterol (TC) and triglyceride (TG) levels have been measured as diagnostic markers in order to prevent cardiovascular diseases, because these markers have shown the usefulness as the risk prediction for many years. Therefore, the risk factor for the therapeutic target has been developed to reduce TC and TG historically. However, several new diagnostic markers have emerged recently and provided the additional information for the prevention of cardiovascular diseases.

AS shown in Figure 2, there are many subclasses of lipoproteins among chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL).

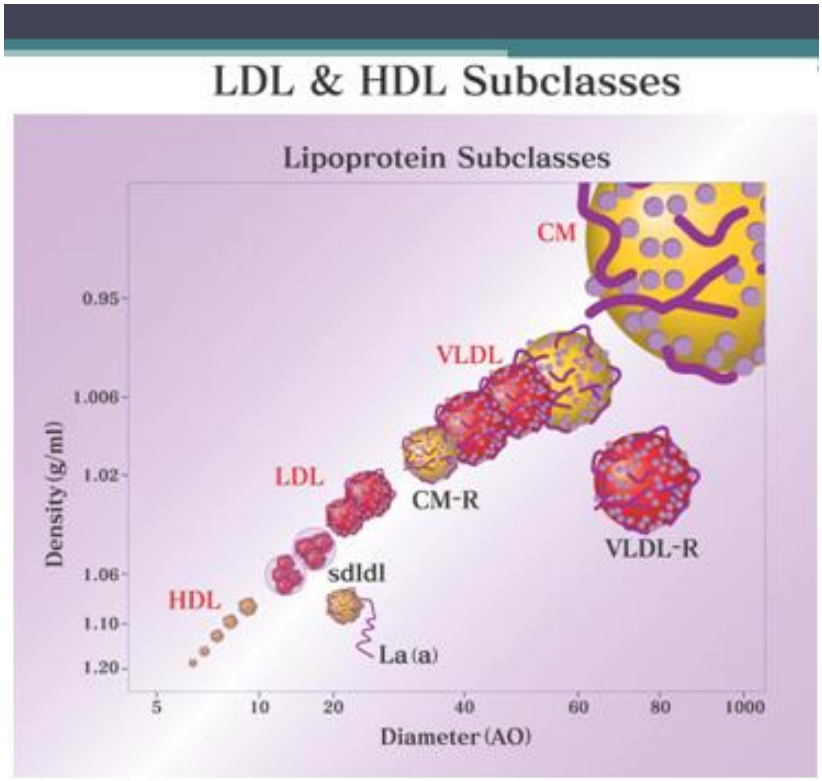

Figure 2. Subclasses of plasma lipoproteins. There are many subclasses of lipoproteins in CM, VLDL, LDL, and HDL, which have different biological and chemical characteristics. Remnant lipoproteins are found in VLDL fraction and Ox-LDL and small dense LDL are found in LDL fraction.

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The analytical methods for each subclasses are now under development. Among them, LDL-C including Ox-LDL and small dense LDL, and remnant lipoproteins are the major new diagnostic markers for the prevention of cardiovascular disease. Lowering these risk factors in plasma has been targeted by the various therapeutic drugs and nutritional treatment.

The most effective target for the prevention of cardiovascular disease may be remnant lipoproteins based on the following evidence we have investigated during the last two decades, which is the most common risk factors for cardiovascular diseases, except familial lipid and lipoprotein disorders. LDL or small dense LDL are the final products metabolized from TG-rich lipoproteins which include atherogenic remnants. Most of the atherogenic properties of lipoproteins belong to remnant lipoproteins rather than LDL in plasma.

3.2. Different Role of Plasma LDL and Remnant Lipoproteins at Coronary Atherosclerosis and Cardiovascular Events; from the Studies of Autopsies in Sudden Cardiac Death Cases

We have investigated the risk factors of sudden cardiac death (SCD), especially Pokkuri Death Syndrome (PDS) (sudden cardiac death without coronary artery atherosclerosis observed mostly in Southeastern Asian young males), during the last two decades [96] and found the different roles between LDL and remnant lipoproteins as cardiovascular risk factors in plasma. Based on our autopsy studies, more than two thirds of SCD cases were found to be associated with postprandial remnant hyperlipoproteinemia [97-101]. The occurrence of sudden cardiac death has been observed prevalently at midnight (Figure 3), which is highly correlated with the highest TG and remnant lipoprotein levels in plasma during the day (Figure 4). LDL-C levels do not change during the day as remnant lipoproteins.

If severe spasm of the coronary artery is to be a crucial event prior to cardiac death in PDS cases, we may say that the vasospasm is not very likely to occur in coronary arteries with severe coronary artery atherosclerotic lesions due to reduced elasticity and increased stiffness or hardness of the vascular wall. Caucasians experience more severe coronary atherosclerosis than Japanese or other Southeastern Asians.

Accordingly, this might be one explanation why PDS is uncommon among Caucasians.

In view of this background, PDS could be an interesting disease case to study coronary heart disease (CHD), which is independent of severity of coronary atherosclerosis and plaque ruptures in spite of remnant hyperlipoproteinemia. Significantly younger age of PDS cases compared to the other SCD cases may be one of the reasons why PDS cases were not associated with severe coronary atherosclerosis. The prevalence of severe coronary atherosclerosis is known to be strongly associated with age.

We found that plasma lipid (TC, TG) and lipoprotein (LDL-C, RLP-C, and RLP-TG) levels were significantly elevated in these sudden cardiac death cases as compared with those in control death cases when the severity of coronary atherosclerosis was pathologically graded above (1+), reflecting the clinical feature of severe coronary atherosclerosis (97-99). Most of the coronary arteries in PDS cases were pathologically graded as (−) and (±), indicating no coronary atherosclerosis [97].

Plasma LDL-C in SCD cases was shown to be highly correlated with the severity of coronary artery atherosclerosis [99]. This is in line with the perception (albeit by implication) that LDL-C plays a major role in the progression of coronary atherosclerosis in CHD patients.

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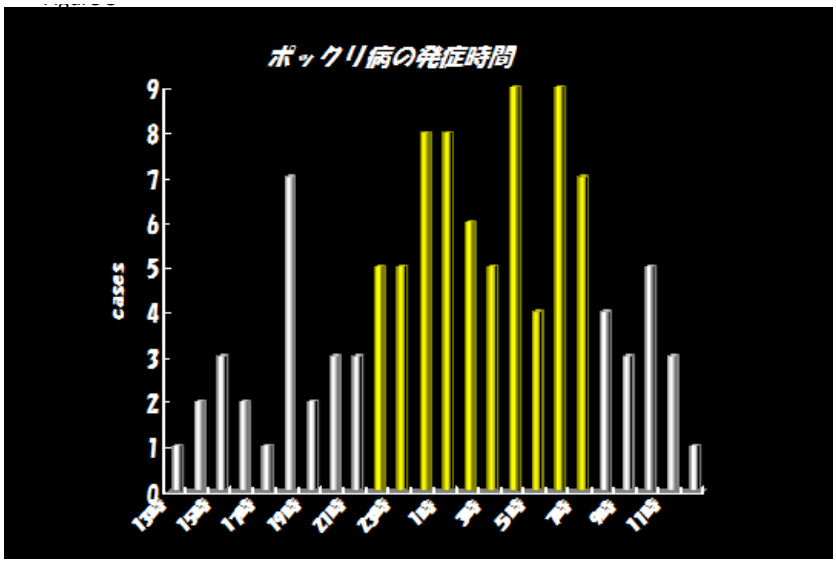

Figure 3. The prevalence of sudden cardiac death occurred during the day time. Sudden cardiac death occurred most frequently at around 2 PM at midnight. (Takeichi et al, *Int. J. Legal Med.* 1997;110: 213-219.).

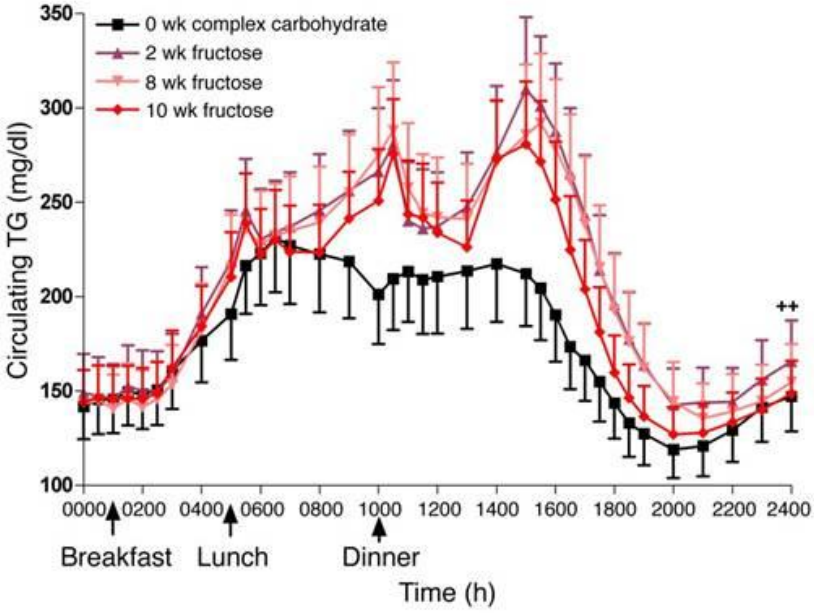

Figure 4. The plasma TG levels were found to be significantly increased during the day associated with food intake. Only in the early morning did the TG levels in all cases returned to the basal levels, and were highest in the middle of the night at regular food intake with additional glucose and fructose beverage (Stanhope et al, J Clin Invest. 2009; 119: 1322-34.).

We found that the incidence of elevated plasma LDL-C was significantly greater in SCD cases with coronary atherosclerosis compared with than in controls and PDS cases. However, plasma LDL-C levels were all within normal range in PDS cases [102].

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Hence, LDL-C did not seem to play a significant role at cardiovascular events in PDS, despite being slightly elevated within normal range, rather the data strongly indicated an association between plasma LDL-C and the progression of coronary atherosclerosis in SCD cases. Elevated plasma remnant lipoproteins (RLP) levels were the most striking observation in PDS (RLP-C likelihood ratio; 3.13, RLP-TG; 2.73, LDL-C; 1.52, TC; 1.30, TG; 1.07) for predicting sudden cardiac death in the fasting (gastric content; absent) and postprandial state (gastric content; present) (Table 2).

Despite the high plasma concentration of RLPs in PDS cases, the progression of atherosclerosis at coronary arteries was not observed. It might be valid to say that increased plasma RLPs may initiate the vascular endothelial damage and this is followed by an influx of large amounts of LDL into the vascular wall. Then it follows to form an advanced atherosclerotic lesion with macrophages and smooth muscle cells as Nakajima et al reviewed previously [6]. PDS cases may be in the early stage of atherosclerosis, which can lead cardiovascular events under certain conditions such as with severe stress without strong morphological changes.

Therefore, we proposed that the occurrence of cardiovascular events at coronary arteries and the severity of atherosclerotic lesions in CHD should be considered as separate factors. Therefore, the intervention should be targeted to suppress the cardiovascular events more aggressively than to slow down the progression of atherosclerosis. Takeichi and Fujita did not observe frequent plaque ruptures in coronary arteries at autopsy in Japanese SCD cases [102].

The literature on atherosclerosis has long been dominated by data in Caucasian patients who in most cases had severe atherosclerosis at the time of fatal clinical events.

Hence, fatal clinical events have been believed to occur in relation to the severity of atherosclerosis in coronary arteries. In contrast, fatal clinical events in PDS cases had occurred in the absence of coronary atherosclerosis or plaque rupture. Plasma LDL-C levels were also within normal range associated with no coronary atherosclerosis in PDS cases.

This again puts more weight on RLP as the causative factor of cardiovascular events. Interestingly, we found that RLP-TG (TG concentration in remnant lipoproteins) was not an indicator for predicting the presence or progression of coronary atherosclerosis even in SCD [102]; however, it was significantly associated with fatal clinical events in SCD including PDS.

Table 1. RLP-C is a CHD risk in females at Framingham Heart Study

Variable	
RLP-C > 75th percentile	
Odds ratio (per year)	2.27
95% Confidence limits	1.37-3.77
P Value	0.0015
TG > 2.25mmol/l (200mg/dl)	
Odds ratio (per year)	1.48
95% Confidence limits	0.71-3.10
P Value	0.30

McNamara JR, Schaefer EJ, et al: Atheroschlerosis 2001;154: 229– 36.

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Table 2. Plasma lipid and lipoprotein concentrations in the presence ro absence of gastric contents in sudden coronary death and control cases

Plasma lipid and lipoprotein concentrations in the presence or absence of gastric contents in sudden coronary death and control cases

Absence

	Control (n=17)		Pekkuri disease (n=21)		
	Median	25%-75% Tile	Median	25%-75% Tile	P value*
Cholesterol (mg/dL)	181	112-211	166	133-200	NS
Triglyceride (mg/dL)	116	62-126	115	100-159	NS
RLP-C (mg/dL)	6.0	3.9-12.3	12.0	6.0-16.3	< 0.05
RLP-TG (mg/dL)	39	21-48	69	51-94	<0.001
VLDL-C (mg/dL)	13	4-34	18	15-37	NS
LDL-C (mg/dL)	104	62-149	89	82-114	NS
HDL-C (mg/dL)	34	32-43	42	30-68	NS

Presence

	Control (n=34)		Pekkuri disease (n=36)		
	Median	25%-75% Tile	Median	25%-75% Tile	P value*
Cholesterol (mg/dL)	154	137-188	187	158-237	< 0.05
Triglyceride (mg/dL)	117	80-136	157	93-258	< 0.05
RLP-C (mg/dL)	12.1	8.7-14.2	14.0	9.5-27.8	< 0.05
RLP-TG (mg/dL)	49	43-72	86	60-167	< 0.001
VLDL-C (mg/dL)	23	8-35	28	16-45	NS
LDL-C (mg/dL)	98	69-126	122	95-140	NS
HDL-C (mg/dL)	40	26-65	41	35-54	NS

*Mann-Whitney U test NS, non statistically significant (p>0.05)

Takeichi et al. *Atherosclerosis* 1999;142: 309-315.

The bioactive components co-localized with triglycerides in RLP such as oxidized phospholipids or their metabolites [103] may enhance the formation of coronary vascular lesions and may induce severe spasm in coronary arteries. These results also suggested that triglycerides in RLP were not associated with the progression of atherosclerotic plaques, but cholesterol in RLP was strongly associated with the severity of atherosclerosis [99, 102]. Therefore RLP-TG could be an appropriate diagnostic marker for predicting cardiovascular events but not the severity of coronary atherosclerosis, whereas RLP-C could be a marker for predicting both cardiovascular events and the severity of coronary atherosclerosis. LDL-C could be a marker for predicting the severity of coronary atherosclerosis, but not cardiovascular events. Elevated Oxidized LDL seems to be associated with the presence of vulnerable plaque at blood vessels [6], not a causative factor for the formation or initiation of atherosclerosis because of it extremely low concentration in plasma.

4. Treatment for Cardiovascular Disease; Statins, CETP Inhibitor and Probucol as Therapeutic Drugs

4.1. Stains Increase LDL Receptor in Liver and Remove Plasma Remnant Lipoproteins More Effectively than LDL

HMG-CoA reductase inhibitors (statins) are known to decrease cellular cholesterol synthesis and consequently reduce the hepatic production of VLDL and increase expression

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of LDL receptor and lower plasma LDL-C levels [3]. Clinical trials have shown that improvements in plasma LDL-C levels are associated with retardation of atherosclerosis and reduction in coronary artery morbidity and mortality [4,5]. The major mechanism of this therapeutic effect has been recognized as the increase of LDL receptor expression in liver to remove elevated LDL-C in plasma by statins. However, recently, remnant lipoproteins have been increasingly implicated in progression of atherosclerosis, with elevated fasting remnant lipoprotein levels shown to predict clinical events independently in coronary artery disease patients [6]. A major target for remnant lipoprotein research has been postprandial dyslipidemia. Postprandial dyslipidemia has been found to be associated with endothelial dysfunction as an early indicator of atherogenesis [104,105]. Elevated remnant lipoprotein levels have also been associated with coronary endothelial dysfunction, with remnants shown to stimulate expression of proathero-thrombotic molecules in endothelial cells [106, 107].

Hence, the prevention and treatment of atherosclerosis merits pharmacotherapy targeted at regulating postprandial dyslipidemia, namely, RLP. Postprandial RLP are the atherogenic lipoproteins that appear and increase in plasma at the initial step of lipoprotein metabolism after food intake and then change to further metabolized lipoproteins, such as LDL. The postprandial state with increased RLP in plasma continues almost throughout the day, except in the early morning, while this is not the case in LDL. Therefore, RLP are atherogenic risks and should be the primary therapeutic target to prevent cardiovascular disease. Increased LDL is not directly associated with the daily food intake like RLP.

Possible mechanisms suggested for abnormal accumulation of lipoproteins postprandially in plasma are defective clearance via receptor-mediated pathways and/or increased competition for high-affinity processes because of increased numbers of intestinally and hepatically derived particles postprandially.

Plasma RLP containing chylomicron and VLDL remnants isolated from postprandial plasma was used to investigate the comparative reactivity to LDL receptor and VLDL receptor cDNA-transfected cells. We studied whether RLP are bound to LDL receptor more efficiently than LDL because RLP is apoE-rich. RLP competed more efficiently with DiI-β-VLDL than LDL in LDL receptor-transfected cells. These results suggest that RLP is more efficiently bound and internalized into LDL receptor than LDL. In VLDL receptor-transfected cells, RLP was more efficiently bound and internalized through VLDL receptor than β-VLDL particles even though we did not show any difference of binding ability between RLP and β-VLDL in LDL receptor-transfected cells. In contrast to TGRLs, LDL was not recognized by the VLDL receptor as Takahashi et al first reported [108].

It seems that VLDL receptor preferentially binds apoE-rich RLP rather than β-VLDL. These findings indicate that plasma RLP are removed by hepatic LDL receptor and muscular VLDL receptor, and the up-regulation of the expression of these lipoprotein receptors may be a therapeutic approach for anti-atherosclerosis. The fact that LDL receptor preferentially binds TGRLs rather than LDL was elucidated by Kita et al. [109].

They concluded that LDL receptor deficiency induced the primary deficiency of the removal of TGRLs (VLDL and IDL), and subsequently the enhanced conversion of VLDL to LDL was a cause of high plasma LDL level in homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits. As statins decrease cellular cholesterol synthesis and consequently reduce the hepatic production of VLDL and increase expression of LDL receptor[110], these properties suggest that statins may be potential agents for regulating the plasma levels of both RLP and LDL-C. Recent studies showed the effects of high-dose, long-

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term statin treatment on the metabolism of postprandial lipoproteins in heterozygous FH [101, 112, 113]. Statins may be able to induce half of normal LDL receptor in heterozygous FH that enhances the removal of RLP and LDL.

However, it has remained unknown whether RLP and LDL are removed by only increased LDL receptor expression with statin treatment or whether other lipoprotein receptors are working. It is likely that normal LDL receptor expression in heterozygous FH has already been up-regulated maximally by low-dose statins, and other mechanisms for reducing TGRLs (including RLP) and LDL particles may be working in the case of strong statins. In this study, we found that pitavastatin (NK-104) induced VLDL receptor expression in skeletal muscle cells (L6 cells) at significantly high concentration (approximately 1,000-fold) compared with the effect of NK-104 on LDL receptor expression in HepG2 (114)

The direct comparison between RLP and LDL has shown that RLP have superior binding and internalization reactivity to LDL receptor, which is similar to the reactivity with VLDL receptor. These results suggest that RLP may be more primarily and efficiently catabolized in liver than LDL through increased LDL receptor expression by statin treatment. The removal of TGRLs (including RLP) by LDL receptor may induce decreased plasma LDL-C level because of the removal of precursors of LDL. Additionally, the induction of muscular VLDL receptor expression by strong statins may be one of the therapeutic targets for reducing plasma RLP, but we need to be cautious about the strong statin-induced muscular VLDL receptor expression in terms of rhabdomyolysis.

4.2. CETP Inhibitor

4.2.1. CETP Inhibitors Inhibit the Formation of Remnant Lipoproteins Primarily and then Increase HDL-C as the Result

RLP is known to be formed by catabolizing of TRL (CM, VLDL) with a decrease in TG and an increase in cholesterol, so it is predicted that lipase (LPL, HL) and CETP are involved in the RLP formation [115]. Previous studies have shown that in vitro lipolysis of VLDL by exogenous LPL enhanced CETP-mediated CE transfer from HDL to VLDL [116], and CE in HDL is preferentially transferred to VLDL-1 in the postprandial state of type IIB hyperlipidemia [117]. Moreover, it has been reported that plasma CETP activity was increased coincidently with the increase of postprandial plasma TG [118] and was closely correlated with RLP-C level in patients with nephritic range proteinuria [119]. Although these reports suggest that CETP is involved in the formation of cholesterol-rich remnant particles, we have performed the study to obtain more direct evidence of the involvement of CETP in it [120].

The present study demonstrated that RLP-C is increased by 37 °C incubation of human plasma. As the amounts of RLP-C increase were positively correlated with plasma TG levels (r^2 = 0.555), the RLP-C increase was suggested to indicate the RLP formation from TRL. Also, the 37 °C incubation of plasma promoted the CE transfer from HDL to RLP. As there was a closely relationship between the RLP-C increase and the CE transfer to RLP (r^2 = 0.908, plasma TG levels of >100 mg/dl), the CE transfer from HDL to RLP lead to the RLP-C increase. Furthermore, exogenous r-CETP of physiological concentrations (0.5-1.5 μg/ml) promoted the RLP-C increase dose-dependently, and inhibition of endogenous CETP by JTT-705 (CETP inhibitor, Japan Tabaco) and the monoclonal antibody JHC1 suppressed the RLP-

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C increase and the CE transfer to RLP with IC_{50} values similar to those for the inhibition of CETP activity. These all results demonstrate that CETP is essential to the RLP-C increase in this in vitro study.

Guerin et al. have reported that elevated rates of CETP-mediated CE transfer from HDL to TRL are intimately associated with the enhanced formation and accumulation of CE-rich RLP in type IIB hyperlipidemin subjects during the postprandial phase [117]. Therefore, we have examined CE transfers from HDL to RLP and non RLP in TRL fraction ($d < 1.006$ g/ml) and the effects of JTT-705 on the transfers. Although the amounts of CE transfer to RLP and non RLP were not so different, the inhibitory effect of JTT-705 was stronger than that on non RLP. The result suggests that CETP did not only transfer CE to RLP and non RLP, but also CETP promoted the novel RLP formation from non RLP in TRL through the CE-TG exchange, namely, JTT-705 inhibited the increase of RLP-C directly and indirectly.

As the result of examination of CE transfer from HDL to IDL (VLDL remnants), CE transfer to IDL was observed and CE transfer was also inhibited by JTT-705. Although IDL was obtained by the ultracentrifugation, these results were same as that observed in RLP measured using the immunoaffinity gels and, therefore, support that the increase of RLP-C in this study corresponds to the increase of cholesterol in remnant lipoproteins.

On the other hand, it has been reported that plasma LCAT activity was also increased in 2 h or 6 h [118] postprandially, so the effect of LCAT inhibitor on RLP-C increase was examined. As LCAT inhibitor DTNB did not affect on RLP-C increase up to 1 mM, LCAT was supposed to be less involved in the RLP-C increase in the present study. In conclusion, the results of this study demonstrate that CETP promotes the formation of cholesterol-rich RLP through the transfer of CE from HDL to TRL and the inhibition of CETP activity is expected to decrease RLP-C level effectively. Recently, plasma CETP levels were shown be significantly correlated with CAD risk among subjects with high TG levels [121], and it is possible that these subjects with high CETP and high TG levels have high levels of RLP-C. Although HMG-CoA reductase inhibitors have been reported to decrease RLP-C in patients with hypercholesterolemia [122] and Type-2 diabetes [123], CETP inhibitors [124] are also useful for the reduction of RLP-C level.

4.2.2. The Role of CETP and ANGPTL3 for Increasing Plasma HDL-C Levels. ANGPTL3 Inhibit HTGL Activity and Increase HDL-C More Prevalently than CETP Inhibition

We determined serum ANGPTL3 and CETP levels in HALT cases (HDL>90 mg/dl) and compared the abnormal frequencies of these proteins which are known to be associated with increased HDL-C [125]. This study did not focus on the gender differences in these proteins, because the normal range of the standardized parameter “HDL-C” is known to be the same in men and women as seen in Japanese reference range, which is different from USA reference range for HDL-C. To determine the normal range of ANGPTL3 in Japanese population, we developed our own sandwich ELISA of ANGPTL3 for this study. ANGPTL3 determined by other ELISA methods showed either higher serum levels [126] or different clinical significance when compared with our method [127]. Also we recruited cases with very high HDL-C (HALT) at the health check-up centers randomly and expected to find either low CETP [128] or high ANGPTL3 [129-131]. As previously reported [131], these trends were not so clear in moderately high HDL-C cases, however we found significantly high ANGPTL3 levels in cases with HDL-C above 90 mg/dl in this study. Therefore we focused

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on the analysis of ANGPTL3 and CETP in HALT cases for finding a convincing difference between the two proteins on HDL metabolism. High HDL-C cases like above 100 mg/dl seem to be difficult to find in Caucasians [132], while such cases are prevalent in Japanese population [133]. One of the causes is suspected to be a high frequency of CETP polymorphism in the Japanese population [134, 135]. However, this study may present a different mechanism for the high frequency of HALT in Japanese population. ANGPTL3 is now known to be a major inhibitor of EL [131]. Therefore, plasma ANGPTL3 concentration may reflect EL activity in plasma via the ANGPTL3-EL pathway [136]. Our preliminary data obtained with the newly developed EL activity assay showed that ANGPTL3 concentration correlated inversely with EL activity (r=−0.27, Pb0.005) and HDLC (r=−0.13, Pb0.05) in post-heparin plasma, but not in pre-heparin plasma, indicating that high ANPTL3 plasma levels are associated to low EL activities and high HDL-C (authors' unpublished observations). Badellino et al. [137] recently developed an ELISA for measurement of human plasma EL. In this study, median EL mass in pre-heparin plasma was 442 ng/ml (interquartile range=324–617). Median postheparin mass was approximately 3-fold higher, 1313 ng/ml (888– 1927). The correlation between pre-heparin EL mass and postheparin EL mass was 0.46 and both assays showed inverse correlation with HDL-C. These results showed similar trend with those of the new EL activity assay method we are now developing. Comparative studies between EL mass and EL activity including the concentration of ANGPTL3 in plasma need to be studied further as LPL and HTGL assays [138]. Recently, CETP inhibitors [124] have been developed and several reports from clinical trials have already been published [139-141], reporting significant anti-atherogenic effects in experimental animals, but not yet in humans. HDL-C levels were significantly increased by CETP inhibitors, but these HDL particles became very large in size like those in CETP deficient cases [142]. Further, CETP inhibitor molecules adhere to and are carried on HDL particles in the blood, which could be considered as an unwanted interaction [140, 141]. Therefore the efficacy of CETP inhibitors is yet to be accepted [143]. The frequency of CETP deficiency or polymorphism seems to be very rare in Caucasians, but quite frequent in Japanese. Therefore, if the ANGPTL3-EL pathway unlike CETP is not physiologically impaired in Caucasians, it could be a better target for raising HDL-C. This study showed approximately 10 fold higher frequency of abnormally increased ANGPTL3 in HALT cases as compared with the frequency of low CETP. For sometime, we have been speculating that the prevalence of CETP polymorphism or deficiency may be a major cause of high HDL-C in Japanese population [144, 145]. The CETP assay kit we used in this study showed comparatively lower serum levels of CETP than the levels assayed by Dai-ichi ELISA kit (the most frequently used assay kit), which also indicated a high correlation between activity and mass of CETP in average Japanese population [146].

Therefore, we have calculated the normal range of CETP independently in cases with normal HDL-C in this study subjects and determined the low cut-off value as mean-2SD. We also compared the abnormal low CETP and high ANGPTL3 frequencies in HALT cases by 10–90 percentile analysis. CETP less than 10% tile was 21.3% and ANGPTL3 above 90% tile was 76.4% in this study subjects, respectively. Another reason for 10% tile of CETP as the lower cut-off value was the frequency of CETP common mutation (approximately 10%) in average Japanese population [134].

Both mean±2SD analysis and 10–90 percentile analyses showed significantly greater frequency of abnormally high ANGPTL3 than those of low CETP. Therefore we could

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predict the possibility that the ANGPTL3-EL pathway, including hepatic preprotein convertase [136], may have a major physiological role in HDL metabolism. As Ishida et al. [147] proposed EL as the major pathway for HDL metabolism in mouse, we also suggest that ANGPTL3- EL or HTGL is the major pathway for HDL metabolism in humans regardless of the presence or absence of CETP. Interestingly, there was a high correlation between ANGPTL3 and HDL-C in seven CETP deficient cases in this study.

This means that ANGPTL3-EL or HTGL pathways may independently associate with the increase of HDL-C from that of CEPT. CETP deficiency cases were found to be not always associated with high HDL-C from these cases. We have been interested in finding the mechanism of HDL-C decrease after probucol treatment. Miida et al. [148] recently reported that probucol significantly reduced serum ANGPTL3 levels and increased preβ1-HDL, indicating the possibility of increasing the EL activity by reducing ANGPTL3, the inhibitor of EL. However, as yet, an EL activity assay has not been reported, probably due to the catalytic activity in the human EL being inhibited by ApoC-II in serum.

This makes it difficult to distinguish EL activity from LPL/HTGL activity in the human post-heparin plasma [138]. Therefore, ANGPTL3 concentration may take the place of EL or HTGL activity, similar to ApoC-III for LPL activity [138] and remnant lipoproteins [149]. These drawbacks will continue until a direct plasma EL activity assay has been developed to compare directly with CETP activity. Further studies on the polymorphism of ANGPTL3 are warranted for investigating a more direct relationship between ANGPTL3 and EL activity.

4.3. Probucol, a New Life for Old Drug

Probucol has demonstrable anti-inflammatory actions that contribute to a reduction in experimental atherosclerosis. Also, probucol reduces the adhesion of inflammatory cells *in vivo* as demonstrated by the inhibition of mononuclear cell adhesion and reduced expression of vascular cell adhesion molecule [150] following balloon injury in hypercholesterolemic rabbits. Similarly, in LDL receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits, the fibrous cap in the lesions of animals fed probucol had lower macrophage content, but an increase in vascular smooth muscle cells [151]. Taken together, these studies demonstrate that probucol reduces atherosclerosis and cardiovascular disease while improving the state of inflammation and heightened oxidative stress in the affected blood vessels.

4.3.1. Protective Effects of Probucol on Vascular Endothelial and Smooth Muscle Cells

Endothelial dysfunction is associated with an increased risk of cardiovascular events and a vasoconstrictor response to acetylcholine indicates the presence of endothelial dysfunction[152]. The endothelium-dependent vasomotor response to acetylcholine is significantly attenuated in humans by oral treatment with probucol. In support of this, probucol promotes the growth of endothelial cells and promotes endothelium-dependent arterial relaxation and functional re-endothelialization following aortic balloon injury, as measured by the extent of re-endothelialization, nitric oxide production and nitric oxide-mediated vasodilatation [153]. There are a number of potential mechanisms by which probucol may enhance endothelial function. First, probucol protects against hypochlorite-

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mediated, endothelium-dependent relaxation of the aorta in rabbits *in vivo* [154]. Second, the effects of probucol on increasing functional re-endothelialization and inhibiting smooth muscle proliferation [153] are similar to biological processes seen with the increased activity of heme oxygenase-1 (HO-1) [155]. Similarly, pharmacological inhibition of heme oxygenase activity blocks the ability of probucol to promote re-endothelialization and to inhibit intimal hyperplasia following vascular injury *in vivo* [155]. These new findings suggest that probucol's effects on endothelial cell growth and function and on inhibition of smooth muscle proliferation are greatly mediated by HO-1. Increasingly, new evidence points to a key role of HO-1 as anti-oxidant property (156-158). Probucol increases the expression of HO-1 and heme oxygenase activity in balloon-injured rabbit aortas and rabbit aortic smooth muscle cells [155]. HO-1 is a redox-sensitive enzyme and the promoter region of the HO-1 gene contains multiple copies of antioxidant response elements that are critical for enzyme induction [159] and that are tightly regulated by the redox-sensitive transcription factor NrF-E2-related factor-2. Unlike probucol, vitamin E does not induce HO-1 in vascular smooth muscle cells *in vitro* [155], consistent with the notion that 2-electron, but not 1-electron, antioxidants may exhibit part of their anti-atherosclerotic effects via induction of HO-1. Similar antioxidant proteins and enzymes such as metallothionein and SOD are also known to be significantly induced by probucol associated with NrF-2 gene expression [159]

4.3.2. Lipid Metabolism and Cholesterol Efflux by Probucol

Probucol's cholesterol-lowering activity was first discovered in 1964 during screening of phenolic antioxidants. The drug is relatively weak when compared to statins, 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor. How probucol reduces plasma and LDL cholesterol still remains uncertain. However, early studies in LDL receptor-deficient WHHL rabbits demonstrated that probucol reduced the atheroma burden independent of the drug's cholesterol-lowering effects [160]. In fact, the ability of probucol to inhibit experimental atherosclerosis independent of the drug's lipid-lowering effect was one of the corner stone of the oxidative modification theory of atherosclerosis. Abundant data support the oxidative modification hypothesis of atherosclerosis, according to which oxidation of LDL is an early event in, and contributes to, atherogenesis [161]. Although probucol was designed originally as a phenolic antioxidant, the peroxyl radical scavenging activity of probucol is only ~16% that of the most abundant phenolic antioxidant and form of vitamin E in humans, α-tocopherol. Yet, early animal studies consistently reported that probucol reduced lipoprotein oxidation in plasma *ex vivo* or in the vessel wall [162]. However, the oxidative modification hypothesis of atherosclerosis has been recently challenged by the failure of antioxidants, in particular vitamin E [163], to reduce disease progression and clinical events in patients at risk of, or with established, atherosclerosis. Subsequent studies have dissociated the anti-atherosclerotic effects of probucol from its ability to affect lipoprotein lipid oxidation. First, in rabbits, probucol inhibited atherosclerosis to a greater extent than probucol analogs which had greater anti-oxidant activity [164]. Furthermore, probucol did not alter the proportion of aortic lipids that are oxidized. Thus, while probucol exhibit anti-oxidant activity, this property does not seem to account for its anti-atherosclerotic properties. Previously, it had been thought that the strong antioxidant properties of probucol arose from the two phenol moieties. Recent evidence suggests, however, that the sulfur moiety in probucol is critical for *in vivo* protection from atherosclerosis [155] Thus, probucol and probucol dithiobisphenol, but not the sulfur-free probucol bisphenol, inhibited disease in apolipoprotein E-deficient mice and

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hypercholesterolemic rabbits following aortic balloon injury. Paradoxically, probucol lowers HDL cholesterol yet inhibits atherosclerosis. The controversial and anti-atherogenic feature of probucol is most likely attributable to molecular mechanisms: promoting cholesterol efflux, and enhancing reverse cholesterol transport (RCT) by activation of CETP [134, 165] and scavenger receptor class B type 1 (SR-B1)[166]. The apparent reduction of HDL-C by probucol may be due to the remodeled function of HDL: increased preβ1-HDL [148] ("lipid-poor" apoA-1) participating in cellular lipid efflux. These mechanisms could be responsible for probucol-induced regression of xanthoma and its anti-atherogenic effects, though probucol is an effective inhibitor of ABCA1-mediated cholesterol efflux [167]. A recent report by Miida et al [148] suggested the possibility that probucol activates the endothelial lipase by inhibiting angiopoietin-like protein3 (ANGPTL3) and enhance HDL metabolism and cholesterol efflux by increasing preβ1-HDL levels. Contrary to the results of studies using WHHL rabbits, some experimental studies using apolipoprotein E-knockout mice reported that probucol demonstrated pro-atherogenic effects [168]. These contradictory findings in mice with no CETP may rather support the hypothesis regarding cholesterol efflux and the RCT pathway of probucol through CETP. An important role of CETP in the mechanism of cholesterol efflux and RCT is evident in the recent negative clinical trial results of a CETP inhibitor [169] as well as the epidemiological reports [170] of increased coronary heart disease in patients with CETP deficiency and the molecular approach to review of CETP deficiency [171]. Marked hyper-HDL2 cholesterolemia associated with CETP deficiency can be atherogenic regardless of elevated HDL-C, which has been indicated in a long-ignored Japanese report by Matsuzawa et al [172]. The CETP activator instead of the inhibitor might provide a partly rational therapeutic approach to prevent atherosclerosis, after a review of the evolving field of pro-atherogenic HDL, a novel role for human CETP in the defense against an exacerbated production of pro-inflammatory mediators [173], and CETP polymorphism among individuals or ethnics groups [174].

Over the past several decades, probucol has established itself as a potent anti-oxidant with a broad spectrum of other pharmacologic actions; further, it has demonstrated significant therapeutic effects on diverse diseases in humans. Its mechanisms of actions at the molecular level have recently been elucidated and are as diverse as its therapeutic effects. More recently, probucol was demonstrated to significantly reduce CHD risks in patients with heterozygous FH who have very high CHD risks [175]. It is anticipated that probucol will overtake agents like statins, which are reported to lower LDL-C, which is a well known CHD risk factor. There is compelling reason to believe that this old and often misunderstood drug has much more to offer than hitherto known even if it reduces HDL-C levels.

CONCLUSION

Oxidative modification of LDL (Ox-LDL) has been widely believed to play a key role in the initiation and progression of atherosclerosis since Steinberg et al. first proposed this hypothesis in 1989 [2]. This concept has provided strong support for the efficacy of LDL-C lowering drugs. Several atherosclerotic phenomena such as the progression of atherosclerotic lesion have been explained by this hypothesis, but it is equally important to address issues which do not support Ox-LDL per se as the most important risk factor for the initiation of

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atherosclerosis. For example, the concentration of Ox-LDL in plasma may be less than 0.5% of total LDL in CHD patients and to which the endothelial cells are exposed. This plasma concentration may not be enough for proatherogenic and proinflammatory activities of Ox-LDL and the initiation of ‘‘response-to-injury’’ in endothelial cells, judging from the results of many in vitro studies.

The most significant role of Ox-LDL in atherogenesis has been explained in the subendothelial space for interactions with macrophages and smooth muscle cells, not in endothelial cells. The discovery that LOX-1 receptor activation by RLP by Shin et al. [8] has opened the window for a new oxidative modification hypothesis that says RLP but not Ox-LDL in plasma is a major ligand for the LOX-1 receptor in endothelial cells, causing endothelial dysfunction and the initiation of atherosclerosis. RLP are already oxidized in plasma and reported the existence of large amount of lysophospahtidylcholine in cylomicron remnants, a constituent of RLP and their proatherogenic and proinflammatory properties. In other words, major lipoproteins oxidized in plasma may be remnant lipoproteins and not LDL, existing as an oxidative modification.

Ox-LDL and RLP revealed common proatherogenic and proinflammatory characteristics, indicating the same biological functions shown at similar concentrations in in vitro studies. This may suggest that the same oxidized phospholipids exist in these lipoprotein particles which mediate their atherogenicity. The isolation method of RLP from plasma developed by Nakajima and co-workers [6] made it possible to verify the oxidative susceptibility of remnant lipoproteins and to compare the proatherogenic and proinfalmmatory properties of RLP with those of Ox-LDL.

Plasma RLP-C concentration has been shown to be more convincingly associated with the increased risk of premature atherosclerosis by many clinical studies than plasma circulating Ox- LDL. Further, endothelial dysfunction is more likely to be caused by RLP than by circulating Ox-LDL per se in plasma, judging from the plasma concentration of these lipoproteins. Taken together, reducing plasma RLP rather than LDL should be the target of hyperlipidemic therapy especially in patients with metabolic syndrome which is highly associated with plasma RLP-C level, but not with LDL-C levels.

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Chapter III

THE FAT-FED APOLIPOPROTEIN E KNOCKOUT MOUSE AS A MODEL OF ATHEROSCLEROTIC PLAQUE RUPTURE

Andrew R. Bond [*] ***and Christopher L. Jackson***
Bristol Heart Institute, School of Clinical Sciences, University of Bristol, Level 7, Bristol Royal Infirmary, Bristol, UK

ABSTRACT

Atherosclerosis is a disease of the medium to large size systemic arteries, and is the leading cause of death world-wide. Although they do not mimic the human disease exactly, a cause of much debate, animal models of atherosclerosis are crucial to our understanding of the risk factors and mechanisms behind the disease's initiation and progression. Various approaches have been taken, be it treatment with drugs or manipulation of the diet, to give us an insight into possible approaches to the prevention and treatment of the disease. The fat-fed apolipoprotein E knockout mouse has proven itself as a valid and extremely valuable model of atherosclerosis, and in particular as a model of atherosclerotic plaque rupture, the cause of the majority of severe clinical cardiovascular-related consequences. It is this model, and in particular its history, development, key findings, and current use in research, that shall be discussed further in this chapter.

INTRODUCTION

Atherosclerosis is a chronic disease affecting the medium and large size elastic and muscular systemic arteries and is heavily implicated in diseases of the circulatory system, which are the leading cause of all deaths. The exact mechanisms behind the formation of atherosclerotic lesions is unknown, hence the large amounts of ongoing research in this field. However, there are many risk factors identified that are thought to be responsible. They can

[*] Address correspondence to: Dr Andrew R. Bond; Email: andrew.bond@bristol.ac.uk.

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be divided into two groups: those that are modifiable, such as obesity, tobacco smoking, physical inactivity, increased systolic and diastolic blood pressure, hypercholesterolemia, diabetes mellitus [1] and infection [2]; and non-modifiable factors such as gender, age, family genetics, and the menopause [3]. Although thought of as a disease of the modern era and a consequence of current lifestyles and diet, evidence has been found dating back to 1500 BC with Egyptian mummies of high priests and priestesses being afflicted with lesions similar to those found today [4]. Unlike in healthy arteries, where blood is free to flow throughout the body oxygenating and perfusing organs, the lumen of atherosclerotic arteries is compromised. The clinical consequences of this can be severe – myocardial infarction, stroke, or critical limb ischaemia. This is caused when the atherosclerotic lesion growth occurs at a more rapid rate than the blood vessel can outwardly remodel to accommodate the increased plaque burden, decreasing the lumen area.

Although primarily thought of as an inflammatory disease, suggesting injury as the precursor to lesion formation, atherosclerosis does not develop randomly as would be expected if injury was entirely to blame. Rather, it is found in consistent regions throughout the vasculature across species, suggesting factors such as haemodynamics and vessel geometries are also involved. As previously mentioned, atherosclerosis is a chronic disease, with its development typically taking decades. However, acute events typically lead to more severe clinical consequences, and these are triggered by rupture of the fibrous plaque as shall be described in further detail below.

HUMAN ATHEROSCLEROTIC LESIONS

Although the more severe consequences of atherosclerosis develop later in life, early evidence of the disease in the form of lipid deposition, known as fatty streaks, is present from a very young age [5]. At these early ages, rather than being thought of as a disease, the process is seen more as a protective response to potential damage to the endothelium and underlying smooth muscle cells [6]. The approximate location of these early fatty streaks is the same as that of more advanced lesions [7], suggesting this early protective response causes damage to the endothelium provoking an inflammatory response that culminates in increased lesion development.

Activation and damage to the endothelium lining the arteries attracts monocytes and lymphocytes to the site of injury, which under the influence of growth-regulatory molecules and chemoattractants, cause an increase in permeability of the artery wall. This allows migration of lipid components in the plasma, such as cholesterol-laden low-density lipoproteins (LDL), into the intima and media.

The American Heart Association (AHA) has developed a classification scheme (designated by Roman numerals) for lesion types, dependent on the stage of development and their histological characteristics. The early lesion types (I and II) are generally only found in children, although they have also been seen in adults. Type II lesions contain atherogenic lipoproteins in large enough quantities to cause an inflammatory response (seen by an increase in macrophages) and formation of scattered macrophage foam cells [8]. They are classified as pre-atheromatous intermediate lesions and evolve soon after puberty. They consist primarily of macrophage foam cells and lipid-laden smooth muscle cells, and hence

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are also termed ‘fatty streaks’. T-lymphocytes have also been observed in these lesions, although they are not as numerous as the macrophages [8, 9]. Type III lesions are the last lesion type thought of as clinically silent and contain, in addition to the contents of Type II lesions, collections of extracellular lipid droplets. Type IV-VI lesions are termed advanced lesions and are clinically significant. Type IV lesions, or atheroma, contain a large dense lipid core that thickens the arterial wall, and are overlaid by macrophages and smooth muscle cells, both with and without lipid droplets, lymphocytes and mast cells [9]. Type V lesions are generally referred to as atherosclerosis, or an atherosclerotic plaque. The term atherosclerosis is derived from the Greek for porridge/gruel *athéré*, signifying the soft lipid-filled core, and *sclerosis* implying hardening and referring to the hard fibrotic cap that separates the blood from the thrombogenic material in the lesion [10]. Atherosclerotic plaques are recognised when prominent fibrous connective tissue has formed over the lipid core, and they can develop disruptions to the lesion surface i.e. rupture, fissures, haematomata, and/or thrombi. Type VI lesions are referred to as complicated lesions as they contain one or more of the aforementioned surface disruptions.

It is interesting to note that atherosclerosis appears to be more severe in the male population [11] and the suggestion has been made that sex hormones are responsible. This correlates with the findings that when oestrogen production stops, either naturally or after surgery, the risk of atherosclerosis increases in woman [12].

CELL TYPES IN ATHEROSCLEROTIC LESIONS

As has already been alluded to, atherosclerotic lesions contain many different cell types. The first cells to infiltrate the arterial wall are monocytes, attracted as part of an inflammatory response to atherogenic lipoproteins accumulating at atherosusceptible sites (as discussed further below), or as a result of physical damage to the endothelium and subsequent platelet accumulation. The monocytes differentiate into macrophages, which then phagocytose oxidised lipoproteins and become large foam cells. It is these foam cells that form fatty streaks, the early atherosclerotic lesions visible to the naked eye. Apoptosis of foam cells perpetuates the inflammatory process encouraging more inflammatory cells to migrate to the site, along with smooth muscle cells from the tunica media to the intima and hence the fatty streak grows into an atherosclerotic lesion. Apoptosis of the lipid laden cells leads to the formation of the necrotic lipid core, and accumulation of smooth muscle cells, macrophages and collagen overlying this forms a protective fibrous cap.

HAEMODYNAMICS AND LESION LOCATION

Hyperlipidaemia and hypercholesterolaemia that lead to increased lipid concentration and metabolism have been seen to initiate and support the progression of lesion formation. However, this does not account for the distribution of lesions in the vasculature [13], suggesting other mechanisms must be involved. Haemodynamics is the study of the relationship between pressure, viscous resistance to flow, and the volumetric flow rate in the cardiovascular system [14]. It is thought that the work of Rindfleisch, in 1872 (in [15]) first

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showed that there was a link between the distribution of atherosclerosis in arteries and "sites that experience the full stress and impact of the blood". Over a century ago it was recognised that when blood flows faster through a vessel, the vessel enlarges, and when blood flows slower it narrows, suggesting a compensatory mechanism is in place (Thoma, 1893, cited in [16]). This mechanism is likely to involve shear stress, the frictional force of the blood flowing over the vessel wall. The endothelium lining all arteries is very sensitive to the blood flow over it, and endothelial cells and their nuclei have been shown to elongate in increasing flow conditions and align to the time-averaged flow direction both *in vitro* [17, 18], and *in vivo* [19, 20]. Steady, laminar (or streamline) flow, in which an incompressible fluid moves as a series of layers in a straight rigid tube of circular cross-section, will have a velocity profile that is parabolic when fully developed (so-called Poiseuille flow) and is a critical factor in maintaining normal physiologic vascular function, and is seen as atheroprotective. Molecules immediately adjacent to the wall do not move at all (the no-slip condition), and those in the centre move fastest. Once this state is reached, there will be no further change in the flow profile down a uniform unbranched tube. However, in the vasculature it is rare to find a uniformly straight, unbranched section of blood vessel: therefore the blood velocity profile is constantly changing, leading to a non-laminar and disturbed flow pattern. It is this turbulent flow pattern that contributes to atherosclerotic lesions being commonly found at bends (e.g. the inner curvature of the aortic arch) and bifurcations (e.g. the abdominal aorta into the iliac arteries).

ATHEROSCLEROTIC PLAQUE RUPTURE

Despite atherosclerotic lesions all having the same basic morphology, they can be split into two main groups: those that have a high chance of rupture (vulnerable, unstable, high-risk, or thrombosis-prone plaques,), and those that are unlikely to rupture (stable plaques). In humans, plaque rupture has been defined as "a lesion consisting of a necrotic core with an overlying thin ruptured fibrous cap that leads to luminal thrombosis because of contact of platelets with a highly thrombogenic necrotic core" [21]. When the plaque ruptures, the blood is able to come into contact with the thrombogenic core precipitating a thrombus. This in itself can severely decrease the lumen size [22] leading to decreased blood flow, and the resulting organ ischaemia can lead to symptoms such as angina pectoris. A further complication of rupture is thrombus embolism, where small bits of clot break away and get carried downstream, where they become lodged in smaller vessels, occluding flow. If this occurs in the coronary or carotid vessels, it can lead to myocardial infarction or stroke respectively. Approximately 75% of the thrombi responsible for acute coronary syndromes are precipitated by rupture of an atherosclerotic plaque [23]. Determining whether a patient has unstable plaques is crucial in determining the course of treatment/intervention, as unstable plaques need to be treated as a matter of urgency. They are thought to account for approximately 70% of fatal acute myocardial infarctions and/or sudden coronary deaths [24]. In a minority of patients suffering acute coronary events there is no classical plaque rupture, but rather disruption of the arterial endothelium overlying a lesion, and these events tend to be termed "plaque fissure" or "plaque erosion" [25, 26]. Vulnerable plaques are not necessarily large plaques with severe stenosis; vulnerability is more dependent on the composition of the

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plaque itself. Based on retrospective analysis of human autopsy blood vessels, a notable trend in vulnerable plaques is that they tend to have a large lipid-rich necrotic core occupying 40-50% of the plaque's total volume, increased macrophage content, and few smooth muscle cells: these are all covered by a thin fibrous cap (<100 μm) [22, 24]. Stable plaques have thick fibrous caps and a relatively small lipid core. In the clinical environment it is important to try to determine both plaque vulnerability (intrinsic disease) and also the events leading up to the rupture (extrinsic forces) [27].

There are several invasive and non-invasive imaging modalities available to cardiologists, such as angiography, angioscopy, intravascular ultrasound and magnetic resonance imaging, which are useful for imaging and assessing the progression and stabilization of atherosclerotic lesions. However, it is not currently possible to characterize or correlate the image parameters with histopathological lesion types, such as those described by the AHA [28]. The most accurate methods of lesion characterization rely on observing diseased vessels after patients have died, therefore missing the critical acute events leading up to death. This approach causes problems, as patients that die of cardiovascular disease will in all likelihood have had previous plaque rupture, skewing data towards the vulnerable plaque phenotype. It is therefore essential that animal models be employed as they can enable longitudinal studies to be performed, allowing the observation of disease initiation and progression at multiple time points, and making it possible to see the important events that precede and initiate rupture of the plaques. This issue of interpretation has been summarised previously as follows [29]:

1. Patients are self-selecting – they have suffered a fatal cardiovascular event – and thus may not be representative of the general population or even of patients with non-fatal unstable plaques. This may bias any analysis towards particular underlying mechanisms.
2. The elapse of time between symptom onset and specimen retrieval will be accompanied by changes in tissue composition
3. Within-subject temporal histopathological analysis is impossible, and cohort-based between-subject analysis is extremely difficult.
4. *Post hoc* interpretation involves speculation that is not backed up by experimental verification.

The chance of a plaque rupturing, or the Plaque Vulnerability Index (PVI), has been formulated as the ratio of plaque area occupied by lipid components to that occupied by fibromuscular components, i.e. dividing the area of macrophages and extracellular lipid deposits by that of smooth muscle cells and collagen [30]. This takes a very broad few of plaque vulnerability as there are other factors that need to be considered, such as the content and types of collagen and matrix metalloproteinases (MMPs). MMPs are a group of at least 23 structurally-related zinc-dependent endopeptidases with the capacity to break down components of the extracellular matrix in the vessel wall, such as collagen and elastin, gelatins and casein [31], leading to remodelling of the matrix. An imbalance in collagen synthesis and breakdown is thought to lead to problems such as arterial stenosis if there is too much collagen, or plaque vulnerability if there is too little. Human atherosclerotic plaques contain mostly collagen types I and III, with an increase in type V collagen in advanced lesions, and increased type IV in the fibrous cap regions (reviewed by [32]). Collagen

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provides physical strength due to its fibrillar triple helix structure: therefore a deficit leads to plaque weakness and vulnerability [33]. An imbalance in collagen may be caused by the MMPs, and in particular the collagenases (MMP-1, -8 and -13) as they have the ability to cleave fibrillar collagen types I, II, and III into fragments which in turn can be degraded by the gelatinases [31]. It has been shown by *in situ* zymography that normal (non-diseased) vessels do not express MMPs [34]. However, in atherosclerotic vessels, levels of MMPs 1 (collagenase 1), 2 (gelatinase A), 3 (stromelysin 1), 7 (matrilysin 1), 8 (collagenase 2), 9 (gelatinase B), 10 (stromelysin 2), 11 (stromelysin 3), 12 (macrophage metalloelastase), 14 (membrane-type 1 MMP) and 16 (membrane-type 3 MMP) are all increased [34, 35]. Of these, MMPs 1, 3, 8, 9, 11, 12, 14 and 16 have all been found in rupture-prone regions of human plaques [31, 34]. This does not mean that MMPs cause plaque instability, and data from knockout mouse models suggest that some of them may be involved in beneficial matrix remodelling that strengthens the fibrous cap [36]. Furthermore, although collagenase (MMP-8) and gelatinase B (MMP-9) have both been found to be associated with an unstable carotid plaque phenotype, gelatinase A (MMP-2) appears to be associated with a more stable phenotype [37]. MMP-12 transcript levels in human carotid plaques are higher in those that have ruptured, than in those with a thick fibrous cap [38], and interestingly MMP-12 levels are higher in tissue from smokers than non-smokers, perhaps suggesting an alternative mechanism as to why smoking is a risk factor for plaque instability [39].

Rekhter summed up the issue of plaque rupture by saying it is “a complication of an already complex atherosclerotic process and precise mechanisms of this complication remain hypothetical” [40]. One way of trying to unravel this complex problem is to use an animal model of atherosclerotic plaque rupture, as shall be discussed further below.

ANIMAL MODELS OF ATHEROSCLEROSIS

As has previously been mentioned, lipid deposition and subsequent atherosclerosis has been found in humans at all ages from neonates up to old age. However, it is not possible to track disease changes and development in humans over time. Atherosclerosis is a multifactorial disease, with a large amount of uncertainty as to exactly when serious clinical events, such as plaque rupture, will occur. There are numerous imaging modalities available for detection of lesion sizes and composition in humans as put forward by Schaar et al [41]. These include intravascular ultrasound (IVUS, composition of plaques), magnetic resonance imaging (MRI, potential to identify components of plaques), optical coherence tomography (OCT, utilises optical interfaces in tissue to build up image), angioscopy (red vs. white vs. yellow plaques, giving information on lipid and thrombus content) [42], thermography (indicator of the metabolic state of the plaque), electron beam computerized tomography (calcification), positron emission tomography (PET, inflammation [43]), Raman spectroscopy (molecular composition of plaque), and near-infrared spectroscopy (identifies lipid loaded plaques). However, at the moment these techniques are predominantly used when a patient has already presented clinical symptoms of atherosclerosis, and the image resolution still needs improving.

In vitro cell culture is frequently used to try and determine the mechanisms and signalling pathways underlying atherosclerosis. It has the benefits of being easy to manipulate, a rapid

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turnaround time, no non-controllable external factors affecting them, and being reproducible, however culture systems will never be able to replicate a living whole system. Animal models of the disease, whereby changes occur in a relatively rapid and reproducible way, with the elimination of many of the external risk factors associated with human disease, are extremely useful for the study of the processes involved, and enable us to accurately obtain morphometric data, as we can look at lesions at various time points and track the lesions' growth and development and look in great detail at cell types. For example, a recent study has shown that exercise in hypercholesterolaemic mice led to a ~33% decrease in aortic lesion size compared to controls. This study removed external risk factors and enabled the very simple hypothesis that exercise reduces atherosclerosis to be tested quickly and simply [44]. Animal models of atherosclerosis have been studied since the early 1900s, starting with the work of Ignatowski [45], despite atherosclerosis not being recognised as a major disease at the time. Once identified as a major disease, animal models of atherosclerosis were developed that included rabbits, mice, rats, hamsters, guinea pigs, dogs, pigs, cats, and non-human primates (reviewed in [46]). A good model of atherosclerosis should enable us to track changes in disease state over a manageable time scale, have lesions that are recognisably similar (but not necessarily identical) to those in humans, breed readily, and be relatively inexpensive. Of the animals mentioned, the mouse has proved itself as the most useful model due to its low cost, ease of husbandry, ability to manipulate the genome easily, and its rapid breeding.

DIET-INDUCED ATHEROSCLEROSIS IN MICE

According to Maeda [47], researchers working in the field of lipoproteins and atherosclerosis were sceptical at first about the use of the mouse as a model of atherosclerosis for two main reasons. The first was that mice are actually resistant to atherosclerosis, possibly due to their plasma lipoprotein profile being protective, i.e. high in HDL and low in VLDL/LDL. The second reason was that mice have a short life span of 2-3 years, which was thought to be too short to develop atherosclerotic plaques. These issues have both been overcome by genetic and dietary manipulation. In the 1960s, a diet-induced mouse model was developed by feeding the C57BL/6 strain a diet containing 30% fat, 5% cholesterol, and 2% cholic acid, (as opposed to a normal chow diet containing ~3.5% crude fat), leading to the development of atherosclerosis. The downside to this diet was that it was highly toxic and the mice lost weight, and tended to develop morbid respiratory infections [48]. This diet was subsequently modified to contain 15% fat, 1.25% cholesterol and 0.5% sodium cholate (the 'Paigen' diet) [49], and provoked lesion development without the toxic side effects. This diet, which contains 10-20 times the proportion of cholesterol of a human diet, produces a sustained plasma cholesterol level of 5-8 mmol/L, with the increase in cholesterol in the non-HDL lipoprotein fractions. When fed a normal chow diet, cholesterol levels are around 1-2 mmol/L, mostly in the HDL fraction [50]. This atherogenic diet enabled research showing that the strain of mouse chosen can drastically affect the atherosclerotic response, providing strong evidence for genetic make-up as a risk factor for the disease. Before the advent of genetic manipulation, the inclusion of cholate was presumed to be essential for hyperlipidaemia-induced atherosclerosis, as it enhances hypercholesterolemia by down-

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regulating cholesterol-7-α-hydroxylase, reducing the conversion of cholesterol to cholic acid (bile acid). However, it may have toxic metabolic side effects that directly promote an inflammatory response, adding further complexity to elucidating the mechanisms behind atherosclerosis. Experiments comparing diets with and without cholate came to the conclusion that it is not necessary for the formation of atherosclerosis, and that supplementing the diet with cholesterol and saturated fat is sufficient for lesion development [51]. As such, it is omitted from the majority of modern atherosclerosis studies [52], though not all [53].

THE APOLIPOPROTEIN E KNOCKOUT MOUSE

Apolipoprotein E (ApoE) was discovered in the 1970s [54, 55] as a part of VLDL, some isotypes of HDL, chylomicrons and chylomicron remnants [56]. Type III hyperlipoproteinaemia in humans is a genetic disease that has long been known to be associated with a deficiency in ApoE that leads to an altered lipid profile and a subsequent increase in atherosclerosis [57, 58]. By 1992 this same principle was translated into the mouse by two distinct research teams, and the ApoE gene successfully knocked out (referred to as $ApoE^{-/-}$) [59, 60]. When fed a normal chow diet, this led to plasma cholesterol levels being elevated from 2 mmol/L in control animals to 12 mmol/L in the knockouts, and when fed a Western-type diet, cholesterol levels were 4 mmol/L and 45 mmol/L in controls and knockouts respectively. In the knockout animals, spontaneous atherosclerosis developed in the proximal aorta, even when fed a normal chow diet [59], and it was shown that lesions develop in the brachiocephalic artery that are consistently calcified, and contain chondrocyte-like cells. However, to facilitate this, much longer feeding periods (45-75 week) are necessary [61]. Deletion of ApoE does not appear to have any effect on fertility or birth weight when compared to wild-type mice. Although $ApoE^{-/-}$ mice have normal heart rate and blood pressure, they show increased pulse wave velocity, aortic and mitral flow velocity, pulse wave reflection (shown by alterations in aortic acceleration), heart-to-body weight ratio (decreased body weight accompanied by cardiac hypertrophy), and decreased haematocrit [62]. A study by Russell Ross's group [63] looked at the predilection sites of atherosclerosis throughout the whole vascular tree after feeding $ApoE^{-/-}$ mice a diet containing 21% fat and 0.15% cholesterol. They found that the first sites to develop lesions after ~8 weeks of fat-feeding included the aortic root, lesser curvature of the aortic arch, the branches of the right common carotid artery, the branches of the superior mesenteric artery, both renal arteries, the aortic bifurcation, and the pulmonary artery. Longer term feeding resulted in lesions in the descending thoracic aorta, lower abdominal aorta, and the proximal coronary, common iliac and femoral arteries. Control mice also on the Western diet did not develop lesions.

Using $ApoE^{-/-}$ mice fed a normal diet has enabled research to be done into the effects of immunological challenge on atherosclerosis. When infected with murine γ-herpesvirus-68 (MHV-68) at 3-4 weeks old, and fed a normal diet for 24 weeks, mice had increased aortic lesion coverage (assessed using a lipid stain, oil red O) compared to non-infected controls. The change was thought to be caused by initiation of endothelial injury, and the effect was reduced by treatment with an antiviral drug [64]. The same effect was seen in $ApoE^{-/-}$ mice infected orally with *Porphyromonas gingivalis* and kept on a high-fat diet for up to 34 weeks. At the end of the study period, the infected mice had increased lesion size, which the authors

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suggest is due to a chronic inflammatory response to the infection [2]. This is of importance because there is mounting evidence for a link between oral hygiene, *P. gingivalis*-mediated periodontal disease and heart disease in humans [65, 66].

VULNERABLE LESIONS IN THE APOE KNOCKOUT MOUSE

The brachiocephalic artery, also called the innominate artery, is a short (~1.5 mm) vessel connecting the top of the aortic arch to the right carotid and right subclavian arteries (Figure 1a). Ultrasound measurements in ApoE$^{-/-}$ mice show that it has a diameter of 0.5 to 0.7 mm (diastolic and systolic dimensions respectively) (unpublished data,) and it develops advanced lesions on the right lateral wall (Figure 1b).

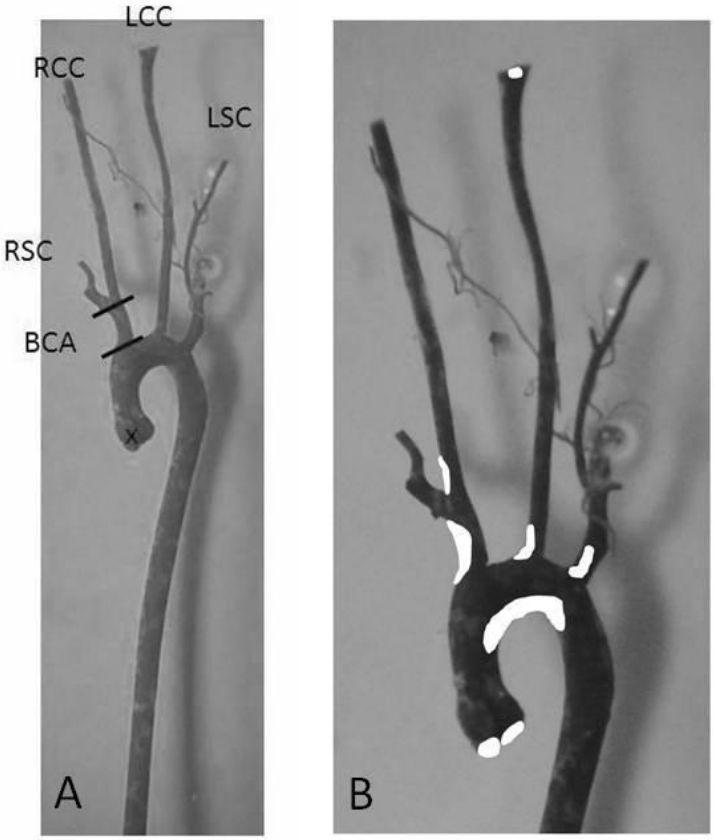

Figure 1. a) Resin cast of murine aortic arch and associated branches. BCA, Brachiocephalic artery; RSC, Right subclavian artery; RCC, Right common carotid artery; LCC, Left common carotid artery; LSC, Left subclavian artery; x, Aortic Root. b) Approximate location of atherosclerotic lesions (white) in murine aortic arch and associated branches.

ApoE$^{-/-}$ mice aged 42-54 weeks, fed a normal chow diet, develop lesions in the brachiocephalic artery that show loss of fibrous cap tissue, intraplaque haemorrhage and fibrotic conversion of necrotic zones [67]. However, despite fibrous cap thinning and the presence of intraplaque haemorrhage, the authors claimed that the plaques did not rupture.

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The findings of an acellular necrotic core through to the lumen may suggest a resemblance to plaque erosion, defined as loss of endothelium, leading to thrombus formation, without any demonstrable fissures or rupture [68]. In 2001, having fed a modification of the Paigen diet containing 21% pork lard and 0.15% cholesterol for up to 14 months, complex lesions were seen in the brachiocephalic artery with clear evidence of plaque ruptures [69]. Serial sectioning along the vessel consistently showed ruptures of up to 60 μm in length, with intraplaque haemorrhage as evidenced by the presence of erythrocytes within the lesion. There was also tight anatomical localisation, with ruptures occurring within the proximal 150 μm of the vessel, enabling comparison across studies [70]. Examples of a stable plaque from an $ApoE^{-/-}$ mouse fed a high-fat diet for 6 weeks, and an unstable plaque with evidence of an acute rupture from a similar mouse fed a high-fat diet for 9 months, are shown in Figures 2a and 2b respectively.

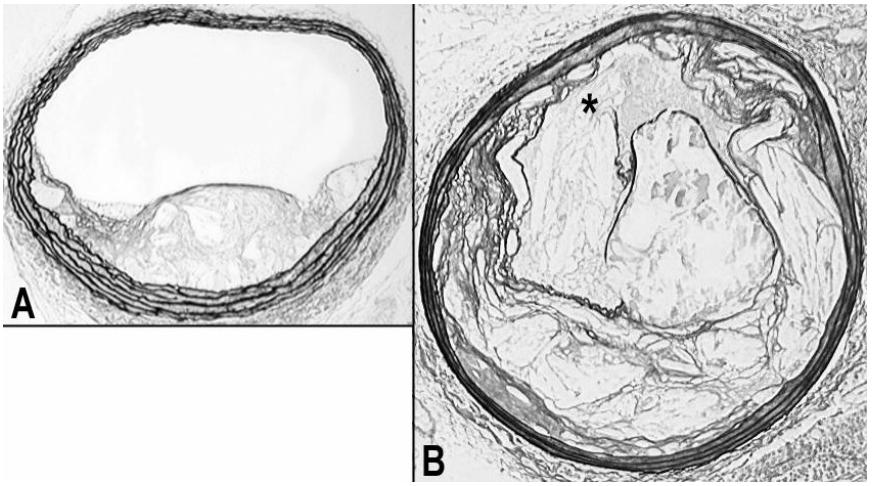

Figure 2. Stable (A) and unstable (B) murine atherosclerotic plaques in the brachiocephalic arteries of male apolipoprotein E knockout mice fed a high-fat diet for up to 9 months. Rupture site indicated with an asterix.

A mouse plaque rupture can be defined as a disruption of the fibrous cap accompanied by intrusion of red blood cells into the plaque itself [71]. This definition enables distinction between true plaque ruptures and damage caused during histological processing, as erythrocytes could only infiltrate the plaque during life when the blood is circulating. It should be noted that this definition is different to that put forward by Virmani [21] for human ruptures, by omitting the requirement that luminal thrombosis be present. Superimposed thrombosis is rarely seen in $ApoE^{-/-}$ mice [72]. One of the main reasons for this is that mice have a very different fibrinolytic system to humans, enabling any thrombi that form to be rapidly degraded and cleared from the blood vessels. Plasma levels of plasminogen activator inhibitor (PAI-1) are 5- to 12.5-fold lower in mice than humans, whereas fibrinogen and tissue-type plasminogen activator (tPA) concentrations are similar [73]. This suggests that the fibrinolytic balance in mice is shifted more towards enhanced lysis. The volume and surface area of a thrombus over a human plaque would be roughly 200-fold and 30-fold greater respectively than in a mouse. Some human coronary thrombi may be present for months [74], so even if we conservatively assume equal rates of fibrinolysis then mouse thrombi will be

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resolved within a few days. This suggests that there is only a relatively small time window after a mouse plaque rupture during which a thrombus will be detectable. It has been observed that the time window to observe acute plaque ruptures is around the 8 weeks of fat-feeding time point [75]. However, it is possible to see evidence of previous plaque ruptures, so-called buried fibrous caps or layers, as smooth muscle cell-rich layers (as shown by α-smooth muscle actin-positive cells), with the incorporation of elastin and fibrin, and usually overlain with foam cells (Figure 3). This striated appearance has been seen in both human [76, 77] and mouse [71] plaques.

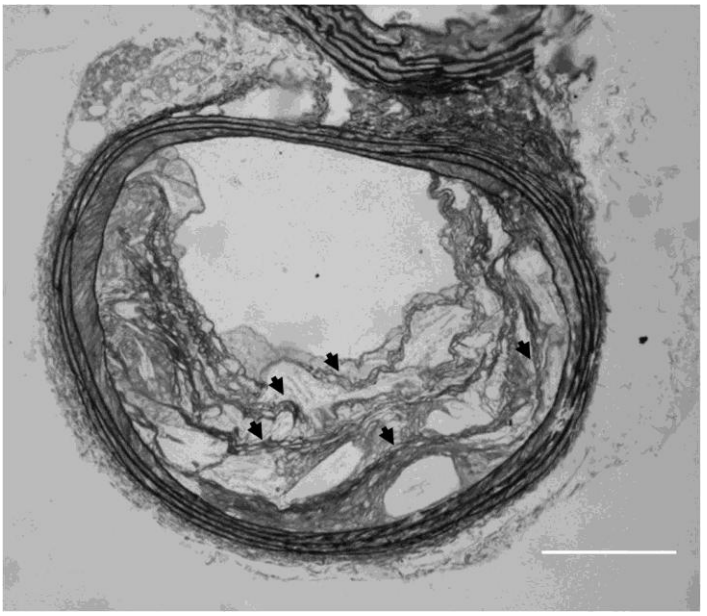

Figure 3. Buried fibrous caps (arrowheads) in a brachiocephalic artery of an $ApoE^{-/-}$ mouse fat-fed for 24 weeks. Scale bar = 200μm.

The issue of buried fibrous caps is a contentious one. It has been suggested that the buried layers are nothing more than layering of the plaque resulting from episodic growth. If this were the case it follows that larger plaques would have an increased number of buried layers, and would not be dependent on the presence of plaque ruptures. The early studies involving diet-induced atherosclerosis noted that the aortic sinus is particularly susceptible to atherosclerosis [78], and this site was originally investigated a great deal more than the brachiocephalic artery. This may have been due to the ease of locating it for histological processing and subsequent comparison between laboratories. Lesions at this site in the $ApoE^{-/-}$ mouse are large and lipid-rich; however, they remain as fatty streaks for an extended period, it is months before the fibrous cap can be discerned [79], and there is no evidence of intraplaque haemorrhage even after extended periods of fat-feeding [75]. To our knowledge, plaque ruptures have not been observed in the retrovalvular lesions of the aortic sinus of $ApoE^{-/-}$ mice, yet the lesions that are found in this region are approximately twice the size of the lesions found in the brachiocephalic arteries of the same mice after 9 months of fat-feeding, thus suggesting that the incidence of plaque rupture is significantly related to the presence of buried layers, but not to plaque area. The lack of rupture in the aortic sinus, even

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when plaque ruptures are present in the same mice in the brachiocephalic artery, suggests that there is some form of protection in the sinus, be it oscillatory blood flow caused by movement of the aortic valves, or the mechanical properties of the retrovalvular vessel wall. This suggests that the sinus, if used at all in plaque rupture studies, should be used as a non-vulnerable comparator site for other vulnerable parts of the arterial tree.

The ways in which the $ApoE^{-/-}$ mouse model is useful in the study of atherosclerotic plaque rupture can be outlined as follows:

1. It is possible to carry out longitudinal studies up to the point of rupture and observe the processes up to (and beyond) this clinically significant event;
2. Mice can be culled at set time points and all tissue can be handled promptly and in a similar manner to minimise changes in tissue composition;
3. It is possible to carry out temporal histopathological analysis and cohort-based between-subject analysis;
4. It is possible to experimentally verify findings as one can go back and manipulate, or modify, one or more factors that may be involved.

ANALYSIS OF LESIONS

A method that has been used for decades, commonly quoted as a marker of the location and surface area of lesions throughout the vasculature tree, is staining with the lipid stain oil red O [80, 81]. The usual, and perhaps most reproducible, method for measuring vessel morphological parameters and atherosclerotic lesion size in the smaller vessels e.g. brachiocephalic artery, carotids, and coronary arteries, is to look at cross-sectional parameters of the blood vessels, using image analysis software. The usual cross-sectional parameters measured are:

- the length of the external elastic lamina (EEL)
- the length of the internal elastic lamina (IEL)
- the area of the plaque (P)

From these parameters it is possible to calculate:

- the total vessel area $EEL^2/4\pi$
- the medial area $(EEL^2\text{-}IEL^2)/4\pi$
- the lumen area $(IEL^2/4\pi)\text{-}P$

These calculations assume that the vessel is perfectly circular in life, and that it has been sectioned at exactly 90º to its long axis. If necessary, serial sections can be taken throughout a blood vessel to see how lesion and vessel sizes change.

Using elastin-stained sections, features of plaque stability can be assessed. These features are buried fibrous caps and breaks in the fibrous cap overlying the lesions, representing previous healed plaque ruptures, and an acute plaque rupture that has not yet healed, respectively. In the latter case, in order to exclude those ‘ruptures’ caused by histological

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processing, the presence of erythrocytes needs to be observed within the lesion, below the rupture, as evidence of a rupture during life [82].

The gold standard imaging technique would be one whereby lesions would be imaged at high enough resolution, whilst still *in situ*, to be able to see the components of the whole plaque, and whether ruptures have occurred. This is currently not possible *in vivo*. However, techniques have been developed, such as optical projection tomography (OPT), that have enabled *ex vivo* tissue to be observed without the need to cut open the vessel. OPT enables a 3-dimensional representation of the vessel and lesions to be produced, enabling quantification of vascular and lesion morphology, without the need for labour-intensive histological sectioning [83].

BLOOD VESSEL REMODELLING

Throughout their lifetimes, blood vessels are not simply static tubes, but highly dynamic, adapting to their local environment as part of a homeostatically-regulated mechanism. It has been shown that the stress and strain imposed on an artery are the main stimuli for growth and remodelling [84] and that blood vessels remodel in response to changes in the fluid shear stress, the so-called constant wall shear stress hypothesis. This is demonstrated nicely when looking at human saphenous vein grafts whereby a section of vein is removed and grafted in the place of an artery. Once exposed to the arterial flow and pressure the vein 'arterialises' (i.e. the wall thickens), atherosclerosis can develop and is the main pathology that can lead to stenosis and occlusion of the graft [85]. The first outward, or expansive, remodelling was observed and thought to be a compensatory response to increasing lesion burden in coronary [86] and femoral [87] arteries of non-human primates, and subsequently in coronary arteries of humans [88]. This so-called "Glagov's phenomenon" prevents the formation of a stenosis until the lesion occupies ~40% of the area within the internal elastic lamina of the vessels. After this critical point the vessel cannot outwardly remodel further and the lesion will start to encroach upon the lumen, decreasing blood flow through the vessel. However, it has also been observed that in some cases the lumen can decrease with no apparent changes to the lesion size (inward remodelling), suggesting that the arterial wall itself actually shrinks, decreasing the cross-sectional lumen area [89, 90].

Using the $ApoE^{-/-}$ mouse model it has been observed that plaque rupture and vessel remodelling are perhaps part of a homeostatic mechanism that maintains the lumen, and enables blood to carry on flowing along the vessel. A study by Jackson [70] showed that feeding a high-fat diet for a year results in expansive vessel remodelling that occurs alongside an increase in plaque area, as is the consensus opinion. This is not simply an age-related process, as strain-matched, wild-type mice fed the same diet did not develop atherosclerosis, and did not exhibit any expansive remodelling. When plaques from $ApoE^{-/-}$ mice are divided up based on their vulnerability (stable vs. unstable), the story becomes more complex. Stable plaques increased in area, but the vessels did not expansively remodel: unstable plaques showed the same increase in plaque area but vessel area did increase. Therefore, plaque growth itself cannot be causing the increased rate of vessel expansion in the brachiocephalic artery, supporting the finding that there is vessel expansion even in the absence of plaque. This raises the question as to whether plaque rupture is causing expansive remodelling, or

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whether expansive remodelling is causing the rupture. The finding that even in the absence of plaques remodelling still occurs supports the latter explanation. When the vessel expansively remodels, the fibrous caps of plaques are placed under tension and when this force overcomes the physical cap strength a rupture ensues. One hypothesis is that the actual rupture event, if it were not for the potentially detrimental thrombotic consequences, could be seen as beneficial to the patient as it allows the lumen to be restored and maintained, and this could explain why plaque rupture can be asymptomatic. This has also been seen in humans using IVUS; outward remodelling was seen in all the vessels containing lesions, and ruptured plaques had more compensatory enlargement (66%) than non-ruptured plaques (48%) [91]. The effect of outward remodelling is so important it has been defined as a significant independent predictor of major adverse cardiac events in unstable angina patients [92].

Further evidence for this controversial hypothesis can be found in studies using 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins (e.g. pravastatin, simvastatin, atorvastatin, rosuvastatin). HMG-CoA reductase is the rate-limiting enzyme in endogenous cholesterol biosynthesis, and catalyses the reduction of HMG-CoA to mevalonic acid. Statins are used to lower plasma cholesterol levels in humans and have been shown to cause regression of atheroma [93]. A recent clinical study showed that patients that received statins before the onset of ST-elevation myocardial infarction had a lower incidence of plaque rupture, implying stabilising effects [94]. This potential effect on plaque stability in humans led to statins being investigated in mice. Treatment with pravastatin at a dose of 40 mg/kg of bodyweight/day in the drinking water of $ApoE^{-/-}$ mice, started at the same time as high-fat feeding (6 weeks of age), showed that atherogenesis could be impaired at plasma levels of the drug similar to those seen in humans. When pravastatin treatment started after 16 weeks of high-fat feeding had elapsed, in other words when lesions were fully established and unstable plaques had developed, there was no effect on plaque size but there was a reduced lipid content (as would be expected from the lipid-lowering effects) and a 5-fold increase in fibrous cap thickness, leading to a decreased rupture incidence [75]. There was no effect on the rate of vessel expansion, but the amount of remodelling was significantly increased [70], suggesting that thickening the fibrous cap increases its structural integrity, and prevents it from being torn apart. Similar stabilising effects have also been shown in WHHL rabbits given pravastatin for 52 weeks (starting at 10 months old) at a sufficient dose to cause a 25% decrease in plasma cholesterol [30]. They showed that after this period the lipid components (macrophages and/or extracellular lipid deposits) and vulnerability index were both decreased, implying plaque stabilising effects.

COMPUTATIONAL ANALYSIS OF PLAQUE VULNERABILITY

With the advent of higher power computers, computational fluid dynamics (CFD) and stress analysis are being carried out in parallel to *in vivo* models to try and determine the mechanical forces present in the atherosclerotic plaque. CFD used to rely on very simplified geometries, such as 90 degree branching angles, and diameters of branches being simple ratios of the parent vessel, to determine flow patterns [95]. Utilising animal models, such as the $ApoE^{-/-}$ mouse, has enabled vasculature casts such as that in Figure 1a to be made, and scanned using microCT to produce 3-dimensional models of the blood vessels *in silico* that

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have real-life dimensions, branching angles and luminal stenoses (caused by lesions). Using techniques such as Doppler ultrasound on conscious mice, real inflow and outflow velocities can be determined and inputted into the model [96]. This enables changes in the flow field to be mapped throughout the vasculature [97].

Computer modelling also enables us to look at various parameters of the atherosclerotic plaque to try to determine which structures are important with regard to plaque vulnerability. From the literature, using computer simulations, it appears that the following have an effect on plaque stability and shall be discussed further below:

- Lumen shape
- Fibrous cap thickness
- Stenosis
- Necrotic core thickness
- Vessel remodelling
- Microcalcification
- Peak circumferential stresses

The lumen of the vessel can be modelled as either circular or elliptical (major to minor axis ratio of more than 1:1) in shape. The work of Kumar and Balakrishnan [98] showed that by varying these parameters, and also modelling the lesion with varying sizes and shapes of lipid core, they could alter the haemodynamic stresses found in the vessel. In a circular vessel, without any lesion, they predicted that the haemodynamic stresses would be uniform around the wall. However, if the vessel is elliptical, as is usually the case *in vivo*, the stresses were redistributed along the major axis and decreased along the minor axis. Addition of a lesion to the model caused an increase in stresses along the major axis and shoulder region of the plaque, and the presence of a lipid pool increased the stresses on the cap further. Research using statins, as has previously been described, suggests that increasing fibrous cap thickness leads to less chance of the cap rupturing. Simulations of carotid arteries back up this finding. Li et al [99] showed that flow through arteries with a ≥70% stenosis leads to a high risk of plaque rupture (defined as a rupture stress of 300 kPa) at various fibrous cap thicknesses, with a 50% decrease in thickness resulting in a 200% increase in maximum plaque stress [100]. Plaque stresses in arteries with 30% to 70% stenosis increased exponentially as fibrous cap thickness decreased. Adding further weight to the argument that expansive remodelling tears the fibrous cap open is the work of Ohayon et al [101], who found that in the early stages of expansive remodelling the lesions were more prone to rupture, which they suggest could promote the progression and growth of clinically silent plaques. They also showed that in addition to fibrous cap thickness, the thickness of the necrotic core, rather than its area, was crucial in determining plaque stability. Microcalcifications in the fibrous cap are thought to be made up primarily of hydroxyapatite (calcium phosphate) and to form from macrophages that have become calcified. It has been shown that microcalcifications cause an increase in the circumferential stress in the fibrous tissue of the cap, and the highest regions of stress are found wherever the microcalcifications are located, be it central or shoulder regions [102]. The authors suggest that the consequence of this shift in stresses could be the reason that 40% of ruptures are not located at the shoulder regions as would be predicted by other models. Recent work by Vengrenyuk et al [103] could provide an explanation as to why plaque

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ruptures are never found in the aortic sinus, but are found in the brachiocephalic artery. They modelled the stresses found in aortic lesions compared to brachiocephalic artery lesions, and predicted that the average peak stress in the fibrous cap of aortic lesions would be ~2.5-fold lower. They suggest that aortic plaque stresses depend only slightly on cap thickness, whereas the stresses in the brachiocephalic artery increase exponentially with decreasing cap thickness.

CONCLUSION

Atherosclerosis, and in particular atherosclerotic plaque rupture, is an extremely important area of research due to the potentially devastating clinical consequences of thrombosis and embolism. Whilst analysis of post mortem tissue and imaging of cardiac patients provide us with clues as to the processes that occur after a plaque rupture, or the later progression of lesions, it is only with longitudinal studies in animals that we can determine the events leading up to the rupture event. The fat-fed $ApoE^{-/-}$ mouse model of plaque rupture is an extremely valuable resource for looking into the disease, but it is only when used in concert with other techniques available, be they *in vivo*, *in vitro*, or *in silico*, that therapeutic treatments will become available to combat plaque rupture.

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Chapter IV

ATHEROSCLEROSIS: NOVEL RISK FACTORS AND TREATMENT

Dimitris Tousoulis*[*,†], *Emmanuel Androulakis*[†], *Nikolaos Papageorgiou, George Latsios, Anna-Maria Kampoli and Christodoulos Stefanadis
1st Cardiology Unit, Hippokration Hospital, Athens
University Medical School, Greece

ABSTRACT

Atherosclerotic lesions initiate at bifurcations of arteries, regions characterized by low shear stress and reduced activity of endothelial atheroprotective molecules such as nitric oxide and grow towards high shear stress areas. Extensive epidemiological research has established that major, traditional risk factors, such as gender, age, cigarette smoking, family history, diabetes, hyperlipidemia, and hypertension, significantly contribute to the development of atherosclerosis as independent risk factors However, these so called classical risk factors not always explain the initiation and the progression of atherosclerosis. Therefore, novel risk factors have been proposed such as inflammatory biomarkers, lipoprotein (a), infections, triglyceride-rich remnants, homocysteine, thrombotic and haemostatic factors. As regards the treatment of these novel risk factors several drug categories have been proposed, such as angiotensin converting enzyme inhibitors/angiotensin receptors blockers, statins, along with a variety of other agents.

Keywords: Atherosclerosis, risk factors, treatment

[*] Address of correspondence: Prof Dimitris Tousoulis MD, PhD, FACC. 1st Cardiology Unit, Hippokration Hospital, Athens University Medical School, Athens, Greece. Tel: +30-210-7782446. Fax: +30-210-7784590. Email: drtousoulis@hotmail.com. Equally contributed.
[†] Equally contributed.

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INTRODUCTION

Atherosclerosis is a chronic disease affecting the entire arterial tree, in large part, representing an inflammatory response in the vessels [1, 2]. The pathogenesis of atherosclerosis involves complex processes leading to the formation of atherosclerotic plaque [3].

Injury to the endothelial cell of the artery, resulting in endothelial dysfunction (ED), is now considered an important early event in the development of atherosclerosis. Also, risk factors for atherosclerosis represent crucial factors associated with ED [4], which seem to precede atherosclerotic lesions in both conduit and resistance coronary vessels, and even occur in offspring with a positive history for cardiovascular disease [5, 6]. During the initial steps of this process, activated endothelial cells attract several cellular components which produce an excessive amount of connective tissue matrix, leading to the formation of a mature fibrous plaque [7].

A growing number of evidence has suggested that intervention programs are effective in the reduction of traditional and newer risk factors and has enlightened the importance of prevention [8]. Indeed, improving population-level risk factors has been clearly associated with a decline in CVD death rates. Other approaches, such as guidelines compliance, classical and novel treatments through various pharmacologic and non-pharmacological modalities, will be necessary to lower the present high risk burden [9].

In the present chapter we will discuss recent data regarding the pathophysiology of atherosclerosis, and the impact of classical and novel risk factor in the initiation and progression of atherosclerosis. Moreover, we will explain the classical and novel therapeutic modalities.

PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

It is widely known that atherosclerosis is a disease of large and medium-sized arteries, which mainly is characterized by endothelial dysfunction and vascular inflammation, whereas coronary artery disease (CAD), has its origins early in life and progresses eventually to the formation of atherosclerotic plaques [10].

Over the last decade, vascular endothelium has been emerged mostly as a paracrine organ responsible for the secretion of several beneficial substances possessing anti-atherogenic effects. Deceased endothelial function, as a result of decreased nitric oxide (NO) bioavailability plays crucial role in the initiation and progress of atherosclerosis. Traditional risk factors such as hyperlipidemia, diabetes mellitus, hypertension, smoking, as well as novel ones represent important factors predisposing to ED [31, 32].

More specifically, oxidative modification of low density lipoproteins (LDL) in the arterial wall by reactive oxygen species (ROS) leads to atherosclersclerosis and classical risk factors increase the production of free ROS from the endothelial, smooth muscle and adventitial cells [11]. Moreover, mounting data suggest that impaired endothelial function participates in the pathogenesis of atherosclerosis [12];experimental data indicate that prevention of NO-ROS reaction leads to the withdrawal of NO-mediated vascular beneficial effects and to the formation of free radicals with proatherogenic properties [13]. Undoubtedly,

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loss of the functional integrity of the endothelium, and thus, its anti-atherogenic effects play a major role in all stages of atherosclerosis from plaque formation to rupture [14, 15] (Figure 1).

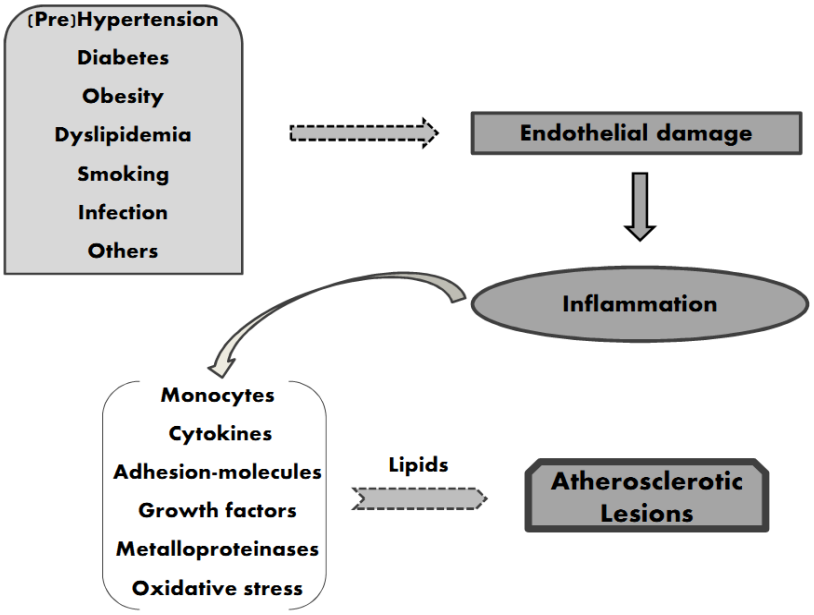

Figure 1. Pathophysiological mechanisms contributing to atherosclerosis.

Hyperlipidemia

Atherosclerotic lesions initiate at bifurcations of arteries, regions characterized by low shear stress and reduced activity of endothelial atheroprotective molecules such as NO and grow towards high shear stress areas [16]. Also, the close relation between hyperlipidemia and/or abnormal lipoprotein metabolism and atherosclerosis has been established since many years, and lipid-laden macrophage or foam cell is recognized as a hallmark of the disease. It has been established that one of the important modifications of LDL which contributed to its recognition by the scavenger LDL receptor on macrophages was oxidative modification [17]. In turn, oxidized LDL (Ox-LDL) was found to have various biological effects on the vessel wall, such as stimulation of cytokine, growth factor production and inhibition of endothelial cell vasodilator function [18, 19]. Obviously, these observations were of major importance, as they provided not only mechanistic links between lipoproteins and the cell biology of atherosclerosis, but they raised the concept that abnormalities of the oxidation-reduction state in the vasculature might be an important pathogenic mechanism in atherosclerosis.

Inflammation

Recently, it has become increasingly apparent that atherosclerosis is an inflammatory disease [20]. In more detail, leukocytes and endothelial cells secrete several factors promoting

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migration and proliferation of smooth muscle cells (SMCs), while macrophages and platelets potentially also contribute to proliferation of SMCs in the intima. Oxidized low-density lipoprotein cholesterol, which is entrapped in the intima and isolated from plasma anti-oxidants, can induce a local inflammatory response [21]. Indeed, regarding the role of lipoproteins, the low to high density lipoproteins ratio (LDL/HDL) has been shown to represent an index of atherosclerosis, and a predictor of coronary heart disease (CHD) [22, 23]. Moreover, chemokines, such as monocyte chemoattractant protein-1 (MCP-1), which are produced in response to modified lipoproteins, participate in the transendothelial migration of adherent monocytes [24, 25]. Subsequently, due to their interactions with endothelial cells (ECs) and the production of matrix metalloproteinase-9 (MMP-9), infiltration of leukocytes through the endothelial layer and basement membrane is allowed [26].

Under the influence of macrophage colony stimulating factor, monocytes mature into macrophages and accumulation of cholesteryl-esters in the cytoplasm converts macrophages into foam cells, which further induce and amplify the inflammatory response through the secretion of numerous growth factors and cytokines [21]. Accordingly, T cells enter the intima and lead T helper 1 responses via various cytokines and CD40 ligand (CD40L) [27]. Furthermore, it has been demonstrated that CD40/CD40L interaction supports mainly T helper lymphocyte 1 cytokine–driven inflammatory processes through several pathways, thereby establishing the immune reaction characteristic for atherogenesis [28]. Notably, cytokines and growth factors provoke SMCs' proliferation and expression of MMPs that degrade elastin and collagen, contributing to an expanding lesion and extracellular matrix destabilization [29]. The final consequence of the evolution of atherosclerosis is the formation of plaques with thin fibrous caps. Thinning of the fibrous cap is the consequence of increased collagen breakdown by MMPs and decreased collagen synthesis by dysfunctional SMCs induced mainly by proinflammatory cytokines. Plaques tend to rupture at sites of increased macrophage content, a fact which is indicative of the important contribution of these cells at the eventual events of atherosclerosis [30-32]. Apparently, the clinical presentations of these phenomena are acute coronary syndromes (ACS) [2, 21] and inflammatory response seems to be involved in many processes associated to atherosclerosis development to plaque rupture.

The Role of Oxidative Stress

It is well-known that impaired endothelial function precedes atherogenesis and is strongly correlated with several risk factors. Thus, endothelial dysfunction, which potentially is induced by oxidative stress, has been proposed as an important diagnostic and prognostic factor for coronary syndromes [33, 34]. Furthermore, oxidative stress links various coronary disease risk factors, such as elevated blood pressure, diabetes and cigarette smoking, contributing to vasoconstriction, thrombosis, plaque rupture and vascular remodeling [35]. Oxidative stress, stimulated by hypercholesterolemia and the production of superoxide anion radicals leads to oxidation of LDL (ox-LDL), which are more easily uptaken by macrophages compared to non-oxidized lipoproteins [36]. Indeed, oxidative stress via hydrogen peroxide (H_2O_2) increases phosphorylation of tyrosin kinases, which stimulate several inflammatory processes [37]. For instance, it affects atherogenesis through the production of transcription factors including nuclear factor-kB (NF-kB) and activator protein-1 (AP-1), which participate in the expression of adhesion molecules, such as vascular cellular adhesion molecules

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(VCAM-1), intracellular adhesion molecules (ICAM-1), E-selectin and other cytokines [38]. Thus, it seems that atherosclerosis is an inflammatory process strongly affected by oxidative stress however, direct evidence for the participation of LDL oxidation in its pathophysiology remains unclear [29].

CLASSIC RISK FACTORS

There is now general agreement that a number of factors commonly characterized as "risk factors" for atherosclerosis have been identified to facilitate the development of atherosclerosis via various mechanisms. Extensive epidemiological research has established that major, traditional risk factors, such as gender, age, cigarette smoking, family history, diabetes, hyperlipidemia, and hypertension significantly contribute to the development of CAD as independent risk factors [39, 40]. Additionally, a number of more recently identified factors have received intense investigation, which could further improve our ability to predict future risk when included along with classic risk factors (Table 1).

Table 1. Classic and novel risk factors for atherosclerosis

	Classic	Novel
Gender	Prehypertension	Lipoprotein(a)
Age	Metabolic syndrome	Homocysteine
Family history	Diabetes mellitus	Thrombogenic/Hemostatic Factors
Hypertension	Impaired glucose tolerance	Inflammatory biomarkers
Smoking	Insulin resistance	Infectious agents
Cholesterol/LDL	Hypertriglyceridemia	Triglyceride-rich remnant lipoproteins

LDL: low-density lipoprotein.

Lipid Factors

Considerable evidence has accumulated to implicate increased cholesterol levels as a major risk factor for atherosclerosis and CAD. Over the last decade it has been suggested that atherosclerosis begins during the early years of life and continues through the adulthood, while increased cholesterol levels and atherosclerosis are strongly associated [41, 42]. Important data also supports the prognostic value of hypertriglyceridemia as an independent risk factor for cardiovascular disease, as suggested by a meta-analysis of 17 prospective trials [43]. Of note, according to PROCAM study hypertriglyceridemia is related to CHD risk, independent of LDL-cholesterol and/or HDL-cholesterol levels [44]. Other lipids exhibit active inflammatory effects, such as high plasma levels of very low density lipoproteins (VLDL), which are associated with increased risk of atherosclerosis. In this regard, it has been demonstrtaed that VLDL activates NF-κB in cultured human endothelial cells [45].

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Metabolic Factors

Significant epidemiological evidence has shown that patients with diabetes mellitus (DM) and glucose intolerance are at increased risk for CHD, while the Framingham Heart Study have demonstrated an increased incidence of cardiovascular disease among men and women with DM [46]. A variety of mechanisms may contribute to the increased risk of developing atherosclerosis in diabetic patients. The abnormal cluster of hyperglycemia and insulin resistance, which characterize DM, acts synergistically to target the endothelial cell, leading to oxidative stress and endothelial dysfunction [47]. Subjects with insulin resistance have demonstrated increased risk for subclinical coronary atherosclerosis as those without [47].

The metabolic syndrome encompasses a range of cardiovascular risk factors and is closely associated with insulin resistance and increased risk of CHD [48]. Accordingly, obesity, as a consequence of sedentary lifestyle, conditioned by environmental and genetic factors, is a major risk factor for atherosclerosis and is associated with increased morbidity and mortality of cardiovascular diseases [49]. Even though the relationship between obesity and atherosclerotic diseases is widely accepted, the related mechanisms remain to be clarified. A growing body of literature have indicated that adipose tissue is an active endocrine organ that secretes various factors namely adipokines, interleukins (such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6)), chemokines (MCP-1) and hormones (leptin, resistin and adiponectin) [50-52]. Leptin is a protein that participates in fat metabolism and has been closely correlated with insulin resistance and other markers of the metabolic syndrome, independent of total adiposity. Thus, it seems logical to assume that it could be proposed as an independent risk factor for CHD [53].

NOVEL RISK FACTORS

Lipoprotein(a)

In the last decade, substantial data have been emerged in the assessment of cardiovascular risk and a variety of recently identified risk factors have received intense investigation. Lipoprotein(a) (Lp(a)), formed by joining a lipoprotein that is structurally similar to LDL, has been strongly associated with CHD. In regard to this issue, a meta-analysis of data available from prospective studies has shown that Lp(a) concentrations are higher in subjects who develop CHD compared to controls, although there is variation in the size of the effect [54]. In turn, the PRIME study, which included 9,133 French and Northern Irish men without manifest cardiovascular disease, confirmed this conclusion [55]. More specifically, Lp(a) increased the risk for myocardial infarction (MI) and angina pectoris, especially in men with a high LDL-cholesterol level and of note, a significant interaction between Lp(a) and LDL-cholesterol levels has been observed. It is also worth-mentioning that elevated levels of Lp(a) have been observed to predict endothelial dysfunction in normocholesterolemic and non-diabetic subjects [56]. Given important data from epidemiologic, in vitro, animal, and genetic epidemiologic studies Lp(a) and ischemic heart disease may even be linked with causal relationships, however randomized clinical trials are presently lacking [57].

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Triglyceride-Rich Remnant Lipoproteins

Considerable amount of interest has been focused on smaller VLDL, intermediate-density lipoprotein (IDL), and LDL particles which have the ability to enter the subintimal space [46]. According to Hodis et al[58], triglyceride-rich lipoproteins play an important role in progression of lesions <50% diameter stenosis in subjects treated with low-cholesterol diet and lovastatin 80 mg/dL. Of note, a prospective, nested case-control study (CARE trial) has demonstrated that plasma concentrations of VLDL particles and apolipoprotein (apo) CIII in VLDL and LDL are more specific measures of coronary heart disease risk than plasma triglycerides [59]. Apolipoprotein (apo) C-III and apoE are components of two major classes of plasma lipoproteins. When compared the distribution of these two apolipoproteins in survivors of MI and control subjects, it was observed that the distribution of apoC-III among lipoproteins may play a role in the susceptibility of the atherogenic process [60].

Homocysteine

Homocysteine has also been implicated as a factor in atherosclerosis and vascular disease, however, it remains elusive the exact mechanism by which impaired homocysteine metabolism could contribute to increased CHD and/or thrombotic risk [61]. It has been previously suggested that homocysteine may be associated with several other risk factors, including cigarette smoking, diabetes, obesity, and hypertension. Potentially, it participates in direct effects on endothelial cells in part via oxidative stress, it also leads to asymmetric dimethylarginine (ADMA) accumulation and to endothelial dysfunction, while folate supplementation may decrease these effects [62, 63]. According to a meta-analysis of 27 studies relating homocysteine to arteriosclerotic vascular disease, elevations in homocysteine levels were considered an independent graded risk factor for arteriosclerotic vascular diseases. The odds ratio (OR) for CAD of a 5-mumol/L homocysteine increment was 1.6 for men and 1.8 for women, while total of 10% of the population's CAD risk appeared attributable to homocysteine [64].

Thrombogenic/Hemostatic Factors

Several data have highlighted the importance of fibrinogen levels as risk factor for atherosclerotic vascular disease. According to prospective epidemiological studies and clinical observations, fibrinogen is correlated with CAD, though with uncertain causal factors [65]. A recant meta-analysis of the Fibrinogen Studies Collaboration [66] reported that the age-and sex-adjusted hazard ratio for 1 g/L increase of fibrinogen levels for CAD was 2.42, stroke 2.06, and other vascular mortality 2.76, Additional information related to the potency of fibrinogen in predicting future CVD have been reported by several other data. This has been nicely showed by Panagiotakos et al[67] who evaluated the prognostic significance of several risk factors on the outcome of CAD in 639 cardiovascular disease-free subjects with familial hypercholesterolemia, during a 15-year follow-up. They have shown that fibrinogen levels are among the strong predictors of CHD.

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Other factors that have been investigated for their potential role in atherogenesis include von Willebrand factor and plasminogen activator inhibitor-1 (PAI-1) [46]. According to the Northwick Park Heart Study, fibrinolytic activity was measured in 1,382 white men aged 40-64, of whom 179 subsequently experienced episodes of ischaemic heart disease during a mean follow-up period of 16.1 years. There has been observed a significant correlation between low fibrinolytic activity and the risk for CAD ($p = 0.02$) in those aged 40-54 at entry [68]. In the Physicians' Health Study, elevated levels of D-dimer have been associated with increased risks of future MI. Even though they do not appear to be an independent predictor when other risk factors are considered, this study has suggested that activation of the endogenous fibrinolytic system occurs many years in advance of CAD [69]. Also, in a prospective multicenter study of 3,043 patients with angina pectoris followed for 2 years (ECAT study), an increased incidence of events was associated with higher baseline concentrations of tissue plasminogen activator (TPA) antigen, PAI-1 activity and PAI-1 antigen, however the underlying mechanisms have not yet been explored [70]. Moreover, platelet aggregation may be related to the risk of atherosclerotic cardiovascular events. Data from the Caerphilly Collaborative Heart Disease Study, based on a large cohort of men (2,398) have demonstrated that there is a twofold increase in the odds of a past MI in subjects of the highest platelet aggregation [71].

Infections

Infections and systemic inflammation have been suggested to play crucial role in the initiation and evolution of CAD [72]. It is well-known that immune cells, predominantly macrophages and T lymphocytes, are present in atherosclerotic lesions however other types of inflammatory cells, such as mast cells, neutrophils and dendritic may also participate [73]. Many seroepidemiology studies have indicated a potential association of infection in the pathophysiology of ischemic heart disease and atherosclerosis. Of note, Cytomegalovirus and Helicobacter pylori have been associated with atherosclerotic lesions, whereas Chlamydia pneumonia seems to be most likely involved in coronary disease through various mechanisms [74-76]. In particular, it has been supported the hypothesis that intracellular infection with Chlamydia pneumoniae may relate to the severity of atherosclerosis in some subjects [75]. Further, recent studies have suggested that dental status and bacterial infections may also be clinically relevant. A growing body of literature has emerged regarding the relationship of periodontitis with classic risk factors and atherosclerosis/coronary heart disease and has even implied that the criteria for causality may be met in the near future [77, 78]. Interleukin-18 (IL-18), for example, could explain the potential association between previous infection and cardiovascular events which induces the production of interferon-γ and T-lymphocytes, found in atherosclerotic lesions [79].

Inflammatory Biomarkers

As result of chronic inflammation, several biomarkers such as C-reactive protein (CRP), IL-6 and -18, TNF-α, ICAM-1 and E-selectin are increased in plasma and may serve as possible risk factors for atherosclerosis [80, 81]. In particular, a growing body of evidence has

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focused on the predictive value of CRP in subjects both with and without cardiovascular disease [81, 82]. It has been shown that high sensitivity CRP (hs-CRP) could be a risk factor independent of traditional risk factors, such as total dyslipidaemia, age, smoking, body mass index, and blood pressure [83]. In the Cardiovascular Health Study, an observational study which included 5,888 subjects, followed up for as long as 12 years for CVD incidence, elevations of CRP levels (> 3 mg/L) were associated with increased risk for CVD death (72%) and all-cause mortality (52%). Importantly, increased levels of hs-CRP have independently predicted advanced carotid plaques in dyslipidemic subjects and early-onset carotid atherosclerosis, as indicated by increased intima-media thickness, has been associated with elevated serum levels of CRP and other inflammatory markers [84-87]. In a prospective study of 1,127 patients with documented CAD, matrix metalloproteinase-9 (MMP-9) concentrations have been also identified as a novel predictor of cardiovascular mortality in patients with CAD, even though this notion requires additional assessment [88]. Specifically, median concentrations of MMP-9 were significantly higher among patients who experienced a fatal cardiovascular event than controls (62.2 versus 47.8 ng/mL, $P<0.0001$) and after adjustment for clinical and therapeutic confounders, hazard risk ratio was 1.3 (95% CI, 1.1 to 1.6; $P=0.005$).

In another large, prospective study with a follow-up period of 3.9 years, serum IL-18 level was identified as a strong independent predictor of CVD death in patients with CAD regardless of the clinical status at admission, which is in line with the concept of IL-18-mediated inflammation leading to acceleration and vulnerability of atherosclerotic plaques [89]. This cytokine has recently been detected in human plaques, while its administration was associated with increased atherosclerosis in experimental models. Recently, Rosso et al[90] have indicated that patients with stable and unstable angina exhibit higher serum levels of IL-18 compared to controls. However, levels of IL-18 did not differ significantly between patients with stable and unstable angina and it was observed no evidence of association with the progression towards plaque instability [90].

TREATMENT STRATEGIES

The goals of therapy of atherosclerosis and its clinical manifestations are supposed to include arresting or even reversing its progression. Recent clinical studies have tested various pharmacologic and non-pharmacological modalities which demonstrated great success in the treatment of patients with atherosclerosis, though patients are often undertreated of the underlying disease.

Antihypertensive Agents

Current anti-hypertensive strategies using angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs) and beta-blockers seem to exert beneficial effects, beyond their blood-lowering properties, on risk factors and atherosclerosis, according to clinical and experimental data. Since the renin–angiotensin–aldosterone system (RAAS) participates in vascular inflammatory responses, these agents may delay inflammatory

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processes and interfere with atherosclerosis [91, 92]. According to several clinical studies ACE-inhibition have improved endothelial function in several disease states, such as hypertension, DM and CAD [93-95]. It has also been demonstrated that ramipril significantly reduces cardiovascular disease and may prevent the progression of atherosclerosis, however, data are inconsistent [96, 97]. Moreover, several prospective, randomized studies have demonstrated vascular benefits with the ARB olmesartan. Specifically, it has exhibited positive properties regarding the reduction of progression of coronary atherosclerosis in patients with stable angina pectoris (OLIVUS), the decrease of inflammatory biomarkers in hypertension (EUTOPIA) and the improvement of IMT and plaque volume in patients with diagnosed atherosclerosis [92]. In addition, third-generation beta-blockers, among the most widely used drugs in the prevention and treatment of hypertension, have been shown not only to avoid increased peripheral resistance, but also to induce vasodilation via various mechanisms [98]. In particular, nebivolol has been associated with improvement of endothelial function, increased cellular superoxide dismutase levels, and beneficial changes of the levels of dimethylarginine [99].

Table 2. Studies assessing statins as pharmacological intervention in atherosclerotic lesions and ACS

Study	Population	Type of study	Results
Nohara et al [105]	348 patients with hypercholesterolemia	Prospective	Rosuvastatin significantly delayed progression of carotid IMT at 12 months
Kovarnik et al [106]	89 patients with stable angina	Prospective	Dual lipid-lowering therapy decreased the percent atheroma volume and increased lumen volume
Briel et al [109]	13,024 patients with ACS	Meta-analysis	Initiation of statin therapy within 14 days after ACS does not reduce death, MI, or stroke up to 4 months
PROVE IT-TIMI 22 [145]	4,162 patients with ACS	Prospective	Reduced primary composite endpoint of UA, CABG, AMI and death
A to Z [146]	4,497 patients post ACS	Prospective	No significant difference combined primary outcome of nonfatal AMI, CV death within first 4 months. After 4 months reduced primary outcome in the high simvastatin dose group
Spencer et al [111]	13,871 patients with non-STEMI receiving statins before hospital admission	Retrospective	Withdrawal of statin therapy in the first 24 hours of hospitalization was accompanied by increased hospital morbidity and mortality

ACS: acute coronary syndromes; IMT: intima media thickness; AMI: acute myocardial infarction; Non-STEMI: non-ST-segment elevation myocardial infarction; CV: cardiovascular diseases; UA: unstable angina; CABG: coronary artery bypass grafting.

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Statins

There is convincing evidence that statins exhibit additive vascular protective properties beyond lipid lowering in cardiovascular diseases, such as potent anti-inflammatory, antiatherogenic activities that explain their benefits in reducing cardiovascular events, even if baseline lipids were already at target levels [100] (Table 2).

Notably, it has been observed that even a low dose of atorvastatin could reduce the levels of several inflammatory biomarkers such as IL-6, TNF-a, soluble vascular cell adhesion molecule-1 (sVCAM-1) and MCP-1 in patients who received statin treatment compared to the placebo group. In addition, a substantial body of evidence has emerged suggesting that statins exhibit beneficial properties concerning endothelial function and arterial stiffness, in CVD [101-104]. Of note, it has been recently demonstrated that intensive lipid-lowering therapy with rosuvastatin delayed progression of the mean-IMT within 12 month in Japanese patients with hypercholesterolemia [105]. Moreover, in patients with stable angina, dual lipid-lowering therapy has been associated with atherosclerosis regression, though it remains controversial whether it leads to significant changes in plaque composition [106]. Also, statins have beneficial properties with respect to the thrombotic process, and more specifically in levels of CD40 ligand-mediators, von Willebrand factor, factor V, protein C and antithrombin III [107, 108].

Importantly, they seem to reduce cardiovascular events, morbidity and mortality in patients with CAD, compared to untreated patients [109, 110]. Pioneering observational studies which included patients treated with statins after ACS, have suggested that there are important benefits in terms of the expected survival. According to the National Registry of Myocardial Infarction, which analyzed the role of statin therapy or discontinuation in 300,000 patients with non-ST elevation acute myocardial infarction (non-STEMI), statin therapy was associated with lower risk of death, whereas discontinuation with increased risk of events, including death, heart failure, ventricular arrhythmia and cardiogenic shock [111, 112]. Furthermore, large, recent, prospective clinical studies have shown strong evidence for the clinical benefits of statins administration as adjunctive therapy in ACS, even though they entail differences in outcome which can be explained by different cohort characteristics. Overall, it has been steadily suggested that early initiation of statin therapy, independent of appropriate revascularization, brings a mortality benefit in clinical atherosclerosis [113, 114].

Peroxisome Proliferator-Activated Receptors Agonists

Peroxisome proliferator-activated receptors (PPARs) agonists (fibrates, thiazolidinediones or glitazones) antagonize angiotensin-II (Ang-II) effects and has been shown to exert antioxidant and anti-inflammatory properties. Several clinical trials have demonstrated their beneficial effects on vascular endothelium, in diabetic and not diabetic subjects in vascular endothelium by elevating NO bioavailability [115, 116]. In addition, Takase et al[117] tested the hypothesis that pioglitazone, a PPARγ agonist, further reduce vascular inflammation in patients receiving ARBs. Specifically, it has reduced CRP, ICAM-1 and VCAM-1 levels within 1 month, an observation which has also been confirmed by experimental data [118]. Even though there is a lack of significant data on the effects of PPARγ agonist on cardiovascular events, due to their interference with key processes of

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atherosgenesis, they could offer additional opportunities to improve cardiovascular risk beyond glycemic control in patients with DM [119].

Antioxidants

Even though oxidative stress plays crucial role in the progression of atherosclerosis and antioxidants have been enthusiastically used in the treatment and prevention of heart disease, the results of prospective, randomized, clinical studies have been discouraging [35].

Several data have illustrated positive responses of vitamin C and vitamin E in hypertensive patients, while combined treatment with vitamins C (1g) and E (400 IU), significantly improved endothelial dysfunction and arterial stiffness, effects which were associated with changes in plasma markers of oxidative stress [120, 121]. Beneficial effects of combined administration of vitamins C and E on endothelial function inflammatory process and thrombosis/fibrinolysis has also been shown in chronic smokers [122, 123]. Moreover, in terms of cardiovascular disease endpoints and MI, several trials using various combinations of antioxidant vitamins have reported encouraging results [35].

However, a meta-analysis of 6 large randomized trials of vitamin E with pooled data from over 77,000 subjects has shown absence of associations with clinical outcome [124]. Moreover, in a large, long-term trial, neither vitamin E nor vitamin C supplementation reduced the risk of major CVDs [125], and similarly, there has not been observed effects of ascorbic acid or β- carotene on cardiovascular events in female at high risk for CVDs [126]. In regard to progression of atherosclerosis, the use of vitamins E, C or β-carotene has also been inconclusive, according to a meta-analysis of randomized controlled trials [127]. Thus, despite the encouraging results from basic-science studies and small case-control trials using several vitamin supplements, it seems that antioxidant treatment is not the ideal strategy to reduce cardiovascular risk [35].

L-arginine

L-arginine is a semi-essential amino acid, substrate for endothelial nitric oxide synthetase (eNOS) and the precursor molecule for the synthesis of NO [128]. Notably, its effects on endothelial function and the myocardium have been thoroughly investigated. In particular, it has been associated with a variety of actions on the cardiovascular system, such as antihypertensive and antioxidant effects, regulation of intracellular pH, impact on the metabolism, either through NO-mediated or NO-independent mechanisms [129-131]. However, further to these well defined effects of L-arginine, in terms of clinical outcome the results are rather disappointing. Importantly, VINTAGE MI study [97], has demonstrated that L-arginine treatment was associated with increased post-infarction mortality when supplemented at a dosage of 3 g x 3 times a day [132]. It has been suggested that under specific conditions, such as in the presence of atherosclerosis, L-arginine supplementation may even be harmful for vascular health [133, 134].

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Tetrahydrobiopterin (BH4) and Folate Treatment

Tetrahydrobiopterin (BH4), an essential cofactor of NOS, has been shown to improve endothelial function in several studies, while with regards to its effect on hypertension, there are indicative, though inadequate data [135, 136]. Significant experimental data have suggested that vascular function is improved by administration of BH4 in models of atherosclerosis, diabetes or hypertension [137].

Hyperhomocysteinemia, representing a novel cardiovascular risk factor, could be treated by folate supplementation which could contribute to the improvement of endothelial function in patients with advanced atherosclerosis and hypertension [138, 139]. Despite these early clinical trials, recent larger trials in patients with stroke, MI, or stable CAD, have demonstrated that folic acid treatment is not associated with the improvement of clinical outcome [140-142]. On the other hand, it is worth mentioning that that 5-methyl-tetrahydrofolate (5-MTHF), the circulating metabolite of folic acid, has been shown to be a strong scavenger for peroxynitrite *in vitro* and to interact with purified eNOS *in vitro*, preventing its 'uncoupling', thereby acting directly on vascular endothelium [143, 144].

CONCLUSION

It is widely known that atherosclerosis is a disease of large and medium-sized arteries, which mainly is characterized by endothelial dysfunction and vascular inflammation. It has been suggested that gender, age, cigarette smoking, family history, diabetes, hyperlipidemia, and hypertension are the traditional risk factors for the atherosclerotic disease. In additional to these classical risk factors, inflammatory biomarkers, lipoprotein (a), infections, triglyceride-rich remnants, homocysteine, thrombotic and haemostatic factors, are proposed recently as novel risk factors. The treatment of the classical and novel risk factors includes, angiotensin converting enzyme inhibitors/angiotensin receptors blockers, statins, and novel therapies. However, further studies and research are required to establish the impact of the novel risk factors for atherosclerosis, and the proposed new therapeutic modalities.

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Chapter V

NOVEL TREATMENTS FOR DIABETES-ASSOCIATED ATHEROSCLEROSIS

Stephen Gray[1], Elyse DiMarco[1], Karin Jandeleit-Dahm[1,2] and Anna Watson[1]
[1]Baker IDI Heart and Diabetes Research Institute,
Melbourne, VIC, Australia
[2]Department of Medicine, Monash University, Melbourne, VIC, Australia

ABSTRACT

It has long been recognised that diabetic patients have accelerated atherosclerotic disease, particularly due to the underlying inflammatory response associated with the diabetic milieu resulting in atherosclerosis. Accelerated atherosclerosis contributes to the high rates of myocardial infarction and stroke observed in diabetic patients. Whilst current treatment regimes do slow the progression of diabetes-associated atherosclerosis they do not prevent it. This lack of effective treatment, coupled with the increasing prevalence of diabetes worldwide makes identification of novel therapeutic targets imperative. This review will outline recent work from other laboratories and our own with respect to novel therapeutic targets including peptide hormones, such as urotensin II and endothelin I. Furthermore we will outline strategies which target generation of reactive oxygen species (ROS) directly by inhibition of the main enzymatic source of ROS, NADPH oxidase. It is now considered that the innate and adaptive immune system also play an important role in diabetes-associated atherosclerosis. This review will therefore also outline potential novel therapeutic targets within the immune response which contribute to the progression of diabetes-associated atherosclerosis. Ultimately identification of new therapies will lead to a reduction in the burden of cardiovascular disease in diabetes.

INTRODUCTION

It is estimated that 7.7% adults in developed countries have type II diabetes and by 2030 this is predicted to increase by 20% in developed countries and 69% in developing countries

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[1]. The incidence of type 1 diabetes is also increasing world wide [2, 3]. Thus, increases in the number of individuals with both type I and type II diabetes will lead to a significant increase in vascular complications. In the longer term the social and economic health burden of the cardiovascular (CV) complications of diabetes is likely to increase substantially. Whilst current therapeutic regimes slow the development of diabetic vascular complications, they do not prevent or reverse them.

Many diabetes associated CV events, including death from CV disease, are linked to diabetes-associated atherosclerosis (DAA; for review of DAA see [4]). Relatively few clinical trials in diabetic patients have examined the effects of new therapies on DAA. Thus the majority of evidence for the anti-atherosclerotic effects of current therapeutic strategies originates predominantly from mouse models of atherosclerosis, in particular the advanced DAA seen in the streptozotocin-induced diabetes in the hyperlipidemic apolipoprotein E (apoE) KO mouse. In this chapter we will outline new novel targets for the treatment of DAA based on recent pre-clinical data from other laboratories and our own.

NOVEL PEPTIDE HORMONE SYSTEMS IN ATHEROSCLEROSIS

Endothelin-1

Endothelin is thought to play a role in endothelial dysfunction and increased inflammation in both the diabetic and non-diabetic context. Elevated plasma levels of endothelin 1 (ET1) serve as a marker for macrovascular disease [5]. Mice over-expressing human prepro-endothelin have impaired vasodilatation, and endothelial dysfunction including increased vascular ROS production [6, 7]. ET1 and endothelin converting enzyme are also expressed in human plaques and plasma ET1 levels increase with the degree of plaque burden in humans [8, 9]. Both endothelin receptor A (ETA) and endothelin receptor B (ETB) show a high expression in cells of the vascular wall critically involved in the development of atherosclerosis such as in vascular smooth muscle cells and in macrophages including in foam cells [10, 11]. ET1 receptor antagonism has also been shown to reduce vascular smooth muscle cell proliferation and fibrosis [12, 13].

Despite both receptors being up-regulated in diabetes, it is generally agreed that the ETA receptor is a more promising therapeutic target due to the role of ETB in clearing ET1 [14].

Trials of endothelin receptor antagonists ("-sentan" compounds) have been shown to decrease DAA in experimental diabetes [15]. In our study we found that the endothelin receptor antagonist avosentan attenuated the development of diabetes-associated atherosclerosis in streptozotocin-induced diabetic apoE KO mice. Diabetes induced atherosclerosis is characterised by a large increase in vascular inflammation in apoE mice. Interestingly we demonstrated both diabetic nephropathy and diabetes-associated atherosclerosis to be attenuated with avosentan treatment, suggesting simultaneous micro- and macrovascular protection with avosentan in this model. Compared to placebo treated diabetic animals, diabetic mice treated with avosentan showed less macrophage accumulation in the aorta as well an attenuation of nitrotyrosine levels, indicative of a reduction in peroxynitrite (ROS scavenging NO- for more see "Reactive Oxygen Species in

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Atherosclerosis" below). Furthermore, ET1 has been shown to induce ROS production via NADPH oxidase in endothelial cells [16], in rat aorta [17], and also to interact with angiotensin II (Ang II) to increase ROS formation [18, 19].

Many, but not all, endothelin receptor antagonists reduce blood pressure [15], thus many of the beneficial effects of ET1 receptor antagonists have been attributed to these antihypertensive effects. Our studies did not show a significant decrease in blood pressure with avosentan treatment in mice, and clinical trials also show that this compound does not significantly lower blood pressure [20]. Thus it is likely that many of the anti-atherosclerotic effects observed with ET receptor blockade are independent of changes in blood pressure [21].

Despite clear preclinical evidence for ETA receptor antagonism reducing DAA few studies in patients with cardiovascular disease have been carried out. An exception is a recent randomized double-blind study conducted in 47 patients with coronary endothelial dysfunction. This study found that patients treated for 6 months with the ETA antagonist atresentan showed significant improvements in coronary arterial responses to acetylcholine when compared to placebo treated patients, suggesting that ET1 plays a role in early endothelial dysfunction which is considered to precede the development of atherosclerotic plaque [22]. These studies suggest that targeting of ETA may indeed attenuate the development of atherosclerosis.

Urotensin II

Another potential inflammatory hormone contributing to the progression of DAA is the vasoactive peptide urotensin II (UII). UII is a small peptide of between 11-14 amino acids in length with a C' ring structure which is conserved, being identical in all species isoforms of the peptide identified to date including fish, rodents and humans. UII binds to a single receptor (UT) which is also highly conserved across species with the rat receptor showing 75% homology to the human receptor [23].

Both UII and its receptor have a wide expression through out arterial and venous vasculature [24], however expression does vary from species to species [25]. In arteries, UII has been characterised as the most potent vasoactive substance identified to date, even more potent than ET1 [26]. However UII induced vasoconstriction does not always occur, probably as a result of receptor occupancy and the 'pseudo-irreversible' nature of UII binding to UT [25]. UII is expressed in endothelial and smooth muscle cells with levels being elevated in (non-diabetic) atheroma [37, 28]. UII receptor is expressed in macrophages in atheroma [28], which is in keeping with its in vitro effects where it is known to increase foam cell formation [29]. Indeed, UII was found to be up-regulated in atherosclerotic human coronary arteries collected during heart transplantation compared to non-diseased coronaries collected from autopsy [30].

UII was also found to potentiate macrophage and smooth muscle cell migration in vitro [31] and UII also can influence adventitial fibroblast differentiation and migration in vitro [32, 33]. Thus UII may potentiate the development of atherosclerosis via increasing the recruitment of macrophages. In line with this UII has been shown to increase leukocyte adhesion in human coronary endothelial cells in vitro [34]. Interestingly there was no change in UII receptor binding when comparing normal and atherosclerotic human coronary arteries,

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indicating UII receptor levels were unaltered [35]. Given the strong binding of UII to UT, it is possible that a large up-regulation in UT expression is not needed for an increased response.

In the aorta of atherosclerosis prone apoE KO mice gene expression of UT and binding to UT were found to be elevated when compared to wild-type (non-atherosclerotic) animals [36]. Another study utilised high-fat fed apoE KO mice, which develop advanced atherosclerotic lesions. Ten weeks of treatment with the UT antagonist SB-657510 resulted in a decrease in atherosclerotic lesion area [37].

In contrast it has also been shown that high-fat fed apoE KO mice which lacked the UII receptor developed increased atherosclerosis [38]. It should be noted that UT/apoE double KO mice showed many other physiological abnormalities, including significant increases in plasma cholesterol, serum triglycerides, serum insulin, a decrease in hepatic steatosis (and liver mass), as well as hypertrophy of the heart, kidney and spleen when compared to single apoE KO mice [39]. The main cause of these abnormalities in the double KO is likely to be a decrease in hepatic cholesterol uptake and/or handling which could have affected atherosclerosis development [38].

Little is known of the effects of UII in diabetes. Plasma UII levels are known to be elevated in type II diabetic patients, although concentrations are low, measuring in the fmol/ml range [40-42]. It is likely that UII acts more as a paracrine or autocrine factor in the vessel wall, with plasma levels reflecting spill over.

UII stimulates inflammation via ROS generation through increased expression and activation of Nox4, at least in vitro [43, 44]. Given the pro-inflammatory nature of diabetes, it is possible that UII plays an important role in the diabetic vasculature and the development of diabetes-associated atherosclerosis. Due to the disappointing effects of the UII receptor antagonist palosuran in diabetic nephropathy [45], enthusiasm for such treatments has decreased. To date the effects of UII antagonism on vascular function and atherosclerosis development in diabetes have not been investigated in the clinical setting. Given the effects of UII in activating macrophages and inducing inflammation, partially via Nox4, such treatments may prove beneficial in reducing diabetes-associated atherosclerosis.

REACTIVE OXYGEN SPECIES IN ATHEROSCLEROSIS

In diabetes there is an increased production of vascular ROS which contributes to the development of atherosclerosis. The most important chemical reactions pertaining to enzymatic ROS production begin with superoxide (O_2^-) by various enzymes, including isoforms of NADPH oxidase, or Nox (see below). Spontaneous or catalytic dismutation of O_2^- via superoxide dismutase (SOD) enzymes results in the generation of the more stable yet biologically active hydrogen peroxide (H_2O_2) which is detoxified to water or converted to the highly reactive hydroxyl radical (OH*) or the anion (OH^-) [46, 47]. The hydroxyl radical can then go on to form reactive nitrogen species (RNS) by reacting with NO (in its neutral, radical or protonated form). Production of NO, an important vasoactive molecule, is by nitric oxide synthase (NOS) enzymes [46]. The reaction between O_2- and NO* (radical) produces ONOO- (peroxynitrite), itself a powerful oxidant [48]. It also results in the loss of biologically available NO and a loss of the beneficial actions of NO such as endothelium-dependent

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vasodilation [49, 50]. Increased ROS generation contributes to the progression of atherosclerosis therefore targeting ROS is a potentially promising therapeutic avenue.

ROS promote a pro-inflammatory state through direct activation of adhesion molecule expression, by inducing pro-inflammatory gene activation and by reducing NO availability [51]. The effects of ROS appear to be mediated through activation of redox-sensitive MAP kinases (ERK1/2, p38MAP kinase, JNK, pro-inflammatory kinases, ERK5, involved in protein synthesis, cell cycle progression and cell proliferation), tyrosine kinases (c-Src, EGFR, PI3K) and transcription factors including those that have been extensively linked to inflammation (NFκB, AP-1 and HIF-1).

NADPH Oxidase (Nox) Enzymes

There are many sources of ROS, however in the vasculature the NADPH oxidase (Nox) family of enzymes produce ROS as their primary function, thus it is thought that these enzyme isoforms are the main source of the pathological increase in ROS seen in diabetes-associated atherosclerosis [52, 53]. Nox are now established as disease-relevant sources of ROS and represent exciting potential targets for limiting oxidative stress in vascular tissue [52, 54, 55].

The first isoform of Nox described was the phagocytic form of NADPH oxidase which is involved in host-defence processes. It comprises of a membrane-bound flavocytochrome b558 made up of gp91phox (Nox2) and p22phox as well as three modular cytosolic regulators (p47phox, p67phox, p40phox) [56]. In activated cells, p47phox is phosphorylated, leading to cytosolic complex formation and translocation to associate with cytochrome b558 to assemble the active enzyme [53].

Over the past 5 years several novel Nox2 homologues have been recognized (Nox1-5). Within the vascular wall there are principally three isoforms of the Nox family: Nox1, Nox2 and Nox4. The Nox5 isoform is only present in humans and its precise role within vascular disease is relatively unknown [57]. The (patho)physiological functions of these different isoforms of the Nox family are unknown however, certain isoforms play an important role in vascular pathobiology in diabetes inducing inflammation and fibrosis.

Nox4 is ubiquitously expressed in vascular cells whereas Nox1 and Nox2 mRNA have been detected primarily in smooth muscle and inflammatory cells, respectively [58-60]. Nox4 constitutively produces ROS in the vasculature whereas Nox1 mediates agonist-induced O_2^- production [52, 57, 60-63]. There are many agonists of vascular Nox, including peptide hormones such as Ang II, ET1, UII (see above), and also TNF-α, IL-1, oxLDL, TGF-β and IFN-γ, which have been shown to have pro-atherogenic functions, particularly in the context of diabetes [47, 52]. Basal Nox1 expression is low in vascular cells, but is induced by stimuli such as platelet derived growth factor (PDGF) and Ang II and is increased in pathological conditions such as diabetes, atherosclerosis and hypertension [60, 64].

Various studies in the absence of diabetes have produced conflicting results as to the role of the different Nox isoforms in models of vascular injury. This is an area of ongoing investigation, especially within the context of the pro-inflammatory environment seen in diabetes [52].

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TARGETING NADPH OXIDASE (NOX)

Nox1

Basal Nox1 activity within the vascular wall is much lower in comparison to other Nox isoforms. In many studies Nox1 gene expression is undetectable by RT-PCR and has yet to be demonstrated by immunohistochemistry [65]. However, under disease states the expression of Nox1 is significantly increased, as demonstrated in balloon injury model of atherosclerosis and in models using pro-atherogenic LDL and Ang II [65-6]7. Studies employing the use of genetic deletion of Nox1 are limited however there has been a causal association between Nox1 and a role in the pathophysiology of hypertension and in neointima formation [68-70]. Nevertheless, further studies are warranted in order to delineate the role of Nox1 in atherosclerosis. It is suggested that Nox1 associates with p47phox to form a functional complex leading to early proliferative changes within the neointima [59].

Nox2

Nox2 deletion in apoE KO mice on a high fat diet reduced plaque area in the aorta, reduced lesion size and was associated with decreased aortic ROS production. In contrast, additional studies failed to find a difference in atherosclerosis development in the aortic sinus of the Nox2 KO mice [72]. As the authors noted, these observations were made within the sinus, and as such cannot rule out changes in the development of atherosclerosis within other regions of the aorta [71]. These studies highlight a Nox2 mediated response in the development of atherosclerosis.

p47phox

Deletion of the p47phox subunit of the Nox1 complex in the apoE KO mouse model resulted in a reduction in the development of atherosclerosis within the descending aorta irrespective of diet. Furthermore the p47phox/apoE double KO did not show altered serum lipid concentrations [73]. However, there was no change in the development of atherosclerosis within the aortic arch, where there was evidence of advanced lesion development. Potentially generation of ROS through the Nox4 isoform could be contributing to this disparity in atherosclerosis development, as the p47phox subunit is not required for Nox4 activity. Thus deletion of p47phox should not have affected Nox4 activity. Furthermore, disruption of the p47phox gene reduced superoxide production in the vessel and inhibited proliferation of vascular smooth muscle cells [73, 74]. This experiment highlights an important role for the subunit p47phox in the development of atherosclerosis.

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Nox4

In the normal state, Nox4 expression is relatively high and is found within all cell types of the vascular system [75-78]. The primary role of Nox4 within the vasculature is to produce H_2O_2 at a constant rate and under pathological conditions this rate of production is increased [79]. Hypertension is a precursor for the development of atherosclerosis and Nox4 expression and activity are increased in animal models of chronic hypertension [80]. Hypertension itself causes endothelial dysfunction, causing an increase in shear stress which induces H_2O_2 production by endothelial cells. Further to hypertension, diabetes and the dyslipidaemia associated with diabetes are crucial factors in the process of endothelial dysfunction, in which oxidative stress is significantly increased [81, 82]. The process of endothelial dysfunction is considered a key step in the promotion of atherosclerosis as it leads to the infiltration by monocytes and secretion of chemokines such as PDGF and Ang II, which both mediate further production of ROS through both Nox1 and Nox4 [70, 83-85].

PHARMACOLOGICAL INHIBITION OF ROS

Inhibition of ROS to reduce vascular disease has been met with limited success primarily due to the use of relatively non-specific ROS inhibitors. Classical ROS inhibitors such as diphenylene iodonium (DPI) and apocynin have been shown to have Nox-independent effects [55, 86]. DPI was first identified as an NADPH inhibitor *in vitro* [87]. It was subsequently found to inhibit the activity of the NAD(P)H dehydrogenase mitochondrial complex I in the rat liver [87] and in the bovine heart [88]. Later it was shown to inhibit the respiratory burst that occurs within neutrophils as part of the immune response to pathogens [89]. Further experiments later identified DPI as a non-specific inhibitor of ROS due to cross-talk with NO production via the inhibition of IL-1 inducible NO synthesis, thus causing a reduction in NO bio-availability [90]. Furthermore, DPI has pro-oxidant activities which may lead to an increase in lipidperoxidation and oxidative stress [91]. Thus DPI is now considered as a non-specific ROS inhibitor.

In 1990, apocynin was first identified as a strong inhibitor of neutrophil $O_2^{\bullet}$- radical production *in vitro* and to exert anti-inflammatory activity *in vivo* [92]. The actions of apocynin on Nox activity are largely unknown, however, it is suggested to react with essential thiol groups in the Nox subunits, preventing complex assembly [93]. For many years apocynin was used as a specific inhibitor of Nox, as it was shown to inhibit all isoforms of Nox in many different experimental models and tissues [63, 94-97]. However, recent studies have demonstrated that apocynin not only inhibits Nox activity but also inhibits cytochrome P450 activity in endothelial cells and interferers with actin polymerization and cytoskeletal rearrangement. Furthermore, it has been identified that apocynin can have pro-oxidant activity as it causes ROS generation in rat vascular fibroblasts [98]. Subsequently DPI and apocynin are currently considered to be suppressers of oxidative stress but not specific Nox inhibitors.

In contrast, the highly novel triazolo pyrimidine VAS3947 shows promise as a specific Nox inhibitor as it has recently been shown to inhibit Nox activity in primary rat vascular smooth muscle cells and human umbilical vein endothelial cells [99, 100]. Furthermore, in

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zebra fish it has been found to inhibit the H_2O_2 burst at wound margins without obvious toxicity, suggesting that it may inhibit Nox4 activity [101]. Subsequent pharmacological profiling of VAS3947 found that it inhibited H_2O_2 production in addition to inhibiting the production of superoxide and perioxynitrate [55]. One drawback is that VAS3947 targets multiple isoforms of the Nox family. It is becoming evident that each isoform has different roles in the pathophysiology of disease development thus the development of isoforms-specific Nox inhibitors is important. Other Nox inhibitors such as the pyrazolo pyridine derivative compound GKT136901 specifically inhibit Nox1/Nox4-mediated ROS production [55, 100, 102, 103]. While this drug has yet to be used in the context of vascular disease, it has been shown to have renoprotective effects in nephropathy in that treatment resulted in a reduction in oxidative stress and attenuation of the progression of disease [103].

IMMUNOMODULATION IN ATHEROSCLEROSIS

Innate and adaptive immunity are the two arms of the immune system which, via shared mediators and direct cell-cell contact, operate in concert to deliver a highly integrated and efficient response to infection and injury [104] (see Table 1). Innate immunity is the first line of defence against invading microbes, comprising of physical barriers, germline-encoded biochemical mediators and constitutively expressed cell types to deliver a fast yet blunt response [104, 105]. Adaptive immunity is characterised by immunological specificity and 'memory,' reflected in both humoral (mediated by soluble antibody proteins secreted by activated B-cells called plasma cells) and cellular (effector T-cells) responses [105, 106].

When inappropriately triggered or not properly controlled, the same immune mechanisms involved in host defence cause tissue injury and disease [105]. Atherosclerotic lesions represent an excessive inflammatory, fibroproliferative response against different noxious stimuli [107].

TARGETING RECRUITMENT OF IMMUNE CELLS TO THE INJURED VASCULATURE

Immune cell activation and recruitment has long been known to play a key role in the pathogensis and progression of atherosclerosis. Some of the potential therapeutic targets in the immune system have been investigated (Table 2).

VCAM-1

Adhesion of immune cells to the inflamed endothelium is a multi-step process that begins with rolling aided by E- and P-selectins, followed by stronger binding between immunoglobulin (Ig)-like molecules on endothelial cells, such as vascular cell adhesion molecule-1 (VCAM-1), to their reciprocal integrin ligands on leukocytes [108-111]. VCAM-1-mediated adhesion of monocytes has been observed in atherosclerotic vessels of apoE KO

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mice and interruption of these interactions effectively reduced atheroma formation [109, 112, 113].

MCP-1

Of particular importance is CCL2 or monocyte chemotactic protein-1 (MCP-1) that promotes recruitment, migration and activation of monocytes, T cells, and smooth muscle cells in the aortic wall. These effects represent critical events in the development of atherosclerotic lesions [114]. MCP-1 expression is up-regulated in both human and rabbit atherosclerosis, particularly in macrophage-rich areas of plaques [115, 116]. Patients with diabetes exhibit increased levels of inflammatory markers such as MCP-1 while the anti-atherosclerotic effects of rosiglitazone in streptozotocin-induced diabetic apoE KO mice have been associated with reduced mRNA levels of MCP-1 in the aorta [117, 118]. Moreover, apoE KO mice lacking the receptor for MCP-1, CCR2, show reduced monocyte/macrophage accumulation in addition to impaired Th1-T cell responses leading to decreased atherosclerotic lesion formation [115]. Novel gene-based strategies targeting MCP-1/CCR2 signalling are currently being investigated as a potential anti-inflammatory treatment of atherosclerotic cardiovascular diseases [119].

Table 1. Immune cells potentially involved in the development of atherosclerosis [105, 108]

Innate immune cells	Adaptive immune cells
Granulocytes (polymorphonuclear leukocytes)	Natural killer (NK) T cells
Macrophages	B and T lymphocytes
Dendritic cells (DCs)	
Mast cells	
Natural killer (NK) cells	

APC: antigen presenting cell, IFN-γ: interferon-gamma, IL-10: interleukin-10, dKO: double knockout, MCP-1: Monocyte chemotactic protein-1, M-CSF: Macrophage colony stimulating factor, Rag: recombination activating gene, SCID: severe combined immunodeficiency, SR: scavenger receptor, VCAM-1: Vascular cell adhesion molecule1.

Targeting Macrophages

The progression of atherosclerosis has been linked to the recruitment of circulating monocytes to the arterial intima where they differentiate into DCs or macrophages under the influence of granulocyte macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF), respectively [104, 123]. Atherosclerosis was shown to be drastically reduced in M-CSF-deficient (*op/op*) apoE KO mice, indicating a primary role for monocyte-derived macrophages in atherogenesis [124].

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Table 2. Genetic knockouts of key immune cells and mediators [104, 120-122]

Immune defect (KO)	Immunological effect	Effect on atherosclerosis
MCP-1, CCR2	Reduced transmigration to aortic intima	↓
P-, E-selectin	Reduced leukocyte rolling in recruitment	↓
VCAM-1	Reduced leukocyte adhesion	↓
M-CSF	Macrophage differentiation	↓
CD36, SR-A	Scavenger receptors, less oxLDL uptake	↓
Rag1/ Rag2 dKO	T- and B-cell defect	↓
SCID	T- and B-cell defect	↓
Splenectomy	Removal of atheroprotective antibodies (?)	↑
IFN-γ or IFN-γ receptor	No IFN-γ signalling, suggestive of defective Th1 phenotype	↓
IFN-γ (exogenous administration)	Increased IFN-γ signalling	↑
IL-10	Increased pro-inflammatory cytokines, ↑Th1, less Th2	↑

The majority of macrophages in early lesions form foam cells via scavenger receptor-mediated recognition and high affinity uptake of modified LDL [125-129]. Foam cells actively secrete pro-inflammatory cytokines, growth factors, proteases and ROS that amplify and perpetuate the inflammatory response in the lesion [121, 130]. Deficiency of the class B scavenger receptors CD36 in apoE KO mice decreased atherosclerotic lesion development in conjunction with reduced foam cell formation [131]. A recent study by Bernal-Lopez et al. found that diabetes induced hyperglycaemia altered expression of the scavenger receptor CD36 in patients with atherosclerosis [117]. In order to better understand the potential beneficial and/or harmful effects of vascular CD36 inhibition in atherosclerosis, typical co-morbidities such as diabetes should be considered.

Targeting CD4+ Helper Cells

T cells are abundant in human and murine atherosclerotic lesions, most of which are CD4+ T-cell receptor (TCR) $\alpha\beta^+$ [132, 133]. Experimentally, transfer of CD4+ T-cells from immunocompetent apoE KO mice to immunodeficient apoE KO x SCID/SCID dramatically increased atherosclerosis in the recipient [134]. The CD4+ helper T-cells can be classified into two main functional subsets, Th1 and Th2, characterized primarily by differences in cytokine repertoire [135, 136]. Lesional T-cells largely secrete Th1-type cytokines: IFN-γ, IL-2, TNF-α and TNF-β which cause activation of macrophages and vascular cells, thus promoting inflammation and cellular immunity [121, 137]. Furthermore, both pharmacological and genetic blockade of Th1 differentiation and IFN-γ receptor gene have been shown to reduce lesion development in apoE KO mice [104, 138-140].

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B CELLS- HUMORAL IMMUNITY OF ATHEROSCLEROTIC LESIONS

Relatively few B cells are present in atherosclerotic plaques however the detection of specific anti-oxLDL antibodies in the plaque and circulation of patients and experimental animals suggests an active role for B cells in atherosclerosis [104, 121, 141]. Antibodies (or immunoglobulins, Ig) secreted by differentiated B-cells fulfil a protective role in host immunity by neutralising and opsonising infectious material for its efficient clearance by phagocytes bearing reciprocal Fc receptors [108]. Splenectomised apoE KO mice have shown a 100% increase in atherosclerotic lesion size possibly due to the removal of atheroprotective antibodies produced by spleen B cells [142, 143]. Current evidence suggests that production of natural IgM antibodies by B-1 cells is atheroprotective whereas IgG production by conventional B-2 cells, requiring T-cell stimulation, may be proatherogenic [122, 144].

CONCLUSION

In conclusion, whilst current therapeutic regimes to prevent or treat cardiovascular disease in diabetes include a range of treatments to control blood pressure, lipids and glucose, these interventions are still not able to prevent CV complications in diabetes. Exciting new therapeutic targets, such as the vasoactive peptide hormones ET1 and UII warrant further clinical investigation as treatments for diabetes-associated atherosclerosis. Furthermore, therapies which target ROS generation, especially via Nox isoforms promise to be new treatment options in the treatment of vascular disease, specifically in diabetes.

Finally, it is evident that the immune system contributes to the advancement of atherosclerosis in diabetes, but further studies are required to identify new targets for immunomodulation in DAA. The development of new treatments in order to reduce atherosclerosis and CV disease in diabetes will aim to attenuate the diabetes induced alterations in the immune system without affecting defence immune mechanisms.

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Chapter VI

ANTIOXIDANTS: ARE THEY REALLY BENEFICIAL IN ATHEROSCLEROSIS?

Arvin Parvathaneni, Pramil Cheriyath and Daniel Fischman
Department of Internal Medicine, Harrisburg Hospital, Harrisburg, PA, US

INTRODUCTION

Atherosclerosis, also known as atherosclerotic vascular disease (ASVD), is a prime cause of premature death and long-term disability throughout the world. Various theories like injury hypothesis, retention hypothesis and oxidative mechanism hypothesis were put forward over the years to explain the pathogenic mechanisms involved in ASVD. With so much already known about the etiology and pathology of this disease, the primary focus of investigation in the last two decades has shifted to prevention. One such area of attention has been the role of oxidative mechanisms in the development of ASVD, and the potential benefit of antioxidants in preventing oxidative vascular damage. Despite the presence of compelling evidence that oxidation mechanism plays a major role in atherosclerosis, the precise molecular mechanisms which lead to oxidative changes are still being studied. Although there is an accumulating body of evidence that does suggest that the oxidative mechanisms do play a role in ASVD, several large trials with antioxidants have failed to demonstrate any reduction in mortality from ASVD. In this chapter we discuss the evidence for and against antioxidants having a role in preventing atherosclerotic disease, with special attention to Alpha Tocopheral (vitamin E) and Ascorbic Acid (vitamin C). We also review the literature regarding the role of Uric Acid and Bilirubin in the pathogenesis of ASVD. Finally, we will discuss potential implications of current research regarding the prediction and prognosis of atherosclerotic disease.

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PATHOGENESIS OF ATHEROSCLEROSIS

For more than a century, research efforts have been ongoing to give us a better understanding of the pathogenesis and contributing factors associated with ASVD. Based on these efforts, several clinical, epidemiological and genetic studies have concluded that Low Density Lipoprotein (LDL) is involved in the pathogenesis of atherosclerosis. The "Incrustation hypothesis" of Rokitansky and "Lipid transudation hypothesis" of Virchow were the early theories proposed to explain this ASVD pathogenesis. Both these hypotheses share a common view that passive deposition of lipids is the pathological hallmark in the process of atherogenesis (Stocker & Keaney, 2004). These theories were then modified by Ross and Glomset who described a component of endothelial injury as a vital initiating factor in atherogenesis (Ross & Glomset, 1973). The works of Ross and Glomset modified the concept that atherosclerosis is an inflammatory process rather than a passive deposition. Ross and Glomset explained that injury to the endothelium alters its homeostatic properties, which leads to aggregation of cells and initiation of a series of inflammatory events, which subsequently result in the formation of the atherosclerotic lesion. In further description of these "inflammatory events," macrophage recruitment, resulting from this endothelial damage was considered as an important intermediary step. These macrophage cells have the ability of increasing the uptake of LDL particles and thereby forming foam cells, which mark the formation of an early atherosclerotic lesion. The above process is followed by progressive inflammatory changes in the atherosclerotic plaque leading to cellular death and expansion of the atherosclerotic lesion causing complete blockage of the involved blood vessel (Jonasson et al, 1986). Later studies have demonstrated that intact endothelium forms the lining of various atherosclerotic lesions and that the loss of endothelial lining is not common in the process of development of atherogenesis.

The finding that rate of LDL uptake is uniform across the blood vessel irrespective of endothelial damage, has prompted the proposal of an alternative hypothesis for atherosclerosis. In contrast to the above hypotheses regarding the pathogenesis of ASVD, the retention hypothesis proposed that LDL uptake is the major initiating factor for the development of atherosclerosis (Williams & Tabas, 1995). This hypothesis argues that accumulation of lipoprotein particles in the sub-endothelial vascular layer is the major mechanism which initiates the events occurring in the pathogenesis of ASVD. As per this hypothesis, interaction between various lipoprotein molecules leads to retention of lipids in the arterial wall, which subsequently results in various inflammatory changes believed to be responsible for atherogenesis. Retention hypothesis was later succeeded by oxidation modification hypothesis which emphasized that LDL in its natural form is not atherogenic. The hypothesis of oxidative modification of Low density lipoprotein (LDL) as a vital step in the pathogenesis of atherosclerosis evolved from various studies which have been performed in the last three decades. Among all the mechanisms proposed to explain the pathogenesis of ASVD, oxidative modification hypothesis is the most widely accepted one when compared to other theories and is explained in the next section. Although each of the above mentioned hypotheses is unique to itself, LDL uptake and inflammatory changes initiated by various inciting factors form the basic pathway for all these hypotheses.

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OXIDATIVE MECHANISMS IN ATHEROSCLEROSIS

Oxidative modification mechanism is one of the most elaborately studied pathogenic mechanisms implicated in the development of atherosclerosis. As early as 1979, Chisolm et. al. and Goldstein et. al. had proposed that LDL oxidation played a major role in the process of atherogenesis (Hessler et al, 1979). LDL oxidation aids in the development of atherosclerosis by recruiting monocytes, enhancing the uptake of lipoprotein, inhibiting the movement of foam cells from endothelium, and endothelial damage (Stocker & Keaney, 2004). In contrast to previous hypotheses that the presence of elevated LDL levels by itself, was sufficient for the development of atherosclerosis, in vitro studies have demonstrated very little intake of Low density lipoprotein by cultured cells when provided with large amounts of native LDL (Goldstein et al, 1979). Also the fact that atherosclerosis has been reported in people who have familial hypercholesterolemia secondary to absent LDL receptors favors this hypothesis. These findings have led to the hypothesis that LDL is modified in some way, and that this modified molecule undergoes enhanced uptake by macrophages. The proposal that for macrophages to avidly ingest LDL, alteration of LDL must happen is supported by experiments which have shown an increased uptake of Low Density Lipoprotein in the presence of oxidative substances (Fogelman et al, 1980). Furthermore, the hypothesis of an oxidative mechanism is reinforced by in vitro experiments which have shown that antioxidants impede the uptake of LDL by epithelial cells.

Further evidence for oxidation playing a role in the pathogenesis of ASVD comes from the finding of oxidized lipids in atherosclerotic lesions (Carpenter et al, 1993). The presence of oxidized forms of lipids in atherosclerotic lesions has been confirmed by various chemical analytical studies (Glavind et al, 1952 & Gilbert et al, 1969). Oxidized forms of LDL were also identified with the help of antibodies which recognized the epitopes present on the oxidized forms of LDL. LDL isolated from atherosclerotic lesions had properties which are similar to the oxidized LDL in the in vitro experiments performed.

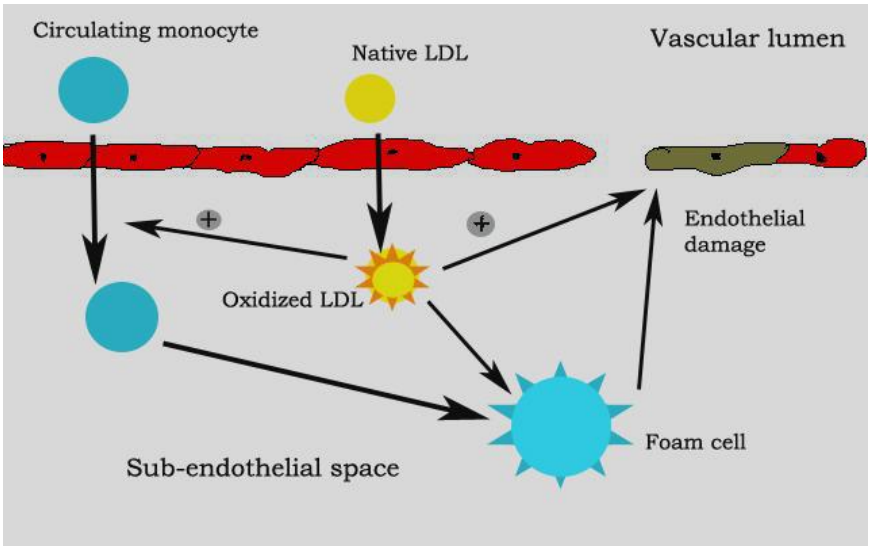

Figure 1. Oxidative modification hypothesis of atherosclerosis.

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All these findings substantiate the role of oxidation in modifying circulating LDL, which fosters the development of atherosclerotic lesions. Moreover, the levels of specific antibodies against oxidized LDL have been correlated with severity of atherosclerosis when investigated in some animal models. Furthermore these experiments demonstrated that inhibiting lipo-oxygenase activity, the enzyme responsible for oxidizing LDL, resulted in an ASVD risk reduction (Steinberg & Witztum, 2002).

Despite the presence of convincing evidence about the role of oxidation in ASVD, there still remains some skepticism regarding the role of oxidation mechanism in ASVD. Most of the oxidative changes to LDL occurred in vascular wall rather than in the plasma owing to the presence of higher levels of antioxidants in plasma when compared to vascular wall. But significant levels of antioxidants were found in atherosclerotic plaques in the walls of blood vessels (Stocker & Keaney, 2004). In vitro oxidation of lipoprotein molecule requires major alterations including the deletion of Alpha Tocopherol but in vivo oxidation changes do not involve depletion of Tocopherol and oxidized lipids are formed in the presence of Tocopherol. Also studies performed using various antioxidants in predicting the risk of cardiovascular diseases have yielded mixed results which do not go in favor of oxidation hypothesis (Diaz et al, 1997).

ROLE OF ANTIOXIDANTS IN ATHEROSCLEROSIS

Due to the presence of mounting evidence in favor of oxidation modification mechanism, multiple studies have attempted to reverse this oxidation mechanism by use of antioxidants with the hope of preventing development of atherosclerosis. It was assumed that these compounds by virtue of their antioxidant nature will reduce the oxidation of LDL which will hinder the formation and progression of atherosclerotic plaques. Animal studies performed using the antioxidants Probucol, N-N diphenyl phenylenediamine and Butylated Hydroxy Toulene (BHT) have demonstrated reduced oxidation of LDL and concurrent decrease in atherosclerotic lesions (Carew et al, 1987). Findings such as those reported by Carew are credited with leading to the speculation that dietary antioxidants such as Alpha Tocopherol, Beta Carotene and Ascorbic Acid could also modify ASVD risk. Numerous studies were conducted on the role of these antioxidants in reducing the risk of ASVD and most of these studies were directed at vitamin E. Not only each dietary antioxidant was investigated separately regarding its role in reducing the risk of ASVD, but also studies were performed which assessed the combined effects of these dietary antioxidants. Various antioxidants which are commonly studied and their sources have been illustrated in the following table.

Vitamin E

Groups of compounds known as Tocopherols and Tocotrienols are represented by the common term vitamin E. Among these compounds Alpha-Tocopherol, has higher biological activity and antioxidant properties. As early as 1946, Shute et. al. hypothesized the protective role of vitamin E in heart disease but were unable to support their suppositions with evidence, thus this hypothesis was not embraced at the time (Vogelsang & Shute, 1946). In support of

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the Shute's findings, the work of Esterbauer et al suggests that LDL depleted of Alpha-Tocopherol undergoes rapid oxidation, and that LDL isolated after supplementation with Vitamin E is resistant to oxidation mediated by metal ions like Copper (Dieber-Rotheneder et al, 1991). In the last two decades, multiple epidemiological studies have been performed to assess the role of Alpha-Tocopherol, the bio-available form of vitamin E, in reducing the risk of developing atherosclerosis. These studies yielded mixed results and did not have concluding evidence about the role of vitamin E.

Table 1. Antioxidants

ANTIOXIDANTS	SOURCE
ASCORBIC ACID (Vitamin C)	Dietary sources
α-TOCOPHEROL (Vitamin E)	Dietary sources
URIC ACID	Purine Metabolism
BILIRUBIN	Breakdown of hemoglobin
GLUTATHIONE	Dietary and biosynthesis
CAROTENE	Dietary sources
UBIQUINOL (Coenzyme Q)	Dietary and biosynthesis

Observational studies have revealed that supplementation with vitamin E reduces susceptibility of LDL to oxidation (Berry et al, 1991). Furthermore, the Nurse's Health Study and Male Health Professionals follow up study, which are prospective studies performed in two large cohorts, investigating the role of vitamin E in primary prevention of ASVD, showed that vitamin E was beneficial at a daily intake of 100 International Units/day and is ineffective at lower doses (Rimm et al, 1993 & Stampfer et al, 1993). Studies which were aimed to assess the role of vitamin E in secondary prevention in atherosclerosis like Cambridge Heart Antioxidant Study (CHAOS), revealed that supplementation with vitamin E is associated with a major reduction in the risk (relative risk=0.53, p=0.005) of experiencing nonfatal Myocardial infarction (Stephens et al, 1996). Also, the protective effect of vitamin E on pre-existing atherosclerotic lesions has been demonstrated by studies performed in middle aged men who underwent coronary artery bypass surgery (Hodis et al, 1995). Vitamin E supplementation has been proven to be effective in reducing the risk of developing myocardial infarction in patients with increased oxidative stress secondary to other co-morbid conditions like renal failure. SPACE study which was performed in a group of patients on hemodialysis demonstrated reduction in the risk of myocardial infarction with vitamin E supplementation (Boaz et al, 2000). In addition to its direct anti-oxidant effects, vitamin E has also been proposed to inhibit thrombin formation, smooth muscle cell proliferation as well as platelet and monocyte adhesion (Chan 1998). Pre-treatment of endothelial cells with Alpha-Tocopherol in cell culture studies has demonstrated reduced expression of ICAM and VCAM, thereby causing reduced adhesion of monocytes to endothelium (Faruqi et al, 1994).

On the other hand randomized control trials like Alpha-Tocopherol Beta Carotene cancer prevention study (ATBC) and the collaborative primary prevention project (PPP) study showed no beneficial effect of vitamin E on risk of development of atherosclerosis (De Gaetano, 2001 & Tornwall et al 2004). Other studies which were performed to assess the role of vitamin E in secondary prevention of atherosclerosis revealed no beneficial effect.

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Furthermore meta-analysis of results available from randomized control studies performed with vitamin E to assess its efficacy in secondary prevention showed no significant reduction in the risk of cardiovascular endpoints like myocardial infarction (Vivekanathan et al, 2003).

Observational studies were able to find an inverse correlation between vitamin E consumption and atherosclerosis but they cannot establish a causal relationship. Clinical trials performed in population with preexisting disease failed to establish beneficial effects of vitamin E beyond the standard therapy followed for coronary heart disease. Certain studies performed on the molecular mechanisms involving vitamin E suggested the possible role of vitamin E as a pro-oxidant and the presence of Ascorbic Acid and ubiquinol in vivo alter Vitamin E rendering it anti oxidant in nature (Neuzil et al, 1997). These findings suggest that further insight on the antioxidant role of vitamin E in prevention of atherosclerosis is necessary to establish a causal relationship.

Vitamin C

Vitamin C, also known as Ascorbic Acid, is an essential nutrient for humans as well as a potent antioxidant, which is readily available through ingestion of fruits with high citric acid content. In discussing its purported mechanism of action, vitamin C is believed to play a vital role in reducing the adhesive nature of monocytes to vascular endothelium. Furthermore, Ascorbic Acid levels were found to be low in chronic smokers and the monocytes isolated from these smokers displaying increased adhesiveness in comparison to monocytes isolated from subjects who received vitamin C supplementation (Carr et al, 2000). Studies performed on the molecular mechanisms of Ascorbic Acid have demonstrated that it plays a major role in promoting endothelial nitric oxide mediated vasodilatation of blood vessels (Carr et al, 2000).

Onen et al have established that vitamin C deficient men are at increased risk of developing myocardial infarction when compared to individuals with higher plasma vitamin C levels. A large prospective cohort study involving 19,496 men and women revealed an inverse relationship between levels of plasma Ascorbic Acid and cardiovascular disease mortality (Teekhaw et al, 2001). Analysis of available data from national health and nutrition examination survey (NHANES) also suggested an inverse association between serum ascorbic acid levels and cardiovascular disease (Simon et al, 1998). In comparing the relative effectiveness of various anti-oxidant vitamins, a major cohort study was performed by pooling data from nine large, prospective studies which revealed that high intake of supplemental vitamin C was associated with reduced risk of cardiovascular disease and that the risk reduction from vitamin C was greater than that from ingestion of vitamin E (Knekt et al, 2004). To further compare the disease preventive effects of these two vitamins, the Antioxidant Supplementation in Atherosclerosis Prevention study assessed the role of vitamin E and vitamin C, both independently and together, in preventing the progression of intima wall thickness; this measurement having been found to be associated with ASVD risk. This study found that the combined action of vitamin E and vitamin C significantly reduced the progression by about 74% than when either of them was used alone (Salonen et al, 2000). In contrast to the above-mentioned findings, a few studies have reported that consumption of vitamin C at levels as high as 1162 mg per day is not associated with significant reduction in

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risk of coronary disease (Rimm et al, 1993). Therefore, a certain level of uncertainty persists regarding the antioxidant effects of vitamin C have a role in preventing atherosclerosis.

Bilirubin

Bilirubin is the breakdown product of heme, which has anti-oxidant properties. Unlike vitamin E and vitamin C, studies involving therapeutic intervention with Bilirubin are not feasible but various epidemiological studies showed that Bilirubin plays a protective role in cardiovascular disease. In-vitro studies have confirmed that Bilirubin protects LDL from oxidation injury, suggesting that Bilirubin may have a role in prevention of atherosclerosis, (Stoscker et al, 1987). The work of Minetti et al (2002) suggests that Bilirubin has the ability to deplete oxidants through a hydrogen donation mechanism which would explain the molecular basis for its antioxidant nature. However, anecdotal evidence taken from study of individuals with elevated bilirubin levels does suggest a protective effect; the incidence of ischemic heart disease has been reported to lower than expected in patients with Gilbert's syndrome, a form of congenital hyperbilirubenemia.

The National Heart, Lung, and Blood Institute Family Heart Study assessed the relation between serum bilirubin levels and risk of cardiovascular disease. The study observed a significant relation between lower Bilirubin levels and coronary heart disease and proposed that the inferred major gene for Bilirubin may be protective against CHD (Hunt et al, 2001). A prospective study which was performed in a large population of men revealed a U-shaped relationship between serum Bilirubin level and the risk of ischemic heart disease. This relationship was consistent even after other risk factors for cardiovascular disease were taken into consideration (Breimer et al, 1995). Prospective epidemiological study of myocardial infarction (PRIME), a large multi-centered prospective study has established low levels of serum Bilirubin as a novel marker for increased risk of cardiac disease among middle aged men. Furthermore, a meta-analysis of the data from various studies investigating the association between bilirubin levels and ASVD has confirmed an inverse relationship between serum Bilirubin and risk of developing cardiac disease. Unlike vitamin E and vitamin C, where some evidence suggests that they might not be beneficial in cardiac disease, all studies performed on the role of Bilirubin indicate that low serum bilirubin levels are a significant risk factor in progression of cardiovascular disease (Novotny & Vitek, 2003).

Uric Acid

Uric acid was once considered an end-product of purine metabolism where increased levels were only associated with gout. Moreover, it was thought that the antioxidant nature of Uric acid was supposed to be protective against cardiovascular disease; however, hyperuricemia has been frequently found to associated with ischemic heart disease. Uric acid has been proposed to cause hypertension, and thereby involved indirectly in the causation of coronary heart disease. Furthermore, several in-vitro studies have established the role of uric acid in stimulating the proliferation of vascular smooth muscle cells. Uric acid is believed to stimulate the major chemo-attractant protein (MCP) in vascular smooth muscle cells which is one of the major mediators of inflammatory changes in atherosclerosis (Johnson et al, 2003).

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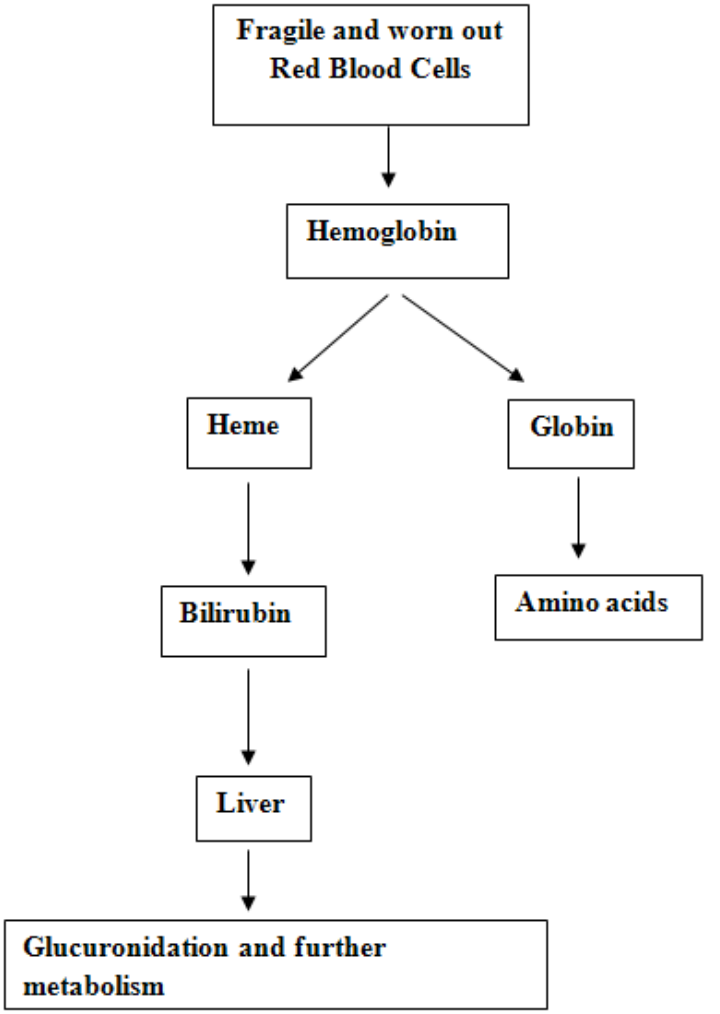

Figure 2. Bilirubin metabolism.

The relation between serum Uric acid and coronary heart disease was established as early as 1951 by Gertler MM and White PD. The relationship was confirmed by a large epidemiological, study in 1967 (Framingham study) which revealed that elevated serum uric acid levels were associated with an increased risk of coronary heart disease in men between the ages of 30-59 (Kannel et al, 1967). The role of uric acid in the pathogenesis of atherosclerosis has been extensively studied during the last few years. Various large epidemiological studies have demonstrated elevated serum uric acid levels in people diagnosed with cardiovascular disease, but it was debated if Uric acid could be considered as an independent risk factor in pathogenesis of the disease. A few studies performed using multivariate analysis models have established that serum Uric acid should not be considered as an independent risk factor in causation of heart disease (Moriarity et al., 2000). However, these studies were criticized for not adequately taking confounders into consider, which has been conjectured to be the reason why no association was found.

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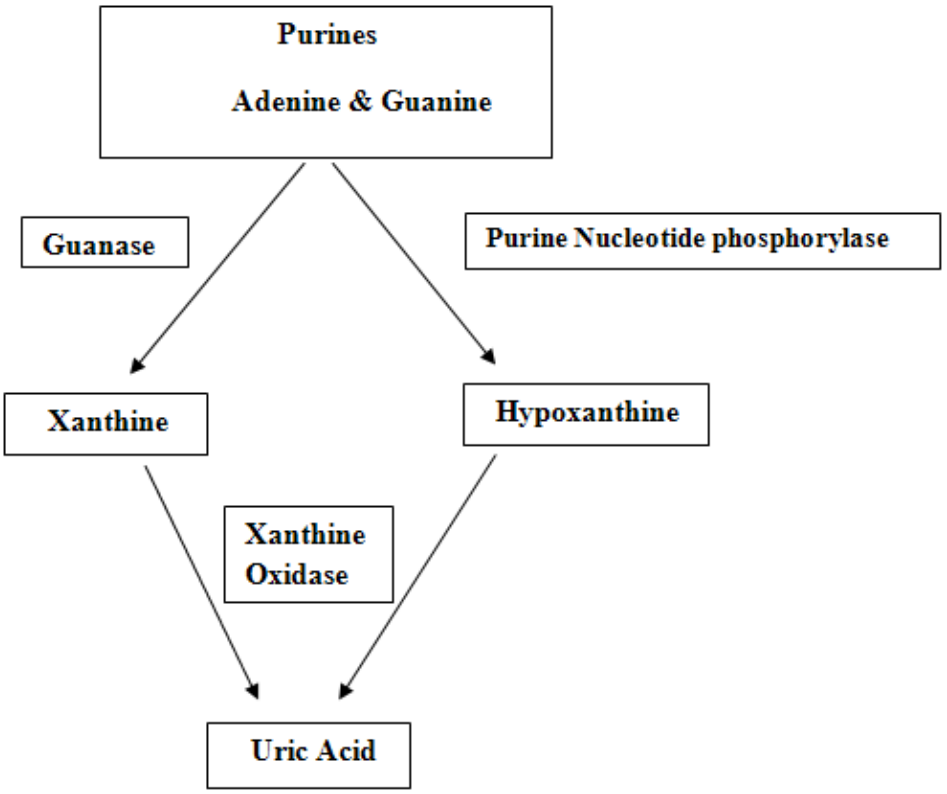

Figure 3. Uric acid metabolism.

A few authors have also hypothesized that elevated levels of Uric acid found in patients with coronary heart disease may be a compensatory mechanism to reduce the associated vascular inflammation. Studies performed by Johnson et. al., and others, after controlling for other risk factors, have established serum Uric acid as an independent risk factor in the causation of ASVD (Johnson et al, 2003). Recent studies have indicated that serum Uric Acid levels can also be used as a predictor for monitoring the progression of atherosclerosis and possibly be used in identifying people at increased risk of vascular disease (Rodrigues et al, 2010). Another interesting aspect of uric acid metabolism is that vitamin C has been found to have a uricosuric effect. Observational studies have shown significant inverse association between the levels of vitamin C and serum uric acid at vitamin C levels of 400-500 mg per day (Gao et al, 2008). Randomized control trials also have shown a significant reduction in the levels of serum uric acid with supplementation of 500 mg per day of vitamin C for duration of two months (Huang et al 2005). Further insight on the role of uric acid in the development of ASVD and monitoring its progression is required before any recommendations to reduce the uric acid levels can be suggested as a clinical guideline.

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DISCUSSION

With increasing evidence about the role of oxidation mechanisms in the pathogenesis of atherosclerotic vascular disease, the role of antioxidants in prevention of this disease has become a primary focus. Despite the presence of evidence that antioxidants can play a significant role in reducing the progression of atherosclerosis, several epidemiological studies have proven otherwise. The levels of dietary antioxidants such as vitamin E and vitamin C can be modified but the evidence that they are beneficial in preventing the prognosis of atherosclerosis is equivocal. On the other hand, various studies confirm that serum Bilirubin level has definite inverse association with atherosclerosis but Bilirubin levels cannot be altered by therapeutic intervention. Levels of serum uric acid can be modified by dietary and therapeutic intervention but existing evidence doesn't prove uric acid as an independent risk factor in the development of atherosclerosis. We conclude that further studies are required to confirm the role of antioxidants in progression of atherosclerosis before recommending any dietary or therapeutic interventions to prevent atherosclerosis.

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Chapter VII

VASA VASORUM HYPOXIA: A POSSIBLE SOLUTION FOR THE CHOLESTEROL CONTROVERSY

Tuohimaa Pentti [*] ***and Järvilehto Matti***
Fimlab Laboratories, Tampere, and Department of Biology,
University of Oulu, Oulu,Finland

ABSTRACT

It is unlikely that cholesterol initiates atherosclerosis, because most of the risk factors (smoking/nicotine, low physical activity, high blood pressure, stress and apnea) or protective factors (high physical activity, vitamin D and alcohol consumption) are minimally linked with serum cholesterol levels. Furthermore, the site of the initial development of atherosclerosis cannot be explained on the basis of cholesterol hypothesis. We propose an alternative hypothesis called "vasa vasorum constriction/hypoxia", which logically covers all previous hypotheses and is in good agreement with risk and protective factors. We postulate that a small constriction of peripheral arteries (including external vasa vasorum) will lead to a progressive hypoxia in the branching areas of these end arteries deep in the smooth muscle layer. This leads to a prolonged contraction and increasing oxygen consumption. Hypoxia will develop to a severe anoxia and capillary damage. Macromolecules (HDL-, LDL-cholesterol= "cholesterol hypothesis", microbes= "microbe hypothesis", matrix vesicles = "microvesicle hypothesis" etc) leak into the wall of the artery (extravasation). An inflammation begins ("inflammation hypothesis"). Hypoxia/anoxia will also cause neoangiogenesis and regeneration.

According to our hypothesis, a high physical activity prevents atherosclerosis, because it causes a peripheral vasodilatation. On the other hand, nicotine is known as peripheral vasocontrictor and therefore it is a risk factor. The beneficial effects of statins might be due to their vasodilatatory properties in addition to their anti-inflammatory action. The hypothesis suggests that peripheral arterial vasodilatation could be more suitable for primary prevention of atherosclerosis than statins, which are not as successful in the primary prevention as in the secondary prevention.

[*] E-mail: pentti.tuohimaa@uta.fi.

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INTRODUCTION

Lipid/cholesterol hypothesis was originally proposed by Rudolf Wirchow in 1856, but Nikolai Anitschkow showed the first experimental evidence for the possible role of cholesterol in atherosclerosis in 1913[1]. Finally, Ancel Keys in 1953 proposed that serum fatty acids and cholesterol cause atherosclerosis [2]. Two Nobel Prices strengthened the significance of cholesterol in atherosclerosis: Konrad Bloch and Feodor Lynen shared the Nobel Price in Physiology and Medicine in 1964 for their discoveries concerning the regulation of cholesterol and fatty acid metabolism and Michael S. Brown and Joseph L. Goldstein in 1985 for the clarification of the signaling pathway. "Framingham study" led to a conclusion that the higher serum cholesterol the highes is risk of coronary disease [3]. The results from the early cholesterol-lowering diet trials were controversial raising doubts about the validity of the lipid hypothesis [4]. This begun a discussion referred to as the "cholesterol controversy". Later some diet studies have demonstrated a positive correlation between a decreased dietary fat consumption and a decreased risk of coronary disease [5]. As a result a worldwide production of low-fat food products was launched, which besides statin drugs is the major consequences of the lipid hypothesis.

It was postulated that clinical trials with statins would finally solve the cholesterol controversy. The Coronary Primary Prevention Trial [6] and Scandinavian 4S study [7] among others supported the cholesterol hypothesis. However several negative studies were ignored [8] and the debate is continuing. Although there seems to be a general consensus on the cholesterol hypothesis as proven [9], from time to time there seems to be criticism against the hypothesis. It has been argued that the hypothesis is based on associations and misrepresented or over-interpreted data, and has not been shown a scientifically validated causal mechanism [10, 11]. In fact, even in the positive studies with statins the decrease of the cardiovascular mortality is not impressive. One would expect more robust decrease in mortality, if cholesterol were the cause of atherosclerosis.

Since clinical trials with statins seem to be important evidence for cholesterol hypothesis, it has been proposed that the pleiotropic effects of statins such as anti-inflammary action and decrease of blood pressure might contribute to the cardioprotection [12]. Statins were developed to inhibit 3-hydroxy-3-methylglutaryl coenzyme A, but they at the therapeutic concentration may interact with vitamin D receptor leading to its activation [13]. This is in good agreement with the result that vitamin D is necessary for nicotine-induced atherosclerosis in rats, a mdel used for atherosclerosis experiments [14]. Also clinical data suggest that vitamin D is involved in the development of atherosclerosis [15]. Here we criticize the logical failures of the cholesterol hypothesis attempting to make a comprehensive synthesis covering the known facts of atherogenesis.

DILEMMAS OF THE CHOLESTEROL HYPOTHESIS

The present cholesterol hypothesis includes the following events: 1. Increased serum (oxidized) LDL cholesterol will lead to an enhanced extravasation of the macromolecules via endothelial inflammation and an increased risk of development of atherosclerosis, whereas serum HDL cholesterol will counteract this development. 2. The lipoprotein particles pass

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through the endothelial cells (transcytosis) either via LDL cholesterol receptors [16] or without them. 3. LDL cholesterol accumulates in the intima. 4. (Inflammation) activated macrophages express scavenger receptors and begin phagocytosis of LDL cholesterol forming foam cells. 4. Lipoprotein plaques are formed, and they are gradually calcified. 5. The plaques induce neovascularization. 6. As the plaque grows, it will rupture into the lumen and blood platelets attach to the damaged endothelium forming a blood clot leading to hypoxia.

The above hypothesis includes several critical problems: 1. It is not proven that high serum oxidized LDL cholesterol can cause endothelial inflammation. 2. Endothelium is one of the tightest membranes in the body. No macromolecules are able to pass through endothelium, if it not seriously damaged. If LDL cholesterol utilizes its receptors, it will internalized via clathrin coated pits and destroyed in lysosomes. 3. Familial hypercholesterolemia (FH) is regarded as an evidence for cholesterol hypothesis, because the associated atherosclerosis can be prevented by statins, although sometimes add-on drugs (cholestyramine, nicotinic acid or fibrates) are needed [17]. The disease is usually caused by mutations in the LDL receptor or apolipoprotein B gene, both leading to a deficient binding of LDL to its receptor. In fact, this is against cholesterol hypothesis, if the receptor is needed for the transcytosis [16]. It is more likely, that the high risk of atherosclerosis in FH is associated with the disease, but caused by other factor(s) [18].

Furthermore, the cholesterol hypothesis cannot explain several phenomena of the development of atherosclerosis: 4. The formation of cholesterol plaques occurs deep in the intima (close to the smooth muscle media)[19, 20]. One of the earliest signs of atherosclerosis is smooth muscle damage [21]. If LDL particles penetrate through endothelium or its junctions, they should first appear immediately under the endothelium. How the particles "jump" from the subendothelial intima to their actual site between intima and media, is not known. 5. The plaques are initially formed at the branching areas of the arteries. It has been speculated that the turbulence of the blood stream at the branching areas might be the cause, but this has not been proven. 6. Plaques contain several different microbes and viruses, which are thought to cause atherosclerosis (microbial hypothesis). In fact, plaques contain a history of the infections of a individual [22]. The cholesterol hypothesis contributes no explanation. 7. The plaques are not present in veins, intramural coronary arteries or in pulmonary arteries. High serum cholesterol should be as fatal to all parts of the vasculature, but it is not. Only the differences in blood pressure and vasa vasorum can explain the fact as described below.

VASA VASORUM CONTRICTION/HYPOXIA HYPOTHESIS

We postulated that a functional hypoxia of the most peripheral vasa vasorum (vv) develops gradually in response to a constriction of the peripheral small arteries and hypertension compressing intramural small arteries and capillaries in the wall of large arteries [23](Figure 1). The external vv originate from the branches of the main artery and they run longitudinally along the media-adventitia border [24]. The branches of vv run circumferentially or retrograde towards the branch point. Vasa vasorum are functional end arteries. The oxygen perfusion of the wall of the main artery comes into the intimal layer directly from the lumen (outward diffusion) and into the adventitia and media from the vasa vasorum (inward diffusion). The putative sequential events of atherosclerosis are:

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1. Peripheral vasoconstriction
2. Hypoxia in the smooth muscle layer
3. Prologed contractions of smooth muscle
4. Increased oxygen consumption, severe hypoxia or anoxia
5. Capillary damage and increased permeability
6. Extravasation of LDL- and HDL-cholesterol, microbes and inflammatory cells
7. Inflammation, neovascularization
8. Plaque formation and calcification
9. Rupture of the calcified plaque

Our vasa vasorum hypoxia hypothesis includes that not cholesterol or microbes are the initial cause of the atherosclerosis but vasoconstriction (and consequent hypertension) begins the fatal process. Our hypothesis includes the cholesterol, microbial and inflammation hypotheses, since accumulation of cholesterol, microbes and inflammatory cells are consequences of damages of capillaries in the arterial wall allowing free efflux of the macromolecules, microbes and inflammatory cells. The inflammation activates macrophages to express scavenger receptors and the process continues as described by the cholesterol hypothesis. According to our hypothesis, serum cholesterol concentration plays no role in the initiation of atherosclerosis, which is in agreement with the poor clinical result in the primary prevention of the disease by statins [25]. On the other hand, the positive results in the secondary prevention by statins might be explained by our hypothesis, because the further development of plaques could slow down with lower serum cholesterol. However, statins do have actions, which better fit to our hypothesis such as vasodilatation, decrease of blood pressure and anti-inflammation. The recurrent branch of the external vv ends to the concave angle of the arterial bifurcation. This is the most vulnerable part of vv to vasoconstriction and hypertension from two sides compressing the intramural artery. According to Lame's law the oxygen perfusion from the vv is limited leading to hypoxia in the oxygen demanding smooth muscle layer. Since the muscle contraction is prolonged in hypoxia, the situation is progressive, unless peripheral vasodilatation increases the perfusion of the most distant vv.

DISCUSSION

Rhytmical contractions of aterial smooth muscles are crucially important for the blood circulation. The contractions need a lot of oxygen. Oxygen supply for the smooth muscle layer comes entirely from the external vasa vasorum. Even a small constriction of the vasa would lead to hypoxia, because these vessels are end arteries. Contractions will be prolonged and more oxygen is needed, therefore hypoxia gets worse and worse leading to capillary damage. Our vasa vasorum constriction/hypoxia hypothesis can explain the conflicting clinical results with fat restricted diet or statin prevention trials even within Scandinavia [7, 25], since cholesterol is not involved in the initiation of atherosclerosis, but may be moderately involved in the progression of the disease. There are several risk factors associated with atherosclerosis including sedentary lifestyle, hyperlipidemia, elevated serum cholesterol and triglycerides, obesity, smoking, hypertension, stress, male gender, sleep apnea, infections and diabetes mellitus [5, 26-29]. Protective factors include active sports,

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female gender (estrogen), vitamin D_3, high HDL cholesterol, caloric restriction, low body weight, moderate alcohol use. All the risk factors seem to be vasoconstrictive, whereas many of the protective factors are vasodilatative, but only few of them affect serum cholesterol levels. Therefore the cholesterol hypothesis cannot explain the situation, whereas our vasa vasorum hypoxia hypothesis is in good agreement [30]. Nicotine is known as high risk factor for atherosclerosis. However it does not affect serum lipid composition [31]. Nicotine is strongly vasoconstrictive for the peripheral vessels [32]. It reduces production and bioavailability of nitric oxide (NO), increases production and release of endothelin. Obesity and metabolic syndrome are well known risk factors of atherosclerosis. Typical lipid profile and hypertension belong to the definition of the metabolic syndrome [33]. Eventhough stress is a clear risk factor for atherosclerosis [34] and it is often associated with changes in serum lipid profile, the mechanism is not known and it is unlikely that stress could directly regulate cholesterol metabolism. In vascular stress, endothelin-1 is released from the endothelium leading to a peripheral vasoconstriction [35]. Endothelin-1 plays an important role in hypertension by increasing peripheral resistance via vasoconstriction [35], but it does not affect serum lipids. Sleep apnea can directly cause hypoxia in vasa vasorum, but it is a high risk factor when it is combined with hypertension [36]. All the protective factors in atherosclerosis seem to be vasodilators. Estrogens may explain the sex difference in serum lipid profile [37], they are also known to dilate peripheral arteries [38]. Vitamin D is proven to be effective in prevention of atherosclerosis [15], it has weak or no effect on serum lipids. Physical exercise seems to lower LDL cholesterol, but it does not affect HDL cholesterol [39], its vasodilatating effects are obvious. Statins were developed to lower serum cholesterol, but their pleiotropic effects include vasodilation [40]. Besides its beneficial effect on HDL, niacin can dilate peripheral vessels causing flush [41]. The effect of alcohol on atherosclerosis is controversial, although its beneficial effects of a moderate alcohol use are well documented [42]. It is clear that acute low doses of EtOH increase both the release of NO and endothelial NOS (eNOS) expression, and augment endothelium-mediated vasodilatation, whereas higher doses impair endothelial functions [43]. It is interesting that the pleiotropic effects of statins include vasodilation and decrease of blood pressure [44-47]. Although the pleiotropic effects of statins have been widely analysed, their significance in the prevention of atherosclerosis has been neglected. The ability of nicotinic acid to strongly increase the plasma concentration of high-density lipoprotein (HDL) cholesterol has in recent years led to an increased interest in the pharmacological potential of nicotinic acid. [41, 48] Flushing is regarded as an adverse effect of niacin, results from GPR109A-mediated production of prostaglandin D2 and E2 in Langerhans' cells which act on DP1 and EP2/4 receptors in dermal capillaries causing their vasodilatation [49]. DP1 receptor antagonist (laropiprant) attenuates the niacin flush in animals and humans. A reformulated preparation of extended-release niacin lowers flushing compared with the extended-release niacin. Aspirin pretreatment seems to attenuate flushing from this preparation. However, these combination drugs prevent most of the peripheral vasodilatation and may, thus, be less effective in decreasing blood pressure, which might be also a beneficial effect of niacin. We propose that it is reasonable to re-evaluate the goals in the primary prevention of atherosclerosis. It seems that vasodilatation might be the most important, whereas lipid-lowering drugs may delay the progression atherosclerosis in the secondary prevention, when combined with vasodilatation. Therefore, statins are optimal in the secondary prevention, but also niacin by increasing HDL should be useful, if its capacity for peripheral vasodilatation is maintained. Since there is a significant comorbidity between

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coronary disease and chronic obstructive pulmonary disease (COPD) and a common risk factor (smoking), we assumed that there could be similar changes in vasa nutritia of the terminal bronchioli as in the walls of coronary arteries (Tuohimaa and Järvilehto in press 2012). In fact, smoking is almost the only cause of COPD and constituents of the cigarette smoke are thought to irritate epithelium to begin inflammation and bronchoconstriction. We expected that LDL cholesterol plaques could be found in the bronchial wall, however, there are no visible plaques, but cholesterol is found inside the bronchial lumen [50]. This intraluminal cholesterol cannot come from the lumen, but it comes from the vasa nutritia, which are similarly contracted by nicotine as vasa vasorum leading to hypoxia and extravasation of LDL cholesterol. In COPD, the intraluminal LDL/HDL cholesterol is degraded and the released cholesterol changes the properties of surfactant leading to emphysema. It can be concluded that all risk factors of atherosclerosis are vasoconstrictive and hypertensive and all protective factors are vasodilatative, but not all of them influence cholesterol levels suggesting that cholesterol cannot explain all the development of atherosclerosis, but vasocontriction and blood pressure might be more crucial. According to our "vasa vasorum hypoxia" hypothesis the vasodilatation would be beneficial in the prevention of the atherosclerosis, which fits well with the clinical experience. NO is a critical endothelium-derived vasodilatatory factor with anti-atherosclerotic properties [51].

Peripheral vasodilatators are not available, because they proved to be unsuitable for clinical practice, since orthostatic hypotension was serious side effect, which was difficult to control. It seems that it would be reasonable to begin a new pharmaceutical development of this class of antihypertensive drugs for the prevention of atherosclerosis.

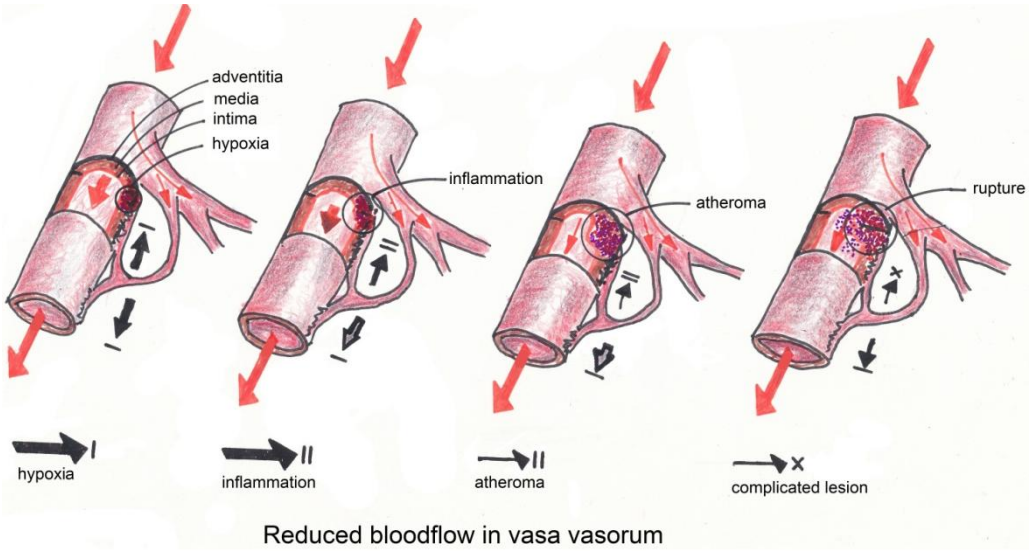

Figure 1. Vasa vasorum constriction/hypoxia hypothesis. Atherosclerosis begins with vasoconstriction of the external vasa followed by hypoxia in the most vulnerable area, intima-media border at the branching area of the artery. Hypoxia leads to a prolonged contraction of smooth muscle and therefore more severe hypoxia and inflammation. After destruction of endothelial barrier, macromolecules extravasate and plaque is formed. Intima= inner layer of artery, media= smooth muscle layer, adventitia= outer loose connective tissue layer containing plexus of the external vasa vasorum.

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Chapter VIII

CORONARY ARTERY ATHEROSCLEROSIS MEASURED BY A HYBRID SPECT/CT CAMERA

Shinro Matsuo*, Kenichi Nakajima and Seigo Kinuya
Department of Nuclear Medicine, Kanazawa University, Kanazawa, Japan

ABSTRACT

Detection of coronary calcification is a highly sensitive marker of underlining coronary atherosclerosis. A hybrid SPECT/CT made it possible to detect coronary atherosclerosis and myocardial perfusion simultaneously. We discuss coronary atherosclerosis, including calcification and coronary endothelial function, to determine whether the combined approach may serve as an important method for monitoring the treatment and making decision in further invasive investigations.

Coronary artery disease remains the leading cause of death in most industrialized countries for both men and women. The prevention and treatment of atherosclerosis require detailed evaluation of the vessels. Coronary artery calcification is a highly sensitive marker of underlying coronary atherosclerotic disease. Calcification is a regulated process prompted by inflammation in the coronary artery. The amount of coronary artery calcium is considered to reflect the patient's coronary atherosclerotic burden and correlates closely with the likelihood of future cardiovascular events [1].

Risk stratification is essential in the management of coronary artery disease. Myocardial perfusion imaging using ECG-gated single photon emission computed tomography (SPECT) can offer important incremental prognostic information on the subjects. Nuclear cardiology techniques are uniquely advanced to address the determinants of prognosis by measuring stress-induced ischemia or function [2] [3] [4]. These measurements examine the extent of infracted myocardium and the amount of jeopardized myocardium. Additional coronary artery calcification (CAC) testing to a prediction model based on traditional risk factors

* Corresponding author: Department of Nuclear Medicine, Kanazawa University Hospital, 13-1 Takaramachi, Kanazawa, 920-8641, Japan. Tel: +81- 76-265-2333, Fax: +81 76-234-4257. Shinro Matsuo, M.D., PhD., E-mail: smatsuo@nmd.m.kanazawa-u.ac.jp.

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significantly improved the classification of risk and placed more individuals in more precise risk categories [5] [6].

A HYBRID SPECT/CT SYSTEM

A hybrid SPECT/CT system has been introduced recently in clinical settings and has been available in many countries [7] [8]. The CAC score can be used to estimate the total magnitude of atherosclerotic burden. A hybrid SPECT/CT system may provide subclinical information of atherosclerosis without any extra-cost. Recently emerging guidelines have acknowledged the strong relationship between the increasing burden of coronary calcification and future cardiovascular events. Detection of coronary calcification using SPECT / CT is a quick check and relatively easy, except for the exposure of the X-ray, which can be enforced without causing pain to the subject.

The electron-beam CT (EBCT) measurement of coronary artery calcification score established by the Agatston methods, quantitative evaluation of calcified plaque in coronary artery calcification area and the CT value (Hounsfield Unit; HU) are calculated. This value can be classified into stages according to the degree of calcification (Figure 1). Instead, a recent advanced SPECT/CT system makes it possible to obtain myocardial perfusion imaging and coronary calcification information at the same time. Combined SPECT/CT acquisition in a one-stop examination is more acceptable, and physicians can obtain perfusion and atherosclerotic information without any extra-cost.

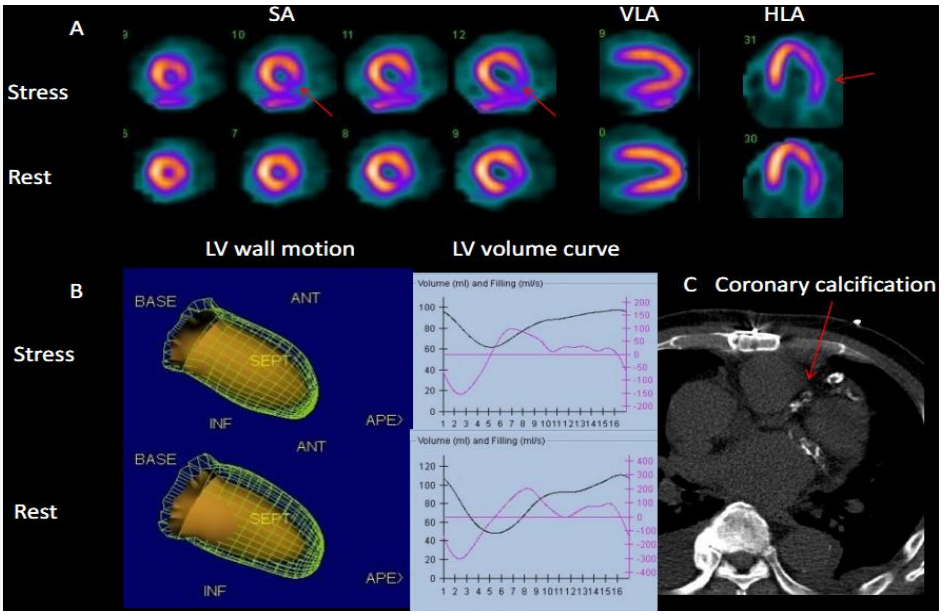

Figure 1. Coronary calcification and ischemia. A 71 year-old male with myocardial ischemia. A) The stress myocardial perfusion imaging demonstrated inferolateral ischemia in the SPECT images. SA short axis, VLA vertical long axis, HLA horizontal long axis. B) Quantitative gated SPECT showed decreased inferolateral wall motion and ejection fraction on the stress image. C) Coronary calcification on the middle portion of the left circumflex coronary artery and the left descending coronary artery was observed.

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RELATIONSHIP BETWEEN CORONARY ARTERY CALCIFICATION AND MYOCARDIAL ISCHEMIA

Evaluation of coronary calcium can be used to predict high major cardiovascular events in patients with a high CAC score. This notion is supported by a large population study [9]. CAC scores are known to be generally predictive of a higher likelihood of ischemia on myocardial perfusion imaging [10] [11]. In our study, grouping the subjects by calcium score clearly demonstrated a trend toward ischemia and infarction on myocardial perfusion imaging in patients with increasing CAC scores, especially among patients with a CAC of 1000. Table 2 shows that the relationship between CAC and myocardial perfusion abnormalities in 105 patients (71±12 years old) with known or suspected coronary artery disease[8]. The subjects without coronary calcification have reportedly very low prevalence of myocardial perfusion abnormalities when it is used for screening of coronary artery disease [10]. However, when it is used to evaluate the populations of suspected coronary artery disease, including the subjects with moderate risk, CAC scores of <10 for subjects does not necessarily mean a normal myocardial perfusion. In fact, the absence of CAC did not necessarily confer normal coronary flow reserve, which limited the predictive value of CAC scoring in patients with known or suspected coronary artery disease. The subjects with inducible myocardial ischemia reportedly had a higher CAC score than those without ischemia [8]. A proportional relationship was demonstrated between the CAC score and the frequency of adverse cardiovascular events [5]. A representative case of evaluating ischemia and calcification is shown in Table 1.

Table 1.

0	no calcium
1-10	minimal
10–100	mild
100–400	moderate
400-1000	extensive
>1000	severely extensive

The calcium scale is a linear scale with 4 calcium score categories: The calcium score correlates directly with risk of events and likelihood of obstructive CAD.

Table 2. Coronary artery calcification and myocardial perfusion abnormalities

CAC	n	SSS	SDS
0	15	1.2±1.9	0.8±1.3
1-10	6	1.2±1.2	1.0±1.1
10-100	17	1.9±3.1	1.1±2.6
100-400	26	4.4±4.7	1.7±2.0
400-1000	16	2.9±5.3	1.8±2.3
1000-	25	5.2±6.3	2.5±2.0

CAC, coronary artery calcification; SSS, summed stress score; SDS.

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SPECT/CT showed that inferolateral ischemia, decreased inferolateral wall motion and ejection fraction on the stress image and coronary calcification on the middle portion of the left circumflex coronary artery and the left descending coronary artery were observed.

CAC IN PATIENTS WITH NORMAL CORONARY FLOW RESERVE

A previous study demonstrated that normal stress myocardial perfusion imaging is associated with low risk of cardiovascular risk with an annual event rate of 0.6-0.9% [12] [13]. Another previous large-scale clinical trial showed a clear association between the extent of perfusion abnormalities and risk of coronary events. However, the wide range of CAC scores in patients with normal MPI suggests that stress perfusion imaging is limited in its ability to delineate subclinical atherosclerosis. There is no evidence to compare the relative short and long-term risk for cardiac events among patients with both myocardial perfusion imaging and coronary calcium evaluation [14]. Another previous study showed that the cardiac event rate in elderly and diabetic patients with normal myocardial perfusion imaging was relatively higher than that in younger and non-diabetic patients. Subjects with normal SPECT may differ in the stage of atherosclerosis and have a different atherosclerotic burden in the coronary artery. Then what about the patients with high CAC and normal myocardial perfusion imaging? We assume such patients might have low risk in the short-term but high risk in the long-term for cardiovascular events, since patients with high CAC have reportedly been regarded as high risk patients [15]. Previous studies suggest patients without ischemia on myocardial perfusion imaging exhibit a stepwise increase in their risk of cardiac events with increasing CAC scores, indicating a warranty period might exist for normal SPECT imaging [16]. Even if the myocardial perfusion imaging is normal, the addition of the CAC score may improve the detection of coronary artery disease, particularly in severe multi-vessel coronary artery disease. The documentation of CAC as a marker of coronary atherosclerosis can be used to target patients requiring more intensive management of coronary risk factors.

INFLAMMATION AND CALCIFICATION

Inflammation may play an important role in atherogenesis. Inflammatory markers such as C-reactive protein (CRP), tumor necrosis factor alpha (TNF-alpha), Interleukin-6 (IL-6) and so on may aid in detecting subclinical atherosclerosis. However the association between inflammatory markers and CAC is weak, since coronary disease attributes concomitantly to coronary risk factors. The approach to use these biomarker as part of a broader risk stratification is a subject of future investigation.

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EVALUATION OF ATHEROSCLEROSIS BY SPECT/CT

CAC score data provide information on the rate of absolute and relative progression of calcification. The value of sequential calcium scoring for documentation of the efficacy of statins in reducing the progression of coronary atherosclerosis was recognized [17]. The combination of CAC and myocardial perfusion imaging can be effectively used for monitoring aggressive risk factor modification and optimal medical therapy. The integration of myocardial perfusion imaging and the CAC score is an interesting strategy for the diagnostic work-up of patients [18]. A recent clinical trial revealed that CAC testing in asymptomatic individuals can be cost-effective [19]. Since high CAC makes it difficult to evaluate coronary artery stenosis, SPECT/CT might be useful for the decision making process with regard to performing CT angiography. When high calcium is observed, CT angiography should not be performed.

Measurement of CAC is reasonable for cardiovascular risk assessment in asymptomatic patients with intermediate cardiovascular event risk [20]. With the uncertainty in cardiovascular disease, we believe that the only way to determine the best strategy is to conduct SPECT/CT testing to identify coronary artery disease in selected patients for more or less intensive treatments.

CONCLUSION

Hybrid SPECT/CT might be useful to conduct diagnostic assessment in one session in patients with known or suspected coronary artery disease. The integration of myocardial perfusion imaging and the CAC score may offer additional benefit for the diagnostic work-up of patients.

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