Dyslipidemia
Causes, Diagnosis and Treatment
DYSLIPIDEMIA:
CAUSES, DIAGNOSIS
AND TREATMENT
ENDOCRINOLOGY RESEARCH AND CLINICAL DEVELOPMENTS

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DYSLIPIDEMIA: CAUSES, DIAGNOSIS AND TREATMENT

MIROSLAVA KARAPETROVIĆ
AND ZLATKO AČIMOVIĆ
EDITORS

Nova Science Publishers, Inc.
New York

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Dyslipidemia is an abnormal amount of lipids in the blood. In this book, the authors present topical research on the causes, diagnosis and treatment of dyslipidemia. Topics discussed include diet and nutrition therapy in dyslipidemia management; fish proteins for coronary artery disease; qualitative and quantitative characteristics of low-density and endothelial lipase and its role in the metabolism of HDL-cholesterol.

Chapter 1 - Abnormal blood lipids is a condition of elevated cholesterol, low-density lipoproteins, triglycerides or low high density lipoproteins leading to hypercholesterolemia with risk of atherosclerosis or diabetes. Status of dyslipidemia changes in different age groups and ethnicities in different lifestyles. Dietary low fat intake or nutrition supplementation is the key of lowering blood lipids as art for keeping dyslipidemia in control and manageable. Use of wild and traditional foods is less known in keeping good health at low risk of dyslipidemia or cardiac diseases. Ancient view of low fat, vegan dietary intake, exercise and active life style puts forth evidence of long life expectancy at low risk of cardiovascular disease as observed among tribals in present times. Extensive clinical trials suggest ambiguous state of strictly medical treatment of dyslipidemia to completely cure the disease while it causes more side effects. Recent clinical trials show clear benefits of dietary and nutrition therapy in lipid lowering and management of dyslipidemia in order to keep cholesterol in normal range. New guidelines suggest the search of new more sensitive biomarkers of dyslipidemia or cardiovascular disease to assess the disease growth, need of active life style, better knowledge of lipoprotein metabolic control to fine tune the dosage and selection of drugs combined with dietary or nutraceutical regimens under medical supervision. Ancient traditional ways of active life style suggest benefits of ‘spiritual
acceptance’ or no affluence, no use of processed foods or discipline of regular fasts to keep low fat diet and good health with high life expectancy.

Chapter 2 - It is recognized that substantial cardiovascular (CV) risk remains even after lowering LDL-C, and that low HDL-C is associated with this residual risk. The anti-atherogenic role of HDL-C is supported by its ability to mediate reverse cholesterol transport, reduce oxidation and inflammation. This has led to substantial interest in HDL-C as a therapeutic target. However, several lines of evidence suggest that HDL-C concentration may be an inadequate representation of its anti-atherogenic effect. Considerable variation is found in cholesterol efflux capacity, a measure of HDL function, amongst individuals with the same HDL-C levels. There have also been situations in which increased CV risk was not observed in individuals with low HDL-C, such as in certain genetic conditions of low HDL-C and ApoA-1. These observations highlight the limitations of HDL-C concentration as a surrogate for anti-atherogeneity. It also points to a need for a more refined measure for use as a therapeutic endpoint. Our ability to quantify the anti-atherogenic effects of HDL-C more accurately will become increasingly important as more HDL-C based therapies are developed. There are two proposed approaches to address the limitations of measuring HDL-C levels. The first is an assessment of HDL-C heterogeneity. HDL-C exists as a heterogeneous population of subfractions of different densities, charges, sizes and apolipoprotein composition. These subfractions have been found to correlate differently with CV risk. The second approach is to quantify HDL-C function, using measures such as cholesterol efflux capacity and anti-inflammatory activity. In this chapter, we will review the existing methods of evaluating HDL-C heterogeneity and function. We will also discuss their limitations and potential clinical applications in terms of refinement of cardiovascular risk prediction, and assessment and monitoring of response to HDL-based therapies.

Chapter 3 - Cardiovascular disease (CVD) is leading cause of morbidity and worldwide. Higher levels of low-density lipoprotein cholesterol (LDL-C) are associated with an increased risk of coronary heart disease (CHD), myocardial infarction, and stroke. Guidelines of the Adult Treatment Panel III emphasize intensive reduction of LDL or non-high-density lipoprotein cholesterol in patients at high risk of CHD. Restriction of dietary saturated and trans-fat and cholesterol, along with increased intake of soluble fiber, can achieve LDL-C lowering. However, pharmacological treatment is needed in most patients. Statins are highly efficacious as LDL-C lowering agents and have more modest effects on very LDL-C, triglycerides and HDL-C levels.
The effects of statins on plasma lipids result from their ability to both increase the efficiency with which very LDL-C and LDL-C are cleared from the circulation and reduce the production of apoB-containing lipoproteins by the liver. Placebo-controlled intervention studies of statin drugs for lowering LDL-C provide clear evidence of CVD prevention. In addition, improvement of survival with statins may be due to other pleiotropic effects beyond LDL-C lowering. Side-effects, myopathy and hepatotoxicity, are infrequent and usually mild, but use of lipid-modifying medication demands caution because of the possibility drug interactions. Many recent studies suggest that low levels of HDL-C are a major independent risk factor for CVD. According to several clinical trials, a 1% increase in HDL-C is associated with a 0.7%–3% decrease in CHD events. The direct link between high levels of triglycerides and CHD is less well defined. Fibrates reduce triglycerides levels and may be beneficial for CHD prevention. Fibrates acid derivatives and niacin are primarily used to increase HDL-C levels, although with side effects. Low-dose n-3 fatty acids could be used routinely after a myocardial infarction, but the value of higher doses of n-3 fatty acids in reducing cardiovascular risk remains to be demonstrated. In conclusion, achieving low lipid levels appears safe, but the generalizability of these findings to broader populations and the clinical benefit on the reduction of cardiovascular complications remains to be proven.

Chapter 4 - Recent recommendations of federal and regulatory agencies suggest revisit the claims of fish oils rich in omega-3 fatty acids and benefits in prevention of cardiovascular disease. We propose the importance of fish proteins in prevention of CVD to offer better opportunity of lowering blood pressure and coronary artery disease burden. Fish proteins are analyzed by protein electrophoresis after extraction from fish. The nutrition value of fish protein components are estimated and compared with recommended daily allowances (RDA). However, benefits of fish proteins remain less known due to poor knowledge of clinical outcomes, endpoint calibrations and their food value. The current status of fish dietary research and possibility of their use in coronary artery disease as cardioprotective food is presented if the fish protein intake may be better approach over omega fatty acids as reliable, relevant dietary recommendations for CVD in future.

Chapter 5 - Elevated serum low-density lipoprotein cholesterol (LDL-cholesterol) concentration is firmly established as a risk factor for atherosclerosis and cardiovascular disease (CVD). Yet, a number of CVD patients have LDL-cholesterol levels within the recommended range, suggesting the need for advanced lipid testing to determine residual risk. In this manner, further improvement of risk assessment might be accomplished...
through a more detailed insight into qualitative and quantitative characteristics of LDL particles. Plasma LDL population comprise complex spectrum of particles of different size, density and lipid composition. There is now ample of evidence that certain LDL subclasses, particularly small, dense LDL particles, are superior to LDL-cholesterol in terms of CVD risk prediction. Furthermore, elevation of small, dense LDL particles is also observed in various atherosclerosis-related conditions, such as end-stage renal disease or ischemic stroke. Whereas the measurement of LDL-cholesterol concentrations has proven clinical utility, the usefulness of LDL particles characterisation in clinical practice needs to be further explored. Understanding of structural complexity of LDL particles and their functional consequences might improve prevention and prognosis of atherosclerosis-related diseases, leading toward more specialized therapeutic approaches. Therefore, the questions of whom, when and how to asses small, dense LDL particles still remain open.

Chapter 6 - It is known that the metabolic syndrome (MS) leads to serious cardiovascular disease which continues to be the number one cause of death in developed countries. MS is characterized by dyslipidemia, hypertension, glucose tolerance, and obesity. The MS distribution is growing catastrophically, but molecular mechanisms responsible for developments of complex impairments in MS still remain basically poorly investigated. The formation of complex MS symptoms suggests systemic impairments in lipid and carbohydrate metabolism; it appears that these impairments should have a common basis at the level of expression of appropriate genes. Expression of genes involved into lipid and carbohydrate metabolism is regulated by various transcription factors, including those referred to the superfamily of nuclear hormone receptors: peroxisome proliferator-activated receptors (PPAR), liver X receptors (LXR), pregnane X receptors (PXR), and constitutive androstane receptors (CAR). PXR and CAR are mainly known as the sensors of xenobiotics. Since these transcription factors are ligand-activated, they represent perspective targets for pharmacological treatments. In the recent time, the search of natural and synthetic PPAR ligands is in intensive process. There is a cross-talk between signal transduction pathways of PPAR, LXR, PXR, and CAR; this suggests their integrated role in regulation of genes of lipid and carbohydrate metabolism. In our work at the model of rat strain with Inherited Stress-Induced Arterial Hypertension which demonstrates all signs of MS development it was shown that an increased content of triglyceride, VLDL and LDL cholesterol, a decreased content of HDL cholesterol, a high level of apoB-100, and a decreased level of apoA-I, increased body weight and blood glucose level are associated with increased DNA-binding activity of PPAR,
LXR, PXR, and CAR in the liver; this suggests involvement of these transcription factors in the development of MS in ISIAH rats. We have also shown that stress can affect the DNA-binding activity of these transcription factors. In this review, the role of ligand-activated transcription factors PPAR, LXR, CAR, PXR and the role of stress in the development of metabolic syndrome will be discussed.

Chapter 7 - The endothelial lipase (EL) is an enzyme with lipolytic activity against phospholipids of high density lipoprotein-cholesterol (HDL) in plasma. The EL is synthesized by vascular endothelial cells. In particular, it is synthesized mainly in heart, muscle, and adipose tissue and then transported to the luminal surface of endothelial cells where it hydrolyzes lipoprotein triglycerides and phospholipids. In addition, the endothelial-derived EL can be a direct regulator of HDL metabolism increasing the catabolism of HDL particles, thereby reducing the levels and the size of the tank and impacting recycling. Both experimental in vitro and in vivo studies have revealed remarkable information with regard to the physiological role of EL in inflammatory conditions and its potential role in modulating lipoprotein metabolism under inflammatory conditions, including atherosclerosis. EL also seems to have local actions in the vascular wall and the regulation of circulating lipoproteins, and it seems to be associated with established and novel cardiovascular risk factors like hypertension, diabetes, dyslipidemia, obesity, and leptin, which potentially contributes to the progress of atherosclerosis and therefore could be regarded as a biochemical marker of atherosclerosis. The EL inhibition is considered to be an attractive intervention target in order to favorably modulate the atherosclerosis process by improving the HDL levels. This article reviews the effect of EL on the metabolism of HDL-C, its association with cardiovascular risk factors and the potential perspective to arrest or even improve atherosclerosis by EL inhibition.

Chapter 8 - Microvascular damage is an important pathophysiologic mechanism underlying peripheral and autonomic neuropathy. Microvascular injury is mediated by various vascular risk factors, which are also components of the metabolic syndrome. There is increasing evidence for the role of dyslipidemia in the development and progression of diabetic peripheral and autonomic neuropathy. Although small fiber neuropathy has been reported in non-diabetic patients with hypertriglyceridemia, the role of dyslipidemia in peripheral neuropathy is often examined in the context of other vascular risk factors which may confound the association. A number of long-term studies of large cohorts of patients with diabetes have been completed. In these studies over the course of seven or more years of follow-up, it is not glycemia but
serum lipids, hypertension, and body mass index that are independently associated with the risk of developing diabetic neuropathy. There is emerging evidence that the aggregate effect of all the vascular risk factors may induce microvascular damage resulting in peripheral neuropathy. Oxidative stress provoked by oxidized LDL amongst other mechanisms may play a major role and constitute a therapeutic target. Statins and fibrates may play a role in the prevention and management of diabetic peripheral neuropathy.

Chapter 9 - Objectives: Fenofibrate exerts multiple pleiotropic effects as a selective peroxisome proliferator-activated receptor alpha (PPAR-α) agonist in addition to its well-characterized lipid-lowering effects. Fenofibrate suppressed the production of pro-inflammatory cytokines through the inhibition of NFκB pathway and ubiquitin-proteasome system, leading to the decrease in the incidence of atherosclerosis and type II diabetes mellitus (DM) that are tightly associated with chronic inflammation. Anti-inflammatory and anti-oxidant activities of fenofibrate have been studied in the chronic inflammatory diseases such as rheumatoid arthritis (RA) with a higher risk of atherosclerosis-related death. Methods: In this chapter, our long-term follow-up study on the anti-inflammatory and anti-oxidative activities of fenofibrate in RA were compared with the parallel study of statins therapy and control concerning to the changes in the serum levels of lipid profile and inflammatory parameters including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), matrix metalloproteinase-3 (MMP-3), pentosidine and homocysteine. Results: Both serum lipid profile and the most inflammatory parameters such as CRP, ESR and MMP-3 remained basically stable in all the 3 groups during the 18 months study. Although serum pentosidine and homocysteine levels elevated significantly in the control group without lipid-lowering agents, the two parameters did not change significantly in fenofibrate and statins groups. Prednisolone dosage decreased significantly in all the 3 groups. Conclusions: Lipid-lowering agents such as fenofibrate and statins may play a role as anti-atherogenic and anti-oxidant agents in the chronic inflammatory disease like RA.
Chapter 1

HDL HETEROGENEITY AND FUNCTION: IMPLICATIONS FOR THERAPEUTICS

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ABSTRACT

It is recognized that substantial cardiovascular (CV) risk remains even after lowering LDL-C, and that low HDL-C is associated with this residual risk. The anti-atherogenic role of HDL-C is supported by its ability to mediate reverse cholesterol transport, reduce oxidation and inflammation. This has led to substantial interest in HDL-C as a therapeutic target. However, several lines of evidence suggest that HDL-C concentration may be an inadequate representation of its anti-atherogenic effect. Considerable variation is found in cholesterol efflux capacity, a measure of HDL function, amongst individuals with the same HDL-C levels. There have also been situations in which increased CV risk was not observed in individuals with low HDL-C, such as in certain

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genetic conditions of low HDL-C and ApoA-1. These observations highlight the limitations of HDL-C concentration as a surrogate for anti-atherogenecity. It also points to a need for a more refined measure for use as a therapeutic endpoint. Our ability to quantify the anti-atherogenic effects of HDL-C more accurately will become increasingly important as more HDL-C based therapies are developed.

There are two proposed approaches to address the limitations of measuring HDL-C levels. The first is an assessment of HDL-C heterogeneity. HDL-C exists as a heterogeneous population of subfractions of different densities, charges, sizes and apolipoprotein composition. These subfractions have been found to correlate differently with CV risk. The second approach is to quantify HDL-C function, using measures such as cholesterol efflux capacity and anti-inflammatory activity. In this chapter, we will review the existing methods of evaluating HDL-C heterogeneity and function. We will also discuss their limitations and potential clinical applications in terms of refinement of cardiovascular risk prediction, and assessment and monitoring of response to HDL-based therapies.

**INTRODUCTION**

Clinical and epidemiologic studies have consistently shown a strong inverse correlation between HDL-cholesterol (HDL-C) levels and the risk of coronary heart disease [1-3]. Indeed, even in patients treated aggressively with statins to lower levels of the primary target of cholesterol treatment, LDL cholesterol, low HDL-C remains a significant predictor of major cardiovascular events [4]. Hence, it is recognized that substantial cardiovascular disease (CVD) risk remains even after lowering LDL-C, and that low HDL-C is associated with this residual risk. Furthermore, hypercholesterolemic mice with genetic defects in HDL metabolism are markedly atherosclerotic [5], providing compelling evidence that HDL is a key modulator of the disease in animal models. These observations have led to sustained interest in the development of therapies that raise levels of HDL-C.

However, despite the abundant evidence for the inverse association between HDL-C levels and the risk for CVD, certain drugs, such as niacin and fibrates that elevate HDL-C levels, show inconsistent clinical benefit [6, 7]. Moreover, it is not completely understood how HDL in humans interacts with the artery wall to influence the progression or regression of atherclerosis. The proposed atheroprotective properties of HDL are multifaceted. A central hypothesis revolves around the role of HDL in macrophage reverse cholesterol...
transport (RCT), the process by which excess cholesterol in foam cells in atheromatous vessels, is effluxed to HDL and ultimately delivered to the liver and excreted into bile [8, 9] (Figure 1). However, whether RCT is the most important mechanism for atheroprotection by HDL remains to be conclusively established. HDL has also been shown to reduce oxidation and inflammation [10-13], inhibit platelet aggregation and coagulation, promote endothelial nitric oxide production [12] and maintain endothelial integrity through anti-apoptotic effects [10, 11] (Figure 2).

Figure 1. HDL metabolism and reverse cholesterol transport. The liver synthesizes lipid poor ApoA-I, which acquires cholesterol via the hepatocyte ABCA1 transporter. Lipid poor ApoA-I (nascent HDL) also promotes the efflux of free cholesterol from macrophages via ATP-binding cassette subfamily A member 1 (ABCA1). Lecithin cholesterol acyltransferase (LCAT) esterifies free cholesterol to cholesteryl esters to form mature HDL, which promotes cholesterol efflux from macrophages via the ATP-binding cassette subfamily G member 1 (ABCG1) transporter. Mature HDL can then transfer its cholesterol to the liver directly via cholesteryl ester transfer protein (CETP)-mediated transfer to ApoB-containing lipoproteins, with subsequent uptake by the liver via LDL-receptor. Hepatic cholesterol can then be excreted directly into bile as cholesterol or after conversion to bile acids and, unless reabsorbed by the intestine, is ultimately excreted into feces. HDL can be remodeled by lipases such as hepatic lipase (HL) and endothelial lipase (EL), which hydrolyze triglyceride and phospholipid, respectively.

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Figure 2. HDL Functions and Properties. Important HDL functional properties include promoting cholesterol efflux and reverse cholesterol transport (RCT), as well as its role in anti-inflammatory processes. These functions are distinct but may be overlapping.

These multiple actions of HDL make it an extremely complex therapeutic target, albeit one with tremendous anti-atherogenic potential.

Given this complexity, it is not surprising that several lines of evidence suggest that static measures of plasma steady state HDL-C concentrations alone may not necessarily correlate with HDL function. Plasma HDL-C levels do not provide a measure of RCT rate, nor do they reflect the other properties of HDL; and hence may be an inadequate representation of its potential anti-atherogenic effect. For example, considerable variation is found in cholesterol efflux capacity, amongst individuals with the same HDL-C levels [14, 15]. There have also been situations in which increased CV risk was not observed despite low HDL-C, such as in certain genetic conditions of low HDL-C and ApoA-1 like LCAT deficiency and Apo AI Milano mutation [16, 17]. Torcetrapib, a CETP inhibitor that dramatically increases HDL-C levels was found to be associated with an increase in CV mortality, resulting in the early termination of the ILLUMINATE trial [18]. However, this was attributed to off-target effects of increase in blood pressure and aldosterone caused by torcetrapib, and was not observed with anacetrapib, another CETP inhibitor.

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These observations highlight the limitations of depending on HDL-C concentration alone as a surrogate for anti-atherogeneity. It also points to a need for a more refined measure of HDL as a therapeutic endpoint. Our ability to quantify the anti-atherogenic effects of HDL-C more accurately will become increasingly important as more HDL-C based therapies are developed.

There are two proposed approaches to address the limitations of measuring HDL-C levels. The first is an assessment of HDL-C heterogeneity. HDL-C exists as a heterogeneous population of subfractions of different densities, charges, sizes and apolipoprotein composition. These subfractions have been found to correlate differently with CV risk. The second approach is to quantify HDL-C function, using measures such as cholesterol efflux capacity and anti-inflammatory activity. We will discuss each of these approaches in turn, highlighting potential clinical applications and limitations in terms of refinement of cardiovascular risk prediction, and assessment and monitoring of response to HDL-based therapies.

**AN OVERVIEW OF HDL-C PARTICLE COMPOSITION AND METABOLISM**

HDL is composed of a phospholipid surface with apolipoproteins and unesterified cholesterol, and a core that carries lipids. Normal HDL also contains high levels of antioxidant molecules, such as paraoxonase/arylesterase1 (PON1) and lecithin cholesterol acyl-transferase (LCAT). Approximately 40-60% of HDL is composed of lipids, such as cholesterol, cholesteryl esters, phospholipids, and triglycerides. The major apolipoproteins of HDL are apoA-I (approximately 70%), apoA-II (20%), apoA-IV, apoCs, apoE, and other apolipoproteins and enzymes (10%) [10]. The other apolipoproteins and nonlipoprotein proteins that associate with HDL are poorly characterized, though recently, Vaisar et al. [21] found forty eight HDL-associated proteins by shotgun proteomics. Interestingly, many of these proteins are not classical apolipoproteins, but rather are complement factors, acute phase proteins or proteases, supporting the concept that HDL’s function(s) may not be limited to lipid metabolism.

HDL starts its life cycle as poorly lipidated Apo-A1 (nascent HDL) which collects cellular cholesterol to avoid receptor mediated degradation in the renal tubule [22, 23] (Figure 1). Apo-A1 collects membrane cholesterol through multiple mechanisms, including specific ones requiring physical engagement...
with transmembrane lipid channels such as ABCA1, ABCG1 and SR-BI [24].
It is believed that ABCA1 transfers phospholipids and cholesterol to the
nascent HDL, whereas ABCG1 connects with the larger HDL particles [25].
SR-BI contributes significantly to lipid transfer from cells that are overloaded
with cholesterol, such as lipid laden arterial macrophages (ie. the foam cell),
and therefore potentially play an important role in atherogenesis [26].

As free (unesterified) cholesterol translocates across the cell membrane,
HDL esterifies it by adding a fatty acid chain via the action of LCAT. The
non-polar cholesteryl esters are then stored in the particle core. The core
expansion leads to compositional maturation, yielding “mature HDL” particles
that predominate in plasma HDL. HDL triglyceride content may vary as they
undergo lipolysis by hepatic and endothelial lipases to form smaller HDL
particles. These sequential changes means that HDL is heterogeneous in size
because of variations in the cargo of lipids, apolipoproteins, enzymes, and
other functional proteins that can exist on its surface. In plasma, mature HDL
switches from cholesterol acquisition to cholesterol delivery. This is achieved
mainly via CETP-mediated transfer of cholesterol to triglyceride-rich
lipoproteins and via SR-BI mediated unloading of the cholesterol cargo back
to the liver. Plasma HDL that eliminates its lipid cargo can initiate a new cycle
of peripheral cholesterol acquisition. This process, termed reverse cholesterol
transport (RCT), also encompasses the notion that the anti-oxidant and anti-
inflammatory effects are linked to the collection and removal of oxidized
lipids from cellular membranes and LDL in the arterial wall [27].

METHODS FOR MEASUREMENT OF HDL HETEROGENEITY: POTENTIAL CLINICAL APPLICATIONS AND THEIR LIMITATIONS

From the above overview of HDL metabolism, it can be appreciated that
HDL exists as a heterogeneous mixture of subfractions of different sizes and
composition. These subfractions can be separated based on different
physicochemical properties such as density, size and charge, with different
terminology ascribed to these subfractions depending on the method of
separation used.
Analytical Ultracentrifugation

The earliest method of separation of HDL particles was developed by Lindgren et al. in 1951, and involved separation based on density differences [28]. Separation was achieved based on different flotation rates in a high-salt solution. This initially separated HDL into 3 major subclasses: HDL1, HDL2 and HDL3, in order of decreasing size and buoyancy. The HDL2 subclass was subsequently further resolved to yield another 2 subclasses, HDL2a and HDL2b. The optical schlieren profiles obtained after ultracentrifugation can be used to determine mass concentrations of the individual HDL subfractions. This technique formed the basis of the earliest method of HDL quantification before the era of direct homogenous assays [29].

The major subfractions in humans are HDL2 and HDL3. HDL2 is composed of larger cholesterol-rich particles with a density in the range of 1.063-1.125 g/ml, while HDL3 particles are smaller and relatively lipid-poor, with densities in the range of 1.125-1.210 g/ml. HDL1 is a minor subfraction in humans. Several studies have shown that HDL2 and HDL3 have a differential relationship with CHD. However, it remains controversial as to which subfraction is more protective. In a prospective study over 29 years, HDL2 was found to have a stronger inverse relationship with CVD than HDL3 [30]. This echoed findings in several previous studies [31, 32]. However, the reverse was found in other studies such as the Physician’s Health Study and the Caerphilly study [33, 34]. The differences in the results could be a result of different assay methods. In view of the conflicting results, routine determination of HDL2 and HDL3 subfractions in clinical practice cannot be presently recommended.

Vertical Analytical Profile

This is another method based on separation by ultracentrifugation, with the difference that the separation is performed in a vertical rotor. This method allows quicker separation of subfractions as it occurs across the horizontal axis of the tube rather than the longer vertical axis. Although the shorter turnaround time makes it more suitable for routine clinical practice than the more laborious analytical ultracentrifugation, there is few data correlating the subfractions obtained from this technique with CVD.
Non-Denaturing Polyacrylamide Gradient Gel Electrophoresis

This technique was developed in 1981 by Blanche et al., and is based on the separation of HDL subfractions by size. HDL is first isolated by ultracentrifugation, and then subjected to gradient gel electrophoresis. This method isolates 5 HDL subfractions of different diameters. They are HDL3c, HDL3b, HDL3a, HDL2a and HDL2b in order of increasing size [35]. There is some, but limited data suggesting that HDL2b is the subfraction that is most strongly and inversely correlated with CVD. Johansson et al. reported that HDL2b was inversely related to the severity and progression of coronary atherosclerosis on angiogram [36].

Two-Dimensional Gel Electrophoresis

This technique separates HDL subfractions by size and charge. HDL typically migrates as an α-band on nondenaturing electrophoresis. Two-dimensional gel electrophoresis further separates HDL into additional subfractions: 1) pre-β-1 HDL, 2) pre-β-2 HDL, 3) pre-α 1,2,3 HDL, and 4) α HDL. These particles have different sizes, chemical composition, and physiological functions. Pre-β-1 HDL is the predominant form of HDL produced by the liver. It is a very small flat disc-like precursor of HDL (diameter 5.6 nm) that contains apo AI, phospholipid, and minimal cholesterol. It is an efficient activator of ATP binding cassette transporter A1 (ABCA1), and is thought to be the most effective HDL particle in terms of promoting cholesterol efflux [37]. Intravenous administration of delipidated HDL particles similar to pre-β-1 HDL in animal models of has been found to lead to regression of atherosclerosis [38].

After incorporation of cholesterol, pre-β-1 HDL enlarges to form α HDL. α HDL can be further resolved by 2D electrophoresis to α-4 HDL, α-3 HDL, α-2 HDL and α-1 HDL, in order of increasing size. α-4 HDL are made up of apo AI, phospholipid and free cholesterol. α-3 HDL and α-2 HDL contain apo AI, apo AII, phospholipid, free cholesterol, cholesterol ester and triglyceride. α-1 HDL, unlike α-2 HDL and α-3 HDL, have very little apo AII. Among the α HDL particles, α-1 HDL and α-2 HDL interact the most efficiently with the liver scavenger receptor B1. [39] The function of Pre-β-2 HDL and pre-α HDL is unclear at present.
The results from several prospective studies suggest that the quantification of HDL subfractions by 2D electrophoresis may be superior compared to HDL measurements alone. In the Framingham Offspring Study, α-1 HDL had the strongest inverse correlation with CVD. CV risk was also inversely correlated with pre-α-3 HDL levels, and positively correlated with pre-β-1 HDL and α-3 HDL levels [40]. Similarly, in the Veterans Affairs HDL Intervention Trial (VA-HIT), α-1 was inversely correlated with CVD, while α-3 was positively correlated. In addition, α-2 was also found to be protective against CVD [41].

**Nuclear Magnetic Resonance (NMR) Spectroscopy**

The use of NMR spectroscopy to measure lipoprotein particle concentrations was first developed in the 1990s at the North Carolina State University [42]. This method allows the measurements of HDL particle numbers without need for prior physical separation of individual subfractions. It is based upon the principle that different HDL subfractions of varying sizes produce distinct NMR signals at different frequencies, and these signals are directly proportional to the number of particles. The signals used for quantification are emitted from the terminal methyl group protons found in phospholipid, unesterified cholesterol, cholesterol ester and triglyceride, which form the lipoprotein particle. These combine to form a composite signal that is distinct for each subclass of HDL [43]. The overall NMR signal obtained from a plasma sample is a combination of signals from subpopulations of HDL, LDL, VLDL and chylomicrons. This signal is deconvoluted computationally to extract individual subclass amplitudes. Using this method, HDL particles are subclassified as small, medium and large particles, and reported in moles of particles per liter.

There have been several studies published correlating HDL particle number quantified by NMR spectroscopy and CVD. In a case-control study involving the EPIC (European Prospective Investigation into Cancer and Nutrition)-Norfolk cohort, total HDL particle number was found to be an independent predictor of CVD [44]. Total HDL particle number was found to be more strongly correlated with recurrent CHD in the VA-HIT trial, and with carotid intima-media thickness in the MESA (Multi-ethnic Study of Atherosclerosis) trial, compared to HDL cholesterol measurements [45, 46]. In terms of HDL particle size distribution, an inverse relationship between large and small HDL particles have been observed, with large HDL particles being
positively associated with CVD and small particles being negatively correlated [47].

Small HDL particles were also found to be an independent predictor of recurrent cardiovascular events in the VA-HIT trial [41]. Unexpectedly, in the JUPITER trial, neither HDL-C nor HDL size, determined by NMR, were associated with residual CVD risk in patients after treated to goal (LDL-C < 70 mg/dl) with statins, although statins selectively increase the large HDL2/a-1 subclass [48]. The result was in contrast with others’ findings [49-51] and the validity of the statistical analysis has been argued [52]. One problem estimating the influence of HDL size on CVD risk is that patients with low concentrations of large and high concentrations of small HDLs also have many other atherogenic factors including increased concentrations of triglycerides, small dense LDL particles and C-reactive protein [53]. Without conducting statistical analyses that address these confounding correlations, misleading conclusions may be reached about the clinical importance and potential functional differences among HDL subclasses [54].

Nonetheless, NMR spectroscopy shows promise for future development for clinical use, as it facilitates accurate and reproducible measurements with a rapid turn-around time. This is especially so as more data correlating these measurements with clinical outcomes are accrued. This method will also contribute to refining HDL measurements in clinical research and provide new information into the physiology of HDL.

**Ion Mobility**

Ion mobility, also known as gas-phase differential electrical mobility, separates HDL particles based on differences in time of flight through a voltage gradient. The sample is first subjected to an albumin removal step and ultracentrifugation before being introduced into an electrospray unit. The electrosprayed particles then undergo neutralization of charges, separation in an electric field, and are counted by a condensation particle counter [55]. This method currently separates HDL into large HDL particles (HDL 2b) and small HDL particles (HDL 2a and 3).

As this is a relatively new method of HDL separation and quantification, there are limited studies correlating these HDL measurements by this technique and CVD outcomes. The Malmö Diet and Cancer study examined the association of particles measured by ion mobility and CVD risk. It found using principal component analysis that large HDL particles in association
with an atherogenic lipoprotein phenotype (increased triglycerides and small LDL particles), was associated with increased CVD risk [56].

**Proposed Uniform Nomenclature**

The use of different separation methods has led to multiple ways of defining HDL sub-populations, and there is limited comparison of data across methods. A new HDL nomenclature was proposed by Rosenson and colleagues recently [57], with the aim of aligning the HDL subfractions measured using different methods. This new classification is based upon the density and size of HDL and classifies HDL into 5 subgroups: very large HDL (HDL-VL), large HDL (HDL-L), medium HDL (HDL-M), small HDL (HDL-S), and very small HDL (HDL-VS) [57]. The HDL-VL class include HDL-2b species (measured on density gradient ultracentrifugation, gradient gel electrophoresis, and ion mobility), α-1 HDL (measured on 2D gel electrophoresis), and large HDL particles (measured on NMR spectroscopy). On the other end of the spectrum, the HDL-VS class include HDL3c (measured on density gradient ultracentrifugation, gradient gel electrophoresis, and ion mobility), pre-β-1 HDL (measured on 2D gel electrophoresis) and small HDL particles (measured with NMR spectroscopy). It remains to be seen if this new nomenclature will be adopted in clinical research literature.

**Apolipoprotein Based-Classification of HDL Particles: Direct Measures of Apolipoprotein AI and AII**

Alaupovic and colleagues proposed classifying lipoproteins based on apolipoprotein composition. Based on this classification, HDL can be subdivided into two groups: LpAI which consists of HDL containing only apo AI, and LpAI:AII which are HDL containing both apo AI and apo AII [58]. These particles can be quantified using immunoprecipitation, two-phase electroimmunoassay, enzyme-linked differential-antibody immunosorbent assay, or differential electroimmunoassay [59]. Differential immunoassay is the most commonly used method in clinical studies. It is still controversial if measuring LpAI and LpAI:AII subfractions are useful clinically. In the Framingham Offspring study and the VA-HIT trial, these measurements were not found to provide prognostic information beyond lipid profile and other CVD risk factors. [40, 41]. It is also unclear which particle, LpAI or LpAI:AII
was more inversely correlated with CVD, with some studies suggesting it was LpAI and other studies suggesting both [60, 61]. The classification of HDL particles into subpopulations using this methodology may not sufficiently resolve the heterogeneity of HDL, and this may account for the variable results from clinical studies.

Apo AI is the most abundant protein in HDL, followed by Apo AII. It has been proposed that measuring apolipoprotein levels, especially apo AI, may be superior to measuring HDL-C in terms of CVD risk prediction. This is in view its physiological role in mediating cholesterol efflux via the ABCA1 transporter. Part of the challenge in demonstrating this superiority is that apo AI and HDL-C levels are statistically very highly correlated. There have been a few prospective studies demonstrating that apo AI levels were more predictive, such as in the EPIC-Norfolk cohort and the AMORIS study [62, 63]. It has also been demonstrated that apo AI may undergo oxidation in vivo, which may compromise its anti-inflammatory activity, and ability to mediate reverse cholesterol transport [64, 65]. The measurement of Apo AI is currently not recommended in routine clinical practice. However, there is a potential for it, as well as oxidized apo AI to be developed as prognostic factors for CVD in future. On the other hand, there is still controversy surrounding whether apo aII is pro- or anti-atherogenic. Data from the EPIC-Norfolk cohort and the Ludwigshafen Risk and Cardiovascular Health Study have suggested that it may have a mild anti-atherogenic effect [66, 67]. However, animal studies as well as other human studies have suggested the opposite [68, 69].

**APPROACHES TO ASSESS AND MODULATE HDL FUNCTION**

It is evident that HDL particles are heterogeneous in size and composition; and despite the substantial epidemiological data suggesting a cardioprotective role for HDL, much remains unclear about the anti-atheroclerotic and anti-thrombogenic properties of different particles that comprise this class of lipoproteins.

Methods for measurement of HDL subfractions and compositional assays (as described above) may be superior to HDL-C in predicting coronary heart disease risk. We now move on to discuss additional measures for the attributes of HDL, including its role in reverse cholesterol transport, oxidation and inflammation (Figure 2). These aim to provide advantages to current analytical
measures of HDL to further understand, validate, and quantify the diverse roles of HDL particles in the atherosclerotic process to improve diagnosis, prevention and treatment of CVDs.

**Macrophage Cholesterol Efflux and Reverse Cholesterol Transport**

Promotion of cholesterol efflux from macrophages and the return of this excess cholesterol to the liver, bile, and feces, is termed “reverse cholesterol transport” (RCT), and is thought to be one of the most important mechanisms by which HDL protects against atherosclerosis (Figure 1). Measurement of steady state HDL-C level may not be representative of flux through the RCT pathway. Hence, evaluating the flux of cholesterol (the rate and magnitude of inter-compartmental shifts) provides a more dynamic measure of RCT effectiveness and is potentially a more informative way of assessing the efficacy of a novel HDL-targeted intervention.

To assess cellular efflux, donor macrophages are first incubated with 3H-cholesterol [70, 71]. Incubation with a medium containing an “acceptor” (lipid-free apoA-I, isolated HDL, or diluted human serum) is carried out, and after multiple washings, scintigraphy quantifies the radioactivity in the medium and associated with the cells. Cholesterol efflux is then expressed as the amount of label released into the medium divided by the total label present. Acceptors in the medium and donor cells can be manipulated to examine the effects of genetic and pharmacologic manipulation on efflux potential. Manipulation of donor cells may augment cholesterol efflux. For example, mouse peritoneal macrophages over-expressing ABCA1 efflux cholesterol to apoA-I faster than do wild-type control cells, an effect associated with smaller, less complex aortic valvular atherosclerotic lesions in transgenic ABCA1 mice [72]. In humans, investigation of families with ABCA1 mutations demonstrated an inverse correlation between cholesterol efflux and carotid intima-media thickness [73].

The most applicable approach of ex vivo efflux assays to drug development would be to use whole serum or isolated HDL from subjects treated with an experimental drug and test its ability to promote cholesterol efflux from a defined cell system ex vivo. Pre-clinical studies support the concept that augmenting the ability of serum or HDL to promote the efflux of cholesterol from cells may confer an atheroprotective benefit. Administration of recombinant apoA-I Milano or oral D-4F, a small apoA-I mimetic peptide,
significantly increased the cholesterol efflux-promoting capacity of plasma taken from treated apoE-deficient mice [74]. These ex vivo findings were associated with decreased macrophage and lipid content of aortic atheroma, indicating enhanced mobilization of tissue cholesterol. Interestingly, treatment with the CETP inhibitor, torcetrapib has been shown to enhance efflux capacity of HDL, whether normalized to volume of serum or to HDL mass [75].

However, until recently, there was scarcity of data that link serum efflux capacity to atherosclerosis or cardiovascular events in humans. A significant step forward in this regard was made when Rader and colleagues measured the ability of human serum HDL to promote cholesterol efflux from cultured macrophage foam cells in two large, independent groups of subjects, and correlated this with coronary disease status [14]. It is important to note that the association was inverse, which is consistent with studies in animals that show that HDL is atheroprotective because it promotes cholesterol efflux from macrophages. Further, the ability of serum HDL to promote cholesterol efflux was not determined simply by measuring HDL-C levels, as the association between efflux capacity and the risk of coronary artery disease remained significant even after adjustments for levels of HDL-C and apo A-I. These observations provide important evidence that the ability to promote cholesterol efflux from macrophage foam cells is a key property that explains, at least in part, the inverse relationship between HDL and the risk of atherosclerotic coronary artery disease in humans. This supports the concept that HDL efflux capacity is a measure of HDL function that is relevant to the pathogenesis of atherosclerosis and that dysfunctional HDL contributes to the risk of coronary disease. However, a limitation of efflux potential as a surrogate measure is its inability to assess terminal segments of the RCT pathway or effects of interventions that are directed toward the cells themselves. There is a need to determine the different pathways that contribute to total efflux to serum and how these vary among individuals. Overall, there is also a need for additional data linking serum efflux capacity to atherosclerosis progression and clinical outcomes.

Further, robust and sensitive methods for assessing integrated RCT in humans are needed in order to advance insights gained in animals into the human realm and to assess novel therapies targeted toward HDL and RCT. One potential approach is simply to measure the mass of fecal sterol excretion as a surrogate for RCT. For example, an acute intravenous bolus infusion of pro-apoA-I in humans was found to result in a significant increase in fecal sterol excretion, suggesting promotion of RCT [76]. However, this approach is

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not macrophage specific, is unlikely to be very sensitive, and may have limited utility in the chronic steady-state setting due to counter-regulatory pathways involved in biliary cholesterol excretion and fecal sterol absorption.

A steady-state isotope dilution technique has been developed that involves the intravenous infusion of cholesterol labeled with stable-isotope, which is diluted by the efflux of endogenous (unlabeled) cholesterol from tissues into plasma. Presentation of preliminary human studies using 13C-cholesterol demonstrated feasibility, revealing that an 18-h infusion period was required to achieve plateau tracer levels and reproducible measurements of tissue cholesterol efflux [77]. No primary data are yet available in the peer-reviewed literature, but a more detailed discussion of this method can be found in a review [13]. It remains to be established whether this method will have utility in assessing RCT in humans following therapeutic intervention. However, its ability to differentiate between hepatic and peripheral (non-hepatic) tissues as the source of cholesterol efflux has not been definitively proven. Furthermore, as with fecal sterol excretion, this method is not macrophage specific. Nevertheless, it represents an interesting new approach to this problem.

An alternative approach would be to load peripheral tissues with a cholesterol-like molecule that is not endogenously synthesized but is effluxed and transported similarly as cholesterol and then to follow the rate of disappearance of this tracer from tissues that can be sampled repeatedly (circulating cells, skin, adipose) as well as the rate of excretion in the feces. As with the above methods, however, this is not macrophage specific. Ideally, one would like to label cholesterol specifically in arterial wall macrophages in humans and trace its efflux to plasma through to fecal excretion, a goal that is probably unattainable. However, there may be approaches to selectively labeling cholesterol in macrophages using an in vivo targeting approach that could be adapted to RCT studies in humans. Development of a robust assay for RCT in humans, ideally from the macrophage, will be critical to the development of novel therapeutics that may increase RCT as an antiatherogenic strategy.

Indexes of Anti-Inflammatory and Antioxidant Activity

Normal HDL is an effective antioxidant and anti-inflammatory molecule, by virtue of the proteins and lipids associated with HDL that contribute to its anti-inflammatory capacity. HDL particles can exert potentially antiatherogenic effects independent of cholesterol efflux and centripetal

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transport, including inhibiting lipid oxidation, impairing leukocyte adhesion and monocyte activation, and preventing endothelial cell damage and death [12, 78]. These anti-inflammatory activities of HDL have generated significant excitement, prompted by evidence implicating LDL modification and monocyte migration, which are critical processes for atherogenesis.

**Monocyte Chemotactic Activity**

To characterize the anti-inflammatory or proinflammatory potential of HDL, a cell-based assay measures the effect of HDL on monocyte chemotactic activity, which is the propensity of monocytes to migrate into the subendothelial space [79]. This co-culture model provides a simulated arterial wall consisting of a confluent human aortic endothelial cell monolayer and smooth muscle cell multilayer separated by a layer of collagen. The endothelial cell monolayer is first incubated with standard control LDL or oxidized phospholipids, stimulants of monocyte chemotaxis, in the presence or absence of test HDL.

Human peripheral blood monocytes are subsequently added to the endothelial side of the coculture and visualized by the addition of antimonocyte antibodies. Finally, monocytes that migrated into the subendothelial space are counted.

Results obtained from coculture incubation with test HDL can be divided by those following addition of control LDL alone to yield an “HDL inflammatory index,” with values above 1 indicating proinflammatory HDL and values below 1 denoting anti-inflammatory HDL [80].

An interesting human study [80] compared HDL inflammatory indexes in healthy control subjects and patients with coronary artery disease (CAD) before and after statin therapy. High-density lipoprotein cholesterol taken from age- and gender-matched healthy control patients decreased monocyte chemotaxis, yielding a mean inflammatory index of 0.38. Patients with CAD, however, exhibited proinflammatory HDL augmenting monocyte transmigration more than control LDL alone, with a mean index of 1.38. Administration of simvastatin significantly reduced the inflammatory index to 1.08, suggesting improvement of HDL anti-inflammatory function with statin therapy.

In addition, the study evaluated monocyte chemotaxis in CAD patients with elevated HDL-C levels (mean 95 mg/dl) in an attempt to explain the apparent discrepancy. The HDL obtained from this group was pro-
inflammatory with an index of 1.28, highlighting the limitations of HDL-C alone and the importance of functional metrics. Although this assay is conceptually attractive and has been validated in several settings, it may be cumbersome to use as a routine assay for assessing HDL anti-inflammatory function.

**Anti-Oxidant Activity**

Another aspect of HDL function relevant for atherogenesis is the fact that lipid oxidation products in HDL inhibit antioxidant and anti-inflammatory enzymes associated with HDL, including PON1 and glutathione selenoperoxidase [81]. One recently developed cell-free assay [82] measures integrated HDL antioxidant potential, using physiologic phospholipids known to participate in LDL modification. In the presence of dichlorofluorescein, a fluorescent marker of lipid oxidation products, HDL is added to various phospholipids found in oxidized LDL to determine its ability to inactivate or prevent the formation of biologically active oxidized phospholipids. After the reagents are combined, spectroscopy permits quantification of net oxidation, with diminished fluorescence intensity signaling fewer oxidized phospholipids and suggesting a more antiatherogenic HDL. High-density lipoprotein from CAD patients yields significantly higher levels of oxidation products compared with that of healthy control patients [82]. Treatment with simvastatin patients but fails to restore its antioxidant ability [80]. In another small study of immigrants from South Asia living in the USA, carotid intima-media thickness substantially correlated with dysfunctional HDL after adjusting for age, family history of CVD, and hypertension [83]. This assay is an interesting candidate for validation and application to high-throughput assessment of HDL antioxidant function in response to therapeutic intervention.

**Expression of Endothelial Expression Molecules**

Studies of endothelial expression of adhesion molecules may provide additional methods for evaluating HDL-directed therapeutics. To gain entry into the subendothelial space, monocytes must first arrest their passage through the circulation. Adhesion molecules presented on the luminal surface of endothelial cells slow neighboring monocytes, first via loose tethering

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mediated by E-selectin and then by tight association of endothelial intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 with leukocyte integrins [84, 85]. Expression of adhesion molecules represents a dynamic process, activated by inflammatory cytokines [86] and vascular injury [87] and up-regulated in atherosclerosis in both animals [88] and humans [89, 90]. As data implicate increased endothelial expression of adhesion molecules in atherosclerotic disease [91], in vitro assays have been developed to evaluate the effect of HDL on this particular facet of endothelial activation (92). The original experiment using human umbilical endothelial cells (HUVECs) demonstrated the ability of HDL obtained from healthy donors to inhibit cytokine-mediated protein expression of VCAM-1, ICAM-1, and E-selectin [92]. Subsequent in vitro studies have established the ability of native and reconstituted HDL to down-regulate inducible expression of adhesion molecules [93, 94]. Importantly, suppression of endothelial expression of adhesion molecules in vivo, which has been associated with decreased atherosclerosis [95], appears to correlate with in vitro findings. However, the reliability and reproducibility of the in vitro assay of inhibition of endothelial adhesion molecule expression as a surrogate measure of the antiatherogenic functionality of HDL remains to be established, and standardized and validated assays are needed.

**NO Production and Endothelial Function**

Quiescent endothelium produces endothelial nitric oxide (NO), which acts to inhibit cellular pathways of inflammation, proliferation, and thrombosis. HDL-C has been shown to promote endothelial generation of NO in vitro and improve endothelial function and arterial vasoreactivity in vivo [12]. Thus, assaying endothelial NO production in response to HDL could provide the basis of an in vitro proxy of endothelial function. In principle, basal endothelial production of nitrite and nitrate is generated by incubating endothelial cells with L-arginine, the substrate of nitric oxide synthase (NOS), and stimulated production is achieved through simultaneous addition of an NOS agonist. The NO synthase activity may be determined by measuring NOS conversion of [3H] L-arginine to [3H]L-citrulline via scintillation counting [96] or by quantifying production of nitrite and nitrate via ozone chemiluminescence [97].

This approach has been used to assess the efficacy of HDL-based interventions. For example, the apoA-I mimetic L-4F increased endothelial

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HDL Heterogeneity and Function

A subsequent study [99] correlated these in vitro findings with an improved vasodilatory response to acetylcholine in hypercholesterolemic, LDL receptor-null mice. Evaluating upstream effects of isolated HDL after an intervention on NOS activation and localization in caveolae, specialized plasma membrane microdomains containing a variety of signal transduction molecules, may also prove informative [100]. Finally, apart from preserving NO activity, HDL exerts other putatively atheroprotective effects on endothelial cells, promoting their proliferation and migration [101] and inhibiting their apoptosis [102]. Widespread application of these in vitro assays as a surrogate measure in HDL-targeted therapy development, will require further validation in intervention studies to ensure its in vivo clinical relevance following pharmacotherapy.

Vasoreactivity is the most common method of assessing endothelial function in vivo and is usually studied in humans using ultrasound imaging of the brachial artery at baseline and during reactive hyperemia [103]. Longitudinal images are initially obtained after a 10-min equilibration period with patients in a fully recumbent position. A blood pressure cuff is then placed proximal to the transducer on the upper arm and inflated to above systolic pressure. After 5 min of occlusion, the blood-pressure cuff is deflated and the brachial artery scanned continuously for 60 s. Finally, flow-mediated dilation (FMD) is calculated as the maximum percent increase in brachial artery diameter after 1 min of reactive hyperemia. In observational studies of patients with established CAD [104, 105], patients with atherosclerotic risk factors [106, 107], and healthy subjects [108], the extent of FMD has consistently correlated with HDL-C levels. Studies of HDL-directed therapies have utilized FMD as a proxy of endothelial protection [109]. Although FMD has been suggested to have prognostic value [110], change in FMD following pharmacotherapy has never been correlated with cardiovascular outcomes. Ongoing studies will hopefully further elucidate and validate techniques to assess endothelial effects mediated by HDL to clarify their utility as reliable biomarkers in the setting of pharmacotherapy.

**Antiplatelet and Antithrombotic Activity**

HDL-C may provide additional cardiovascular benefits by antagonizing platelet activity and inactivating the coagulation cascade, both through direct inhibition and via restoration of endothelial function [12]. Employing a rat
model of aortic thrombosis, one pre-clinical trial [111] demonstrated that administration of apoA-I Milano significantly delayed time to thrombus formation, inhibited platelet aggregation, and reduced the weight of thrombus produced. In a human study of hyperlipidemic and normolipidemic patients without significant coronary risk factors [112], high HDL-C levels were associated with reduced thrombus in an ex vivo model. Clot formation, evaluated by exposing porcine aortic intima to flowing, nonanticoagulated venous blood from test subjects, varied inversely to HDL-C in univariate and multivariable analyses.

Within the intricate cascade of hemostasis and thrombosis, a multiplicity of interacting factors complicates determination and prioritization of clinically relevant, HDL specific pathways. Studies thus far [113, 114] implicate the serine protease protein C, a major physiologic anticoagulant that inactivates factors Va and VIIIa in plasma. Using a modified prothrombin time clotting assay, one experiment [115] demonstrated that HDL significantly enhanced inactivation of coagulation factor Va by activated protein C and protein S, and levels of apoA-I correlated with the anticoagulant response. There is also evidence to suggest that HDL may exert antithrombotic activity via inhibition of tissue factor expression, factor X activation, and plasminogen activator inhibitor secretion [116-118]. Overall, surrogate measures of HDL antiplatelet and antithrombotic functionality remain underdeveloped, largely due to the absence of consensus regarding the clinically relevant atheroprotective pathways, and none have been consistently employed or correlated with plaque burden or morphology. However, it is important to note the accumulating evidence implicating platelet activation and thrombin generation in the chronic progression of atheroma [119-121], as well as the final, catastrophic event of plaque rupture leading to acute coronary syndrome and other atherothrombotic diseases. These data support a critical role of platelets and the coagulation cascade in the ongoing progression of atherosclerosis and highlight the importance of elucidating HDL-mediated pathways and developing reliable metrics of antiplatelet and antithrombotic activity.

**CONCLUSION**

We have reviewed the approaches currently available for evaluating HDL heterogeneity and function. The dichotomy between quantity and quality is a somewhat loose one; mainly to help conceptualize the strategies targeting HDL for therapeutic purposes. At present, which of these strategies will prove
to be most effective remains unknown and is an intense area of ongoing investigation. Alongside with the development of therapeutics must be the development of assays for evaluating HDL function that can be adapted for widespread clinical use. Clearly, the current practice of measuring a static HDL-C level is not sufficiently comprehensive. It would be helpful to have a validated, widely available assay of HDL function that can be used in clinical trials to assess new compounds as they enter into clinical development, as not all HDL therapeutics increase HDL-C levels and even those that do may not necessarily improve HDL function. Robust assays that can evaluate the function of HDL, thus supplementing the measurement of HDL-C levels in the clinic, will hopefully leave us with multiple, possibly synergistic, HDL therapeutics that will help reduce residual cardiovascular risk in the population.

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Chapter 2

DYSLIPIDEMIA: CAUSES, DIAGNOSIS AND TREATMENT

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ABSTRACT

Cardiovascular disease (CVD) is leading cause of morbidity and worldwide. Higher levels of low-density lipoprotein cholesterol (LDL-C) are associated with an increased risk of coronary heart disease (CHD), myocardial infarction, and stroke. Guidelines of the Adult Treatment Panel III emphasize intensive reduction of LDL or non-high-density lipoprotein cholesterol in patients at high risk of CHD.

Restriction of dietary saturated and trans-fat and cholesterol, along with increased intake of soluble fiber, can achieve LDL-C lowering. However, pharmacological treatment is needed in most patients. Statins are highly efficacious as LDL-C lowering agents and have more modest effects on very LDL-C, triglycerides and HDL-C levels. The effects of statins on plasma lipids result from their ability to both increase the

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efficiency with which very LDL-C and LDL-C are cleared from the circulation and reduce the production of apoB-containing lipoproteins by the liver. Placebo-controlled intervention studies of statin drugs for lowering LDL-C provide clear evidence of CVD prevention. In addition, improvement of survival with statins may be due to other pleiotropic effects beyond LDL-C lowering. Side-effects, myopathy and hepatotoxicity, are infrequent and usually mild, but use of lipid-modifying medication demands caution because of the possibility drug interactions.

Many recent studies suggest that low levels of HDL-C are a major independent risk factor for CVD. According to several clinical trials, a 1% increase in HDL-C is associated with a 0.7%–3% decrease in CHD events. The direct link between high levels of triglycerides and CHD is less well defined. Fibrates reduce triglycerides levels and may be beneficial for CHD prevention. Fibric acid derivatives and niacin are primarily used to increase HDL-C levels, although with side effects. Low-dose n-3 fatty acids could be used routinely after a myocardial infarction, but the value of higher doses of n-3 fatty acids in reducing cardiovascular risk remains to be demonstrated.

In conclusion, achieving low lipid levels appears safe, but the generalizability of these findings to broader populations and the clinical benefit on the reduction of cardiovascular complications remains to be proven.

**INTRODUCTION**

It is well known that coronary heart disease (CHD) is a leading cause of morbidity and mortality worldwide [1]. Only in the United States, one of every five deaths was attributed to CHD in 2002 [1]. Therefore, CHD has a major impact in health economics with the estimated total cost for CHD in 2005 exceeded $142 billion [1].

The National Cholesterol Education Program Adult Treatment Panel’s third report (NCEP ATP-III) focuses on evidence from clinical trials demonstrating the importance of LDL-C reduction to reduce the risk of CHD [2].

Lowering LDL-C to less than 100 mg/dL is recommended for those with known CHD or CHD risk equivalents such as diabetes mellitus (DM). For subjects at high-risk, a target LDL-C of < 70 mg/dL has been added aiming to the benefits of aggressive lipid-lowering.

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CHOLESTEROL AND LIPOPROTEIN METABOLISM AND TRANSPORT

The major lipids are cholesterol, triglycerides (TGs), and phospholipids, which are used for cell membrane formation, hormone synthesis, and bile acid production [3, 4]. Since lipids are not soluble in blood, they are packaged into complexes of lipid and protein called lipoprotein particles [3]. Lipoproteins are composed of phospholipids, free cholesterols, proteins (Apo lipoproteins), cholesterol esters, and TGs [3]. There are 5 major classes of lipoprotein particles: chylomicrons, very low-density lipoprotein cholesterol (VLDL-C), intermediate density lipoproteins, LDL-C, and high-density lipoprotein cholesterol (HDL-C) [3].

Chylomicrons are the largest lipoprotein particles, and transport dietary TGs and cholesterols from the intestine to different parts of the body [3, 5]. The metabolism of chylomicrons is mediated by lipoprotein lipase (LPL), with ApoC-II acting as a cofactor and activator [3, 5]. Chylomicrons are usually present in the plasma for 3–6 hours after eating and are metabolized after 10 to 12 hours of fasting [5]. After LPL metabolizes the chylomicrons and removes a large portion of the TGs, chylomicrons are further metabolized in the liver by hepatic lipase, returning ApoC-II to HDL-C [3, 5]. Chylomicron remnants, which contain Apo-E and ApoB-4, are then taken up by remnant receptors in the liver, which liberates free cholesterols intracellularly [3]. During the whole process of chylomicron metabolism, some of the components of the chylomicron such as phospholipids and some Apo lipoproteins are recycled and used to make HDL-C [3].

VLDL-C is synthesized in the liver in response to a high carbohydrate diet. Excess carbohydrate is converted into TGs and transferred to nascent VLDL-C. VLDL-C delivers TGs to adipose tissue and cardiac or skeletal muscle for storage and energy release by LPL. Almost all LDL-C is derived from VLDL-C [5].

LDL-C carries about 60% to 70% of serum cholesterol [3]. It transports cholesterol from the liver to peripheral tissues. High levels of LDL-C are harmful, as LDL-C can build up on arterial walls, leading to the formation of atherosclerotic plaques. The binding of LDL-C to its receptor in the liver is the major mechanism of removal of LDL-C from the circulation [6]. An increase in intracellular cholesterol inhibits de novo synthesis of cholesterol, resulting in decreased synthesis of LDL-C receptor and increased activity of an enzyme that facilitates cholesterol storage [3].
HDL-C is known to be protective against CHD, while low HDL-C levels increase the risk of CHD. HDL-C is produced in the intestine, liver and plasma as a complex of Apo A lipoproteins, phospholipids and cholesterol. In the plasma, HDL-C is converted to a cholesterol ester by the action of cholesterol ester transferase (LCAT). As they circulate in the blood stream, HDL-C particles acquire more cholesterol from the blood stream. In addition, HDL-C particles remove cholesterol through a reverse cholesterol transport process from peripheral tissues and atheroma within the arteries to the liver, carrying approximately 30% of the serum cholesterol [5]. Women and individuals on estrogen therapy generally have higher HDL-C levels due to the effect of estrogen on the upregulation of the major components of the HDL-C particle, mainly ApoA-1 lipoprotein [5].

The process known as reverse cholesterol transport involves removal by HDL-C of un-esterified cholesterol from peripheral cells, such as macrophages, and delivery to the liver through the interaction of HDL-C with the hepatic HDL-C receptor. Several studies have shown an inverse relationship between blood HDL-C levels and CHD. These studies show that individuals with low levels of HDL-C have worse CHD outcomes. Furthermore, data obtained from several epidemiological studies emphasize that the risk factor of low HDL-C is completely independent of LDL-C; no matter how low the LDL-C, a decrease in the HDL-C would increase the risk for coronary artery disease [3].

Pathogenesis of Dyslipidemia

There are many causes of dyslipidemia, both primary and secondary. The primary causes of dyslipidemia are due mostly to genetic disorders. There are six categories of lipoprotein disorders according to the Fredrickson-Levy-Lees classification: Type I (high levels of chylomicrons), Type IIa (high LDL-C levels), Type IIb (high LDL-C and VLDL-C levels), Type III (high IDL-C levels), Type IV (high VLDL-C levels), and Type V (high LDL-C and chylomicrons levels) [3]. It is also possible that some disease states can be placed into more than one category of lipoprotein disorder.

The term hypertriglyceridemia usually refers to elevations in VLDL-C and chylomicrons, both of which carry and transport TGs [3]. Primary hypertriglyceridemia includes primary chylomicronemia, familial hypertriglyceridemia, familial combined hyperlipoproteinemia and familial dysbetalipoproteinemia [6]. Primary chylomicronemia is a genetic disease.
that is characterized by a deficiency in LPL or cofactor, and results in elevated chylomicrons and VLDL-C and severe elevation of TGs, leading to acute pancreatitis [6]. Familial hypertriglyceridemia is a Type IV disorder in which primarily VLDL-C is affected [3]. It is caused by a number of genetic determinants that result in insufficient removal of TG-rich lipoproteins [6]. Familial combined hyperlipoproteinemia is a disorder characterized by increased levels of VLDL-C, LDL-C, or both. Familial dysbeta lipoproteinemia is a disorder characterized by increased levels of VLDL-C remnant and chylomicron remnant [3, 6].

The term hypercholesterolemia usually refers to elevated serum LDL-C. Primary hypercholesterolemia includes familial hypercholesterolemia, familial ligand-defective Apo lipoprotein B, and familial combined hyperlipoproteinemia. Primary hypercholesterolemia is a Type IIa dominant disorder that involves mutations in the LDL-C receptor gene. Homozygotes usually have a worse prognosis than heterozygotes. Familial ligand-defective apolipoprotein B is also a Type IIa disorder caused by a mutation in ApoB-100 that disrupts the binding of LDL-C to the LDL-C receptor, thereby decreasing metabolism of LDL-C. In both of these disorders, LDL-C receptor-mediated endocytosis in the liver is decreased, resulting in increased serum LDL-C. In addition, genetic evidence confirms the role of a newly discovered serine protease, pro-protein convertase subtilisin-like kexin type 9 (PCSK9), in patients suffering from autosomal dominant hypercholesterolemia. Three gain of function single nucleotide polymorphisms in the PCSK9 gene have been identified that increase LDL-C levels. PCSK9 destroys low density lipoproteins receptors (LDL-C-R) in the liver, thereby regulating the levels of LDL-C in plasma. Lastly, familial combined hyperlipoproteinemia is a Type IIb disorder that can cause elevated VLDL-C, LDL-C, or both.

Some rare genetic disorders can also cause low levels of HDL-C, ie, Tangier disease, disorders of LCAT and familial hypoalphalipoproteinemia. Tangier disease is caused by mutation and loss of function of ATP-binding cassette 1 (ABCA1). ABCA1 transports cholesterol and phospholipids out of cells for pickup by Apo-A1 in the circulation. In the absence of ABCA1, free Apo-A1 does not acquire cellular lipids. This results in accelerated clearance of Apo-A1 from plasma, leading to low HDL-C levels [7]. LCAT is a lecithin cholesterol acyltransferase responsible for catalyzing the formation of cholesterol esters of HDL-C and LDL-C; thus, it is crucial for HDL-C formation [8]. Familial hypoalphalipoproteinemia includes a wide range of disorders that result in low levels of HDL-C (usually below 35 mg/dL).
There are many secondary causes of dyslipidemia, including disease- and medication-induced dyslipidemia. Some of the more common causes of hypertriglyceridemia are DM, alcohol abuse, estrogen, and obesity [3, 6]. Some of the more common causes of hypercholesterolemia are hypothyroidism, anorexia, and excess corticosteroid use [3, 6]. Low levels of HDL-C can be caused by malnutrition, obesity, and drugs such as beta-blockers, anabolic steroids, isotretinoin, and progestins [3].

**CHD and Dyslipidemia**

High levels of LDL-C (and to a lesser extent, VLDL-C) result in the accumulation of LDL-C in the arterial wall, leading to oxidation of LDL-C [3]. Oxidized LDL-C can cause extensive damage to the arterial wall, provoking inflammation responses, promoting coagulation, increasing the activity of mediators that cause vasoconstriction and inhibiting mediators that cause vasodilation [3]. Oxidized LDL-C recruits monocytes, which enter the arterial wall and are activated to become macrophages. The macrophages ingest oxidized LDL-C through the macrophage scavenger receptor to become foam cells, or fatty streak. Foam cells propagate inflammatory responses as well as facilitate deposition of more oxidized LDL-C. Micro-calcification of the vascular smooth muscle cells will take place, which progresses to atherosclerosis [3].

The fatty streak, which consists of cholesterol-filled macrophages, is the first stage of atherosclerosis [3]. Plaques (deposits of fatty substances, cholesterol, calcium, and cell components) will then form and progress, gradually increasing inside the artery and narrowing the arterial wall, resulting in decreased flow of blood and oxygen to tissues. Plaques are usually kept in check by a fibrous cap, which protects and stabilizes the lesion. If the plaque ruptures, then thrombosis will occur and damage will spread to other areas, leading to ischemic heart disease, myocardial infraction, stroke, peripheral arterial disease, other CHDs, and possibly death [3].

HDL-C is believed to help protect against CHD. Its protective effects are in part due to reverse cholesterol transport. As the name suggests, the reverse cholesterol transport process involves the transport of HDL-C from peripheral tissues and transfer to VLDL-C and LDL-C back to liver for secretion in the bile [3]. HDL-C is also believed to have anti-inflammatory, anti-oxidative, platelet anti-aggregatory, anticoagulation, and pro-fibrolytic effects, which can help reduce damage mediated by high levels of LDL-C [7].
Treatment of Dyslipidemia

New classifications of LDL-C, total cholesterol (TC), and HDL-C levels are identified in Table 2. In addition, ATP III defined the desirable TGs level as 150 mg/dL and set non–HDL-C treatment goals in patients with TGs >200 mg/dL.

The NCEP ATP III guidelines stratify patients into 1 of 3 risk categories according to their near-term risk of developing CHD [9]. The first risk category includes patients with preexisting CHD or CHD risk equivalents and patients with a very high risk, defined as a greater than 20% risk for developing CHD in the next 10 years. The second category consists of patients with 2 or more risk factors but not diagnosed with CHD, and the third category includes those who are in the lowest risk category, with 0 to 1 risk factors (Table 1). Treatment strategies are driven by the degree of risk for CHD; the higher the risk, the more aggressive the treatment strategy.

ATP III recommends the use of a Framingham based risk scoring system to quantify the 10-year risk for a coronary event [9]. Point scores are calculated according to the presence of 5 major CHD risk factors—age, gender, TC level, systolic blood pressure, HDL-C, and smoking status—with each risk factor worth a certain number of points. When added together, the sum yields an estimate of the 10-year risk for experiencing a coronary event. A properly conducted assessment places patients into 1 of the 3 risk categories and forms the basis for all subsequent treatment decisions [9]. Patients with documented CHD and CHD risk equivalents are automatically placed in the highest risk category. CHD risk equivalents carry a risk for a major coronary event equal to that of clinically evident CHD and include type 2 diabetes (T2D), peripheral vascular disease, symptomatic carotid artery disease, and abdominal aortic aneurysm [9].

The LDL-C treatment goal for patients in this high-risk category is a level <100 mg/dL. The first step in evaluating CHD risk is to identify patients with clinical evidence of CHD or CHD risk equivalents. All of these patients, by definition, have a >20% 10-year risk of a CHD event. The second step in assessing risk is to count the risk factors present in the remaining patients (ie, those without CHD or CHD risk equivalents). For those with >2 risk factors, the next step is to conduct a Framingham-based risk assessment to determine the most appropriate course of therapy. If the patient presents ≥2 risk factors, he/she may have a 10-year CHD risk which is >20%. If this is the case, this patient should be placed in the CHD risk equivalent category and treated to a target LDL-C goal of <100 mg/dL [9].
Table 1. Risk Assessment Criteria: New Features of NCEP ATP III Guidelines

<table>
<thead>
<tr>
<th>Focus on Multiple Risk Factors</th>
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<tbody>
<tr>
<td>• Diabetes without CHD equal to CHD risk equivalent</td>
</tr>
<tr>
<td>• Framingham-based assessment of 10-year absolute CHD risk used to stratify patients with &gt;2 risk factors into those with &gt;20% risk for more intensive treatment and those with &lt;20% risk</td>
</tr>
<tr>
<td>• Multiple metabolic risk factors indicate candidates for intensified therapeutic lifestyle changes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modifications of Lipid and Lipoprotein Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>• LDL-C &lt;100 mg/dL optimal</td>
</tr>
<tr>
<td>• Raises categorical low HDL-C from &lt;35 mg/dL to 40 mg/dL</td>
</tr>
<tr>
<td>• Lowers TG classification cutpoints to give more attention to moderate elevations</td>
</tr>
</tbody>
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<th>Support for Implementation</th>
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<tr>
<td>• Lipoprotein analysis versus screening for TC and HDL-C alone as preferred initial test</td>
</tr>
<tr>
<td>• Encourage dietary options (plant stanols/sterols and soluble fiber) to enhance lowering LDL-C</td>
</tr>
<tr>
<td>• Promote adherence to therapeutic lifestyle changes and drug therapies</td>
</tr>
<tr>
<td>• Treat to a second, non–HDL-C treatment goal in patients who have a TG ≥200 mg/dL</td>
</tr>
</tbody>
</table>

ATP III indicates Adult Treatment Panel III; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NCEP, National Cholesterol Education Program; TC, total cholesterol; and TG, triglycerides.

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Table 2. NCEP ATP III Classification of Cholesterol: Total, LDL-C, HDL-C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Level</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td><strong>Total Cholesterol (mg/dL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;200</td>
<td>Desirable</td>
</tr>
<tr>
<td></td>
<td>200-239</td>
<td>Borderline</td>
</tr>
<tr>
<td></td>
<td>( \geq 240 )</td>
<td>High</td>
</tr>
<tr>
<td><strong>LDL-C (mg/dL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;100</td>
<td>Optimal</td>
</tr>
<tr>
<td></td>
<td>100-129</td>
<td>Near optimal/above optimal</td>
</tr>
<tr>
<td></td>
<td>130-159</td>
<td>Borderline high</td>
</tr>
<tr>
<td></td>
<td>160-189</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>( \geq 190 )</td>
<td>Very high</td>
</tr>
<tr>
<td><strong>HDL-C (mg/dL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;40</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>( \geq 60 )</td>
<td>High</td>
</tr>
</tbody>
</table>

Patients with a 10-year risk between 10% and 20% require more aggressive treatment, often with lifestyle modifications and drug therapy. Therapy for these patients should be sufficient to enable patients in this category to achieve an LDL-C target of \(<130 \text{ mg/dL}\). Those patients with \(<10\% \text{ 10-year risk}\) are candidates for lifestyle therapy and rarely, drug therapy. The target LDL-C in this group of patients is \(<160 \text{ mg/dL}\) [9].

**Therapeutic Lifestyle Changes**

ATP III continues to stress the importance of non pharmacologic treatment but recognizes its limitations by reducing the trial of these modalities from six months to 12 weeks before considering the use of medications to assist in achieving recommended LDL-C goals [9].

The distribution of the fat allowance has been altered to recognize the value of monounsaturated and polyunsaturated fatty acids. By replacing saturated fats (cheese, whole milk, red meat) with monounsaturated fats (olive,
canola oil) and polyunsaturated fats (corn oil, peanuts), LDL-C is reduced. Although replacing saturated fats with a high-carbohydrate diet results in lower LDL-C, it has the adverse effect of raising TGs and lowering HDL-C. Saturated and trans-unsaturated fatty acids should be avoided [9].

The ATP III suggests the addition of plant stanols (hydrogenated phytosterols) to the patient’s diet when initial attempts to alter the diet have not resulted in reaching the LDL-C goal. Plant stanols interfere with small intestine absorption of intestinal and biliary cholesterol. While they lower LDL-C, they have no significant effect on HDL-C or TGs [10]. Phytosterols can be found in many products, including margarine spreads. Other sources of phytosterols include sesame seeds and peanuts; soybeans are a natural source of phytosterols.

Physical inactivity is an independent risk factor, raising the risk of a cardiovascular event twofold [11]. Aerobic exercise raises HDL-C and lowers TGs. When it results in weight loss, it contributes to LDL-C reduction. Weight loss also improves insulin sensitivity and serum glucose uptake, reducing the risk of DM. Cigarette smoking remains a cardiovascular risk factor. Patients who stop smoking can expect an increase of up to 30 percent in their HDL-C levels [12].

**LIPID LOWERING AGENTS**

**Statins**

The HMGCoA reductase inhibitors, or statins, are the most widely used and arguably the most effective lipid-lowering agents currently available. These drugs work well as monotherapy and in combination with other agents. All statins lower LDL-C by upregulating hepatic LDL-C receptors after transient competitive inhibition of the reductase enzyme responsible for conversion of HMG-CoA to mevalonic acid during cholesterol synthesis. By upregulating LDL-C receptors, statins not only increase LDL-C clearance from the plasma, but also reduce circulating levels of VLDL-C and intermediate-density lipoprotein cholesterol that serve as substrates for LDL-C synthesis. When used as monotherapy, statins reduce LDL-C by 24%-60% and TGs by 22%-45%. Statins can also raise HDL-C up to 12%.

All statins reduce TC in a dose-related manner. Research has shown that doubling the dose of a statin above the minimal effective dose leads to an approximately 6% greater reduction in LDL-C [13]. Within the class of statins,
differences in absorption and metabolism can be observed for each drug. Lovastatin absorption is increased significantly in the presence of food while pravastatin is best taken on an empty stomach. Because rates of cholesterol synthesis increase between midnight and 3:00 AM, patients should be advised to take lovastatin with the evening meal, while pravastatin should be taken at bedtime. The other statins are unaffected by food and can be taken at any time during the day. The statins also differ in their metabolic pathways. Of the 5 currently available statins, 3 are metabolized through the cytochrome P450 3A4 enzyme system—atorvastatin, simvastatin, and lovastatin—while fluvastatin is metabolized through cytochrome 2C9 [14]. Pravastatin is not metabolized via cytochrome P450; it undergoes hepatic sulfation [14]. Metabolism via the P450 system can result in drug interactions as other agents compete or interfere with P450 enzymatic activity.

Statins are well tolerated by most people. The most common adverse effects of the statins include gastrointestinal upset, abdominal pain, flatulence, constipation, myalgia (defined as muscle ache or weakness without elevations in creatine kinase), flu-like symptoms, and headache. Less common problems are an asymptomatic elevation of liver enzymes (defined as >3 times the upper limit of normal) and myositis (defined as muscle pain, soreness, or weakness with serum creatine kinase concentrations of >10 times the upper limit of normal), rash, insomnia, unpleasant or vivid dreams, and difficulty sleeping or concentrating [14].

Elevated hepatic transaminases generally occur in 0.5%-2.0% of cases and are dose-dependent [15]. Whether transaminase elevation with statin therapy constitutes true hepatotoxicity has not been determined and progression to liver failure specifically due to statins is exceedingly rare. Reversal of transaminase elevation is frequently noted with a reduction in dose, and elevations rarely return when challenged with the same or different statin [16]. Furthermore, no direct evidence suggests that statins exacerbate liver disease [17]. The incidence of elevated liver enzymes can be increased in patients receiving drugs metabolized by the cytochrome P450 system. In patients exhibiting elevated liver enzymes, aminotransferase levels usually are elevated 3 times the upper limit of normal [14]. An LDL-C that suddenly declines despite stable dosing is also a sign of toxicity regardless of aminotransferase levels [17].

The ability of statins to produce myopathy (defined as any disease of the muscles) under some circumstances is well established. A common complaint is myalgia or nonspecific muscle aches or joint pains that are generally not associated with significant increases in creatine kinase. In placebo-controlled
trials, the incidence of these complaints is generally similar to that reported with placebo. Myositis is even less common, occurring in 0.8%-0.9% of patients taking statins as monotherapy [17-20]. Myositis is most likely to occur in people with multiple morbidities and/or those taking several medications.

The Pleiotropic Effects of Statins

The progression of stenoses is reduced by statin treatment in CHD and it would be reasonable to assume that this direct effect on the anatomical atherosclerotic lesion is responsible for the reduction in the cardiovascular end-points. However, these effects of statins on the coronary anatomy are less impressive than the observed clinical effects. Furthermore, the onset of the clinical benefits seen with statins occurs early after the start of the statin treatment and before an effect on coronary stenosis can be demonstrated. Accordingly it has been suggested that statins have an effect on CHD that is not related to their cholesterol-lowering effect and this is referred to as the pleiotropic effect of statins.

Endothelial dysfunction is responsible for the instability of the atherosclerotic plaque, leading to plaque rupture and the acute coronary syndromes. Statins have effects on the vascular endothelium, smooth muscle, haemostatic factors and the vascular wall, which counteract the adverse effects of cardiovascular risk factors [21, 23]. It is likely that these pleiotropic effects play a substantial role in the favourable cardiovascular profile attributed to statins. These effects differ between statins and it is not unreasonable to assume that statins may not be equally effective in the prevention of vascular disease.

Nitric oxide (NO) is an important modulator of endothelial function, whereby normal arteries vasodilate in response to augmented demand and ischaemia. Endothelial function is impaired in the presence of hypercholesterolaemia and other risk factors such as smoking and the presence of the insulin resistance seen in T2D. Numerous studies have demonstrated an improved endothelial function after the use of statins. Statins increase NO via numerous mechanisms including an increased activity and induction of endothelial nitric oxide synthase (eNOS), the prevention of the hypoxia-induced down-regulation of eNOS, and the reversal of the inhibitory effect of oxidised LDL-C on NO [21, 24]. Mechanical stress stimulates smooth muscle cells (SMCs) to proliferate and to manufacture macromolecules that contribute to the extracellular matrix of the vessel wall and also to the atherosclerotic
plaque. Cellular proliferation is also a process intrinsic to the atherosclerotic plaque. The lipophilic statins, but not the hydrophilic statin pravastatin, inhibit SMCs proliferation and migration induced by growth factors. The ultimate effect of this inhibition remains to be defined and indeed, the permissive effect of pravastatin on SMCs growth may be beneficial in terms of stability of the plaque’s fibrous cap.

Rupture of the vulnerable atherosclerotic plaque results in the formation of a thrombus, involving platelets and various clotting factors, including tissue factor. Tissue factor expression by cultured human macrophages is suppressed by statins [25]. Platelets of patients with dyslipidaemia are more sensitive to aggregating factors and this is normalised by statins, possibly due to an alteration of the cholesterol content of platelet membranes [26].

Fibrinogen, an important predictor of CHD, is variously affected by statins, with the majority of them lowering the plasma levels [27]. However, several researchers have found that atorvastatin increases fibrinogen levels [28]. Plasminogen activator inhibitor type-1 (PAI-1), another important marker of cardiac events, is also variously affected by statins [29]. Due to a similarity in structure, lipoprotein-a (Lp(a)) competes with fibrinogen for binding on fibrinogen receptors, fibrinogen and fibrin and it is presumed that this effect is responsible for the atherogenicity of Lp(a). Lp(a) levels are increased by statins by as much as 35% [30]. However, after the reduction of LDL-C by statins, this increase in Lp(a) may be of no consequence.

Atherosclerosis has been established as a chronic inflammatory disease involving T-cells and macrophages [22]. These cells secrete proteinases and inflammatory cytokines that contribute to the instability and rupture of the vulnerable plaque. Statins contribute to the stability of the atherosclerotic plaque via reduction of the cholesterol content of the core of the plaque, by reducing the number of T-cells and macrophages, and by increasing the collagen content of the fibrous cap [29]. C-reactive protein has been identified as being an important marker and predictor of the vulnerable atherosclerotic plaque and acute coronary syndromes. Numerous studies including 4S and WOSCOPS have shown that statins are able to reduce C-reactive protein levels. Abundant data is also available to indicate that statins have an inhibitory effect on the inflammatory cells of the vulnerable plaque and inhibit the release of inflammatory cytokines [22, 23].

Statins are also able to reduce the susceptibility of LDL-C to oxidisation, a crucial step in the uptake of LDL-C by macrophages to form foam cells [22]. The use of statins seems to have an antihypertensive effect that may be clinically significant [31]. Statins have been shown to stimulate bone
formation in experimental animals [32]. This has been supported by epidemiological data that links statin use with a decreased risk of fracture. However, other large studies have not been able to show any benefit on bone health with the use of statins [33]. Indeed, it has been shown that the effect of statins on bone varies with the dosage used, that statins increase osteoclast activity and bone resorption and they reduce bone mineral density in rodents [34]. The implications of these findings in humans remain to be seen.

**Niacin**

Niacin (nicotinic acid) is a safe, effective, low-cost, lipid-lowering therapy that has been used to treat dyslipidemia since 1955. Niacin inhibits the mobilization of free fatty acids from peripheral tissues, reducing hepatic synthesis of TGs and secretion of VLDL-C and inhibiting conversion of VLDL-C to LDL-C. The expected maximum reduction in LDL-C and VLDL-C concentrations is 30% and 40%, respectively. Niacin also increases serum HDL-C by up to 30%, surpassing all other drugs in this regard.

The most common adverse reactions associated with niacin result from prostaglandin-mediated vasodilation and include flushing, tingling, itching, rash, and headaches. Although these adverse events cannot be completely avoided, they can be minimized by slowly titrating the daily dose upward or with pretreatment using aspirin or nonsteroidal anti-inflammatory drugs. Gastrointestinal adverse reactions, such as dyspepsia, diarrhea, flatulence, and nausea, are also seen with niacin. At high doses of sustained-release niacin (>2 g daily), elevations in hepatic enzymes and uric acid can occur, although this phenomenon is not observed with high doses (up to 6 g daily) of the immediate-release formulation of niacin.

Impaired glucose tolerance has been associated with niacin therapy. However, treatment with niacin has been shown to be an appropriate treatment option for patients with diabetes. A recently published 16-week, placebo-controlled study randomized patients with diabetes into 2 groups: one receiving 1000 or 1500 mg daily of extended-release niacin and the other, placebo. In addition to noting dose-dependent increases in HDL-C and reductions in TGs, hemoglobin HbA1c levels were unchanged by niacin therapy compared to placebo. The authors concluded that low-dose extended-release niacin therapy was a safe and effective treatment for patients with T2D [35].
The extended-release form of niacin does not appear to cause the hepatotoxicity associated with previous sustained-release forms of the drug. Niacin can be dosed once, or, more commonly, twice daily. However, flushing can still be a bothersome side effect with this product. At the maximum recommended dose of 2 g daily, extended-release niacin reduces LDL-C by 16.7%, TGs levels by 34.5%, and raises HDL-C by 25.8%.

A combination of niacin with laropiprant, a flushing pathway inhibitor, has recently been added to the therapeutic choices of dyslipidemia [36]. Nicotinic acid/laropiprant is indicated for the treatment of dyslipidaemia, particularly in patients with combined mixed dyslipidaemia (characterised by elevated levels of LDL-C and TGs and low HDL-C) and in patients with primary hypercholesterolaemia (heterozygous familial and non-familial). Clinical studies have shown nicotinic acid/laropiprant to reduce LDL-C, raise HDL-C, and decrease TGs. In clinical studies, patients taking nicotinic acid/laropiprant experienced significantly less moderate-to-extreme flushing than with nicotinic acid. In a pool of four active- or placebo-controlled clinical trials of more than 4,700 patients the percentage of patients taking nicotinic acid/laropiprant who discontinued due to any flushing related symptom was 7.2 percent compared to 16.6 percent for the pooled nicotinic acid (prolonged release formulation) alone groups [36].

**Fibrates**

Fibrates (fibric acid derivatives) gemfibrozil and fenofibrate possess minimal LDL-C reducing capacity, but are especially effective in patients who have severe hypertriglyceridemia and low HDL-C. Fibrates are also useful in patients with combined forms of hyperlipidemia. Although the mechanism by which fibrates reduce TGs is poorly understood, it is believed these agents work to increase fatty acid oxidation in the liver resulting in decreased secretion of VLDL-C and increased LPL activity in skeletal muscle. Thus, the lipid-modifying effects of fibrates are secondary to changes in these LDL-C precursors. In patients with very high TGs, fibrates may increase LDL-C.

Fibrates may be used in combination with niacins or bile acid sequestrants as these drugs appear to be additive in lowering LDL-C and TGs and raising HDL-C. When fibrates are given in combination with a bile acid sequestrant, the administration of the 2 drugs must be separated by at least 2 hours to ensure full bioavailability of the fibrates.
Combinations of fibrates with statins are very effective in lowering LDL-C and increasing HDL-C, particularly in patients with mixed hyperlipidemia characterized by elevated TGs and LDL-C. Combinations of statins and fibrates have been historically limited by the increased risk for myopathy. This is particularly true for the combination of a statin and gemfibrozil. This risk appears to be minimized by substituting one of the newer fibrates, fenofibrate, for gemfibrozil. When the choice is made to use a statin and fibrates in combination, these drugs should be used only in the lowest effective doses and only used in patients who have normal liver and kidney function.

**Bile Acid Sequestrants**

Bile acid sequestrants, or resins, are now used primarily as adjuncts to statins or niacin. The resins bind to bile acids in the intestine, impeding their reabsorption.

The interruption of the entero-hepatic bile circulation causes the liver to convert more of the hepatic cholesterol pool into bile acids. It also stimulates cholesterol synthesis, resulting in increased VLDL-C production. Reduction in hepatic cholesterol pools results in an up-regulation of hepatic LDL-C receptors and increased LDL-C clearance from the circulation.

Bile acid sequestrants reduce LDL-C by 15%-27%. When used in combination with a statin, additional lowering of LDL-C of 9%-16% can be seen with as little as 4 g twice daily. Caution should be used when treating patients who have mixed hyperlipidemias, as bile acid sequestrants can increase TGs by 7%. Currently available bile acid sequestrants include cholestyramine and colestipol, which are both supplied as powdered products that require mixing with juices or food to make them more palatable.

The most common adverse reactions are gastrointestinal effects, including abdominal pain, belching, bloating, constipation, gas, heartburn, and nausea. These resins should be administered 2 hours before or 6 hours after warfarin, theophylline, digoxin, and levothyroxine. Colesevelam is a new bile acid binding resin available in tablet form. Compliance may be improved with this formulation, and no drug interactions have been noted between colesevelam and the agents known to interact with cholestyramine and colestipol, which are listed above.
Cholesterol-Absorption Inhibitors

Ezetimibe, the first agent available in this class, reduces plasma cholesterol by selectively inhibiting the absorption of dietary and biliary cholesterol from the intestine. Ezetimibe appears to specifically inhibit free cholesterol uptake into the intestinal enterocyte by interacting with a cholesterol transporter, however its exact mechanism of action remains to be elucidated [36].

Treatment of 820 patients who have hypercholesterolemia with 10 mg daily of ezetimibe reduced LDL-C by 18%, elicited a 12% decrease in TC, a 4% decrease in TGs, and a 1% increase in HDL-C. The safety profile of ezetimibe was similar to placebo, with no clinically significant changes in creatine kinase or hepatic transaminase levels and no effect on the absorption of fat-soluble vitamins A, D, and E. The most common side effects in both the active treatment and placebo groups were headache, upper respiratory tract infections, and back pain, although the back pain was not believed to be related to either ezetimibe or placebo [38].

The combination of ezetimibe and a low dose statin inhibit both the endogenous and exogenous production of cholesterol. Subjects with hypercholesterolema treated with simvastatin 10 mg daily for 14 days achieved a 35% reduction in LDL-C levels, while those treated with simvastatin 10 mg plus ezetimibe 10 mg achieved a reduction of 52%, which is a decrease expected from the much higher simvastatin dose of 80 mg [39]. Additionally, ezetimibe has virtually no effect on statin pharmacokinetics. Thus, combining ezetimibe with a statin may reduce the dose of the latter drug required to achieve target levels, or, in patients who respond poorly to statins, may improve the lipid-lowering effect.

Diabetic Dyslipidemia

The basic feature of diabetic dyslipidemia is the elevation of TGs. Other elements of atherogenic dyslipidemia are to a large extent associated with hypertriglyceridemia. In diabetes the elevated levels of lipoproteines rich in TGs, both originating form the small intestine (chylomicrons) and endogenous (VLDL), result in an increased and prolonged postprandial hypertriglyceridermia, which in turn enhances exposition of the vessel wall to atherogenic action of these lipoproteins. The activity of both lipoprotein and hepatic lipases (LPL and HL) taking part in TG catabolism is altered in

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diabetes – LPL activity is partially inhibited, and HL activity up-regulated – which accelerates the IDLs to LDLs conversion, causing however no significant rise in the levels of the latter.

Insulin resistance is associated with the lack of inhibition of the VLDL synthesis. Normally their production is selectively down-regulated in postprandial state as the result of an insulin activity. This mechanism is distorted in T2D patients – insulin mediated inhibition of large VLDLs (VLDL-1) synthesis in the postprandial period becomes selectively impaired [40, 41].

Another mechanism that may take part in the pathological overproduction of VLDL particles in diabetes is the insulin triggered increased activity of sterol regulatory element-binding protein 1c (SREBP-1c). SREBP-1c activation leads to an increased *de novo* lipogenesis and plays an important role in the lipid accumulation in the liver (steatosis of the liver) thus increasing the amount of TGs available for VLDL synthesis [42].

The abundance in the plasma TGs enriched lipoproteins (TGRL) triggers the cholesterol ester transfer protein (CETP) protein dependant mechanism of lipid transfer between different classes of lipoproteins. In hypertriglyceridemia an “overflow” of TGs from TGRL enhances the CETP mediated triglyceride transport to LDL-C and HDL-C particles in exchange for cholesterol esters. As a result of HLs activity which is increased in diabetes, TGs are then split from these particles. Thus, formed LDL-C particles have a smaller cholesterol ratio per one particle of apolipoprotein B-100. They are called small dense LDL-C (subfraction LDL-B) and are believed to be more atherogenic. The atherogenic action of small dense LDL-C is dependent on the particle size, its architecture, and biological properties. Because of their small diameter, the dense LDL-C penetrate easier to a vessel wall and are more susceptible to oxidation. Small LDL-C has lower affinity to the LDL-C receptor, resulting in a prolonged plasma circulation which enhances their transport into a vessel wall and accumulation in atherosclerotic plaques. Many studies have shown that in diabetic patients the number of small dense LDL-C increases in correlation with triglyceridemia, starting from level just slightly higher than 130 mg/dL.

**Low HDL**

Low level of HDL-C, together with hypertriglyceridemia constitute a dominant feature of diabetic dyslipidemia. Characteristically there is a predominance of HDL-C particles of smaller diameter, belonging to the HDL
3 subclass, and large HDL-C (HDL 2) level is decreased [43]. The diameters of HDL-C correlate with the TGs in the fasting state. Another specific feature is a relatively greater decrease in the level of HDL-C containing apoAI and apoAII (Lp AI:AII). In T2D the level of Lp AI:AII seems to be more important than that of LpAI for the activity of a reverse cholesterol transport from the peripheral tissues. Therefore, it may be speculated that in T2D the low HDL-C is associated with its smaller effectiveness in the process of reverse cholesterol transport (RCT). The key mechanism responsible for HDL-C decrease is the process of lipid exchange between VLDL-C, HDL-C, and LDL-C, described above, and mediated by CETP protein. The factor intensifying this process is an increase in VLDL-C, during prolonged postprandial lipemia. TGs enriched HDL-C undergoes hydrolysis caused by HL. As a result of the interaction with liver lipase the HDL-C particles become like those of LDL-C, smaller and denser. Small dense HDL-C is catabolized faster leading to renal excretion of apoprotein AI [44, 45]. The relative predominance of the subfraction of small dense HDL-C in diabetes is another feature of diabetic dyslipidemia.

**Prolonged Postprandial Lipemia**

An important element of abnormal lipid metabolism in diabetes is prolonged hyperlipemia. A number of mechanisms have been suggested to be responsible for this process. The postprandial increase in concentration of chylomicrons in serum is combined with the increased levels of VLDL-C particles, the production of which, in contrast to normal state, is not inhibited in diabetes. Both types of lipoproteins compete for binding with LPL what prolongs their catabolism. Additionally, LPL activity itself is impaired. The third postulated mechanism is due to the impaired liver clearance of remnants resulting from a disturbed uptake of these particles by LDL-C receptors and LDL-C receptor related protein [46, 47]. It has not been determined whether in T2D the production of apo B-48-containing lipoproteins (chylomicrons) is enhanced. The equal atherogenic potential of remnants derived from VLDL-C (containing protein apo B-100) and remnants containing apo B-48 is emphasized. The increased postprandial lipemia is a state associated with an impaired endothelial function [48, 49]. Many studies have shown, that in the state of insulin resistance, there is the “lipid intolerance”, presenting as substantially higher increase in TGs in the postprandial period as well as long lasting fasting hypertriglyceridemia. Another important pathogenic mechanism
associated with the postprandial hyperlipemia as an increased prothrombotic activity, which can be the trigger factor for an acute coronary syndrome, following fatty meal [50].

Diabetic patients show specific disturbances in their lipid profile – known as diabetic dyslipidemia. It should be emphasized that usually in DM the level of cholesterol contained in LDL-C – widely believed to be the main atherogenic fraction of cholesterol in serum – is only slightly increased. This relatively “normal” level of LDL-C was, until recently, the reason for neglecting hypolipemic treatment of patients with diabetes. Apart from a moderate increase in LDL-C, usually not exceeding 130–150 mg/dL, in atherogenic dyslipidemia there is an increase in TGs to above 150–200 mg/dL and simultaneous decrease in HDL-C to below 40 mg/dL. An important additional feature of atherogenic diabetic dyslipidemia, not evaluated in standard lipidogram, is an increase of subfraction of small dense LDL-C. Recently, several simple laboratory tests have been developed to enable a quantitative assessment of LDL-B (small dense LDL-C) subfraction in daily clinical practice [51].

In hypertriglyceridemia assessment of non-HDL-C may be useful. The ATP III NCEP has decided the non-HDL-C to be the secondary target of intervention in the population of patients with hypertriglyceridemia > 200 mg/dL, especially in patients with diabetes and/or the metabolic syndrome. The level of non-HDL-C reflects better the risk associated with the presence of the increased levels of lipoproteins containing atherogenic apoprotein B (apo B) i.e. VLDL-C, IDL-C and LDL-C, and Lp(a). In diabetic patients the non-HDL-C target should be < 130 mg/dL.

**Effects of Statins in T2D Patients:**
**Clinical End-Point Trials**

The Diabetes Atorvastatin Lipid Intervention study (DALI) compared the effects of aggressive and standard lipid-lowering therapy on fasting TGs in 217 patients with T2D [52] who were randomly assigned to aggressive (80 mg/d) or standard (10 mg/d) therapy with atorvastatin vs. placebo. During the 30-week study, fasting TGs were reduced by 35% with aggressive therapy and by 25% with moderate therapy compared with an increase of 10% with placebo. Results of A Randomized, Double-Blind Study to Compare Rosuvastatin and Atorvastatin in Patients with Type II Diabetes study (ANDROMEDA), in which rosuvastatin (10 or 20 mg/d) was administered to

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450 patients with T2D and dyslipidemia, found that LDL-C was reduced by 51% and 57%, respectively, and 94% and 96% of patients achieved their LDL-C goal [53].

Numerous primary and secondary prevention trials provide strong evidence that statins decrease the risk of cardiovascular events in patients with DM [54-61]. These cardiovascular benefits are closely linked to the lipid-altering effects of the statin and support recommendations by the NCEP ATP III [9] for aggressive use of statins as first-line therapy in the treatment of diabetic dyslipidemia.

Subanalyses of four secondary prevention trials [Scandinavian Simvastatin Survival Study (4S; two analyses), Cholesterol and Recurrent Events (CARE), Long-Term Intervention with Pravastatin in Ischemic Disease (LIPID), and the Heart Protection Study (HPS)] incorporating more than 9000 patients with mostly T2D or impaired fasting glucose indicate that statin treatment significantly reduces the coronary event rate vs. placebo in this population, and that the risk reductions are similar to those in statin-treated nondiabetic patients [54-56]. The relative risk reduction in these studies ranged from 13–55% compared with placebo. In all of these studies, statin therapy was well tolerated.

Among four primary prevention studies [Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT-LLT), Anglo-Scandinavian Cardiac Outcomes Trial–Lipid Lowering Arm (ASCOT–LLA), CARDS, and Die Deutsche Diabetes Dialyze (4D)] including more than 9000 patients, only CARDS reported an unequivocal significant reduction in cardiovascular event rates [57-60]. The lack of treatment effect in ASCOT-LLA is probably attributable to an inadequate number of absolute events in the smaller subgroup with diabetes [57], because the 16% reduction in myocardial infarction was not significantly different from that observed in the overall ASCOT-LLA population. The negative outcome in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial study is probably a result of a high rate of LDL-C-lowering treatments in the usual care group, resulting in a small (11%) difference in LDL-C concentrations between the treated and usual care groups [58]. Finally, the Die Deutsche Diabetes Dialyze trial enrolled patients with end-stage renal disease, and thus the lack of significant improvement in CHD risk rates suggests either that statin treatment may need to be provided to patients with T2D at an earlier stage of disease or that cardiovascular events in patients with end-stage renal disease is not amenable to statin therapy [59]. The results of CARDS, in which a 40% reduction in LDL-C was associated with a 37% reduction in the primary end

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point of time to first acute CHD event, revascularization, or stroke, does provide strong support for the view that primary prevention is beneficial in patients with T2D [60].

**CHOOSING THE OPTIMAL REGIMEN**

A controversy in the lipid community is whether to increase the dose of a statin or add adjunctive therapy for further LDL-C reduction. Proponents of increasing the statin dose argue that keeping the regimen simple will improve adherence, be more cost-effective, and that adjunctive agents may not provide additional pleiotropic effects comparable to higher statin doses. Conversely, others argue that doubling the statin dose may result in only a 6% further reduction in LDL-C with increased side effect potential, whereas the addition of ezetimibe or a bile acid sequestrant may result in approximately a 20% reduction in LDL-C.

However, while increasing the statin dose may be the simplest option in certain cases, statins do have dose-dependent side effects particularly when titrated to the highest doses. In cases such as this, adding a second agent (ie, ezetimibe, colesevelam) with a different site of action will not only provide more LDL-C reduction but also limit potential side effects. The use of fixed combination lipid-altering products (ie, ezetimibe/simvastatin) offers potential advantages and may be preferential to adding a separate second agent or titrating the statin. The attributes of these products compared with statin monotherapy include an overall improved effect on the lipid profile and the possibility of greater cost-effectiveness.

There is less controversy surrounding additional agents for other types of dyslipidemia. Among patients with low HDL-C, attaining the LDL-C goal is the first priority followed by achieving the non-HDL-C goal and maximizing therapeutic lifestyle changes. If HDL-C still remains a concern, therapy with niacin or fibrates may then be considered.

If patients have mixed dyslipidemia, and TGs exceed 500 mg/dL, the first objective is to reduce the TGs in order to prevent pancreatitis. Many practitioners prefer fibrates for hypertriglyceridemia because of the greater effectiveness, lower incidence of side effects, and lesser need for titration compared with niacin. These individuals may require a statin for LDL-C reduction after the TGs are reduced. Additional precautions must be taken with this combination to avoid possible adverse events.
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Chapter 3

**FISH PROTEINS FOR CORONARY ARTERY DISEASE**

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**ABSTRACT**

Recent recommendations of federal and regulatory agencies suggest revisit the claims of fish oils rich in omega-3 fatty acids and benefits in prevention of cardiovascular disease. We propose the importance of fish proteins in prevention of CVD to offer better opportunity of lowering blood pressure and coronary artery disease burden. Fish proteins are analyzed by protein electrophoresis after extraction from fish. The nutrition value of fish protein components are estimated and compared with recommended daily allowances (RDA). However, benefits of fish proteins remain less known due to poor knowledge of clinical outcomes, endpoint calibrations and their food value. The current status of fish dietary research and possibility of their use in coronary artery disease as cardioprotective food is presented if the fish protein intake may be better approach over omega fatty acids as reliable, relevant dietary recommendations for CVD in future.

**Keywords:** Fruits, vegetables, legumes, diet, coronary disease, trials, prognosis, mortality

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1. Introduction

High protein rich fish eating in diet is routine since ages of human origin [Brown 1990]. Since nineteenth century, very low cardiovascular risk of heart disease among Eksimos in Greenland, tribals in Australia and Japan remained an unsolved puzzle. In last half century cholesterol was identified as responsible of heart disease and lipoproteins as main culprit of heart attacks and atherosclerosis [Cote et al. 2004, Kristensen et al. 2001, Singh et al. 2011, O’Dea 1988, 1991]. Presently, omega-3 fatty acids in fish oil are choice in cardiovascular disease prevention while statins show serious side effects. Several omega fatty acid CVD prevention trials in Asia, Europe and West have documented mixed view on benefits of omega fatty therapy. Supplementation of fish protein has emerging as better source than other protein sources in cardioprotection (Bergeron et al. 1989, 1992). American heart Association (AHA), NECP ATP III has evidenced significantly the prospects of lipid lowering by dietary means. Marine fish protein can also benefit the heart [NCEP Report 2001]. Recent studies provide little or no direct evidence about how fish proteins and amino acids taurine, methionine can prolong life or prevent or delay CAD events (Yamori et al. 2009). Today, it is open question. Author proposes ‘one amino acid-one fatty acid concept’ that amino acid (in fish protein) may be alternative approach to keep high HDL or low (LDL) or (LDL+TG+C) lipids.

In light of these recommendations, we explore the possibility of fish proteins as emerging option of cardioprotective food while most of diets and foods remain at large unidentified without scientific reason of their action. Fish proteins in food practice and major mechanistic action in control of lipids is quite unknown [Singh et al. 2010]. Present paper describes the methods and results of proteins in commonly used fish in central India. Fish are excellent source of protein. 100 g cooked fish provides 18-20 g of protein (or one third RDA) containing abundant essential amino acids. The protein content in most of the fish are constant, invariable with season. Author reported the variation of fish proteins based on fish feed and month of breeding in different seasons [Shriniwas et al. 2008].

The present chapter describes introduction to fish food as source of proteins beneficial in cardiac protection, protein and amino acid composition of fish foods, new one amino acid-one fatty acid concept (one amino acid + one fatty acid can lower lipid + stabilize heart), intervention trials and futuristic approach.
2. **FISH PROTEINS AND AMINO ACIDS**

    The essential amino acid compositions of fish are given in Tables 1-3 indicate abundance of lysine, methionine, threonine and low collagen. It enhances the digestibility coefficient near 100 and fish intake 100 g /day supplied around 25% RDA of adult male. However, less known fact is that fish protein consumption how decreases the atherogenic risk of vascular diseases. Very less known is the results of one study showing sole fish diet increased blood HDL lipoprotein relative to other soy proteins or milk (Beauchesne-Rondeau et al. 2010). Nonprotein nitrogen compounds are common in fish mostly in sarcoplasm which includes free amino acids, peptides, amines, amine oxides, guanidine compounds, quaternary ammonium molecules, nucleotides and urea (Ackman, 1995). Creatine is main component in fish protein. Creatine plays an important role in fish muscle in metabolism in its phosphorylated from. Endogenous proteases yield some free amino acids such as Glycine and taurine, histidine (more than 1%) in red muscle of tuna fish. Important fact of proper cooking of mahi-mahi, mackerel, skipjack and tuna fish here contributes as improper cooking only converts histidine to histamine. Histamine is not affected by heat and becomes hazard to consumers due to “scombroid poisoning” (Arino et al. 2003, Food and Drug Administration, 1989, Haard, 1995).

![Figure 1. Nutrition value of fish protein is shown as micronutrients amino acid composition. Notice the scores of nutrition value 41 for micronutrients and 148 for amino acids.](image)
Table 1. Apparent crude protein and amino acid availabilities (%) from various fish in cardioprotection to meet FAO/WHO daily recommended values*

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Atlantic salmon</th>
<th>Coho salmon</th>
<th>Cherry salmon</th>
<th>Channel catfish</th>
<th>Rainbow trout</th>
<th>Herring menhaden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>6.32</td>
<td>6.08</td>
<td>6.35</td>
<td>6.31</td>
<td>6.57</td>
<td>9.13</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.61</td>
<td>5.99</td>
<td>6.23</td>
<td>6.67</td>
<td>6.41</td>
<td>8.54</td>
</tr>
<tr>
<td>Aspartate</td>
<td>9.92</td>
<td>9.96</td>
<td>9.93</td>
<td>9.74</td>
<td>9.94</td>
<td>8.00</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.80</td>
<td>0.95</td>
<td>1.23</td>
<td>1.34</td>
<td>0.86</td>
<td>0.80</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.41</td>
<td>7.31</td>
<td>7.62</td>
<td>8.14</td>
<td>7.76</td>
<td>8.93</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.02</td>
<td>2.99</td>
<td>2.39</td>
<td>2.17</td>
<td>2.96</td>
<td>9.06</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.41</td>
<td>3.70</td>
<td>3.96</td>
<td>4.29</td>
<td>4.34</td>
<td>8.93</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.72</td>
<td>7.49</td>
<td>7.54</td>
<td>7.40</td>
<td>7.59</td>
<td>9.06</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.28</td>
<td>8.64</td>
<td>8.81</td>
<td>8.51</td>
<td>8.49</td>
<td>8.41</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.83</td>
<td>3.53</td>
<td>3.14</td>
<td>2.92</td>
<td>2.88</td>
<td>8.92</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.36</td>
<td>4.14</td>
<td>4.63</td>
<td>4.14</td>
<td>4.58</td>
<td>8.51</td>
</tr>
<tr>
<td>Proline</td>
<td>4.64</td>
<td>4.76</td>
<td>4.33</td>
<td>6.02</td>
<td>4.89</td>
<td>8.51</td>
</tr>
<tr>
<td>Serine</td>
<td>4.61</td>
<td>4.67</td>
<td>4.48</td>
<td>4.89</td>
<td>4.66</td>
<td>8.51</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.95</td>
<td>5.11</td>
<td>4.63</td>
<td>4.41</td>
<td>4.76</td>
<td>8.51</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.93</td>
<td>1.40</td>
<td>0.83</td>
<td>0.78</td>
<td>0.93</td>
<td>7.27</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.50</td>
<td>3.44</td>
<td>3.58</td>
<td>3.28</td>
<td>3.38</td>
<td>8.97</td>
</tr>
<tr>
<td>Valine</td>
<td>5.09</td>
<td>4.32</td>
<td>4.85</td>
<td>5.15</td>
<td>5.09</td>
<td>8.97</td>
</tr>
</tbody>
</table>

* FAO/WHO recommends for 80 kg man/60 kg woman are: Isoleu 800/600; Leu 1120/840; Lys 960/720; Meth/Cys 1040/780; Phenylala/Tyr 1120/840; Thr 560/420; Tryp 280/210; Val 800/600.

Table 2. Energy and fat contents in different fish

<table>
<thead>
<tr>
<th>Fish (84 g/3 oz)</th>
<th>Calories</th>
<th>Total fat*</th>
<th>Saturated fat*</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g/%DV)</td>
<td>(g/%DV)</td>
<td>(g/%DV)</td>
<td>(g)</td>
</tr>
<tr>
<td>Cod</td>
<td>100</td>
<td>½</td>
<td>0/0</td>
<td>20</td>
</tr>
<tr>
<td>Halibut</td>
<td>110</td>
<td>2/3</td>
<td>0/0</td>
<td>23</td>
</tr>
<tr>
<td>Mackerel</td>
<td>210</td>
<td>13/20</td>
<td>1.5/8</td>
<td>21</td>
</tr>
<tr>
<td>Salmon¹</td>
<td>160</td>
<td>7/11</td>
<td>1/5</td>
<td>22</td>
</tr>
<tr>
<td>Salmon²</td>
<td>130</td>
<td>4/6</td>
<td>1/5</td>
<td>22</td>
</tr>
<tr>
<td>Salmon³</td>
<td>180</td>
<td>9/14</td>
<td>1.5/8</td>
<td>23</td>
</tr>
<tr>
<td>Salmon⁴</td>
<td>200</td>
<td>12</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Tuna</td>
<td>320</td>
<td>24/30</td>
<td>1.2/6</td>
<td>21</td>
</tr>
</tbody>
</table>

Serving size: 3 Oz skinless cooked fish portion.
* Nutrient value / % Daily Value of nutrient.
Table 3. Nutritive Profiles of fish (in 100 gm whole flesh) in cardioprotection

<table>
<thead>
<tr>
<th>Fish</th>
<th>Calories</th>
<th>protein</th>
<th>Fat</th>
<th>Ca</th>
<th>Iron</th>
<th>Vit A</th>
<th>Vit D</th>
<th>EPA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon/Sake</td>
<td>167</td>
<td>20.7</td>
<td>8.4</td>
<td>14</td>
<td>0.9</td>
<td>200</td>
<td>1300</td>
<td>492</td>
<td>820</td>
</tr>
<tr>
<td>Tuna/maguro</td>
<td>322</td>
<td>21.4</td>
<td>24.6</td>
<td>11</td>
<td>1</td>
<td>100</td>
<td>720</td>
<td>1250</td>
<td>2880</td>
</tr>
<tr>
<td>Tuna red meat</td>
<td>133</td>
<td>28.3</td>
<td>1.4</td>
<td>5.0</td>
<td>2.0</td>
<td>20</td>
<td>210</td>
<td>27</td>
<td>115</td>
</tr>
<tr>
<td>Sardine/Iwash</td>
<td>213</td>
<td>19.2</td>
<td>13.8</td>
<td>7.0</td>
<td>1.7</td>
<td>60</td>
<td>390</td>
<td>1390</td>
<td>1140</td>
</tr>
<tr>
<td>Bonito/Katsuo</td>
<td>129</td>
<td>25.8</td>
<td>2.0</td>
<td>10.0</td>
<td>1.9</td>
<td>17.0</td>
<td>400</td>
<td>78</td>
<td>310</td>
</tr>
<tr>
<td>Mackerel/Saba</td>
<td>230</td>
<td>19.8</td>
<td>16.5</td>
<td>22.0</td>
<td>1.5</td>
<td>100</td>
<td>440</td>
<td>1210</td>
<td>1780</td>
</tr>
<tr>
<td>Cod/ Tara</td>
<td>70.0</td>
<td>15.7</td>
<td>0.4</td>
<td>42.0</td>
<td>0.6</td>
<td>100</td>
<td>--</td>
<td>37</td>
<td>72</td>
</tr>
</tbody>
</table>

Table 4. Other potential fish/ non-fish foods available in India with their protein nutritive values in cardiac prevention

<table>
<thead>
<tr>
<th>Fish foods</th>
<th>Energy (kcal)</th>
<th>Protein (g)</th>
<th>Carbohydrates (g)</th>
<th>Fat (g)</th>
<th>Ca (mg)</th>
<th>P (mg)</th>
<th>Fe (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bhelki (fresh)</td>
<td>79</td>
<td>14.9</td>
<td>3.0</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crab (muscle)</td>
<td>59</td>
<td>8.9</td>
<td>3.3</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crab (small)</td>
<td>169</td>
<td>11.2</td>
<td>9.1</td>
<td>9.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilsa</td>
<td>273</td>
<td>21.8</td>
<td>2.9</td>
<td>19.4</td>
<td>180</td>
<td>280</td>
<td>2.1</td>
</tr>
<tr>
<td>Katla</td>
<td>111</td>
<td>19.5</td>
<td>2.9</td>
<td>2.4</td>
<td>530</td>
<td>235</td>
<td>0.9</td>
</tr>
<tr>
<td>Mackerel</td>
<td>93</td>
<td>19.9</td>
<td>0.5</td>
<td>2.7</td>
<td>429</td>
<td>305</td>
<td>4.5</td>
</tr>
<tr>
<td>Pomfret Black</td>
<td>111</td>
<td>20.3</td>
<td>1.5</td>
<td>2.6</td>
<td>280</td>
<td>306</td>
<td>2.3</td>
</tr>
<tr>
<td>Pomfret White</td>
<td>87</td>
<td>17.0</td>
<td>1.8</td>
<td>1.3</td>
<td>200</td>
<td>290</td>
<td>0.9</td>
</tr>
<tr>
<td>Surmai Lobster</td>
<td>92</td>
<td>19.9</td>
<td>NIL</td>
<td>1.4</td>
<td>92</td>
<td>161</td>
<td>2.0</td>
</tr>
<tr>
<td>Prawn</td>
<td>99</td>
<td>19.1</td>
<td>0.8</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rohu</td>
<td>97</td>
<td>16.6</td>
<td>4.4</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sardine</td>
<td>101</td>
<td>21.0</td>
<td>NIL</td>
<td>1.9</td>
<td>90</td>
<td>360</td>
<td>2.5</td>
</tr>
<tr>
<td>Shrimp (small, dried)</td>
<td>340</td>
<td>68.1</td>
<td>NIL</td>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Salmon fish is oily fish, high in protein, omega-3 fatty acids and vitamin D. It is abundant in Atlantic, Pacific oceans, farm raised roe and raw sashimi in origin. Salmon fish contains dioxanes, polychlorinated biphenyl compounds. Salmon flesh is red in color but natural color is white due to astaxanthin or canthaxantin carotinoid antioxidant pigments. (Food Standards Agency, 2004, Mozaffarian 2006).
Sardines are an excellent low calorie, low carbohydrate fish food. Small, silver colored fish is also known as pilchard as a healthy snack or regular meal due to the various nutrients of fish. Pilchards are oily fish, very rich in omega-3 fatty acids, no carbohydrates, rich sources of fish protein, vitamins, A, B, C, D and E with various minerals such as iron, calcium, selenium, magnesium, phosphorous and potassium. Sardine fish contains the antioxidant, coenzyme Q10 and supports Heart Health. Oily sardine fish contains omega-3 fatty acids play a vital role in regulation of blood cholesterol levels to promote heart health. Fish intake helps to lower bad cholesterol and maintain good cholesterol levels. The improved ratio between good and bad cholesterol helps to sustain good heart health. It also reduces the risk of stroke and cardiovascular disease. Vitamin B12, also found in high concentrations in the fish, helps to protect the walls of the arteries. It promotes cardiovascular health. Tuna is an excellent source of protein, and while some vitamin and mineral losses occur during canned tuna processing, the protein nutritive values are not dramatically changed. Tuna is an excellent source of the omega-3 fatty acids EPA and DHA, protein, potassium, selenium, and vitamin B12. Every 100 g fish food contains: Carbohydrates 0.00 g Dietary fiber 0.0 g Fat 0.95 g Protein 23.38 g Vitamin A 60 IU Thiamine (Vit. B₁) 0.434 mg Riboflavin (Vit. B₂) 0.047 mg Niacin (Vit. B₃) 9.800 mg Pantothenic acid (B₅) 0.750 mg Vitamin B₆ 0.900 mg Folate (Vit. B₉) 2 mcg Vitamin B₁₂ 0.52 mcg Vitamin C 1.0 mg Vitamin E 0.50 mg Vitamin K 0.1 mcg Calcium 16 mg Iron 0.73 mg Magnesium 50 mg Phosphorus 191 mg Potassium 444 mg Sodium 37 mg Zinc 0.52 mg Manganese 0.015 mg.

Cod, hake, flounder, sole contain less than 100 Kcal per 100 g fish flesh while mackerel, herring, salmon contain 250 kcal in 100 g flesh. Lean type fish have 40-60 mg cholesterol per 100 g muscle has Salmon, tuna, mackerel, sardine fish have plenty of omega 3 fatty acids also which is considered to be beneficial in cardiac prevention. Major beneficial effects of fish eating are long life expectancy, antiarrhythmic, antithrombosis, lowering triglycerides and VLDL cholesterol and antihypertensive action.

3. Fish Proteins in Cardiac Prevention

Fat intake is not culprit at all. We analyze this issue based on recent studies. The Harvard School of Public Health showed that total fat intake bore
no significant relation to the risk of CAD but fish intake in diet reduces the risk. Other studies suggested high omega fatty acid intake with adverse effect or stimulating fibrillation and reducing platelet aggregation or antithrombotic (Saravanan et al. 2010; Aarsetov et al. 2008, Harris 2007, Kowey et al. 2010, Pratt et al. 2010, Din et al. 2004). Investigators clearly advocated that claims of fish omega fatty acids in cardioprotection are fishy and not conclusive (Albert et al. 2010). In fact, several studies over 4 decades suggested that fish diet is rich in proteins with potentials to reduce the risk of CAD (Bernstein et al. 2010, de Leo et al. 2009, Mozaffarian 2008, Das 2000, ADA report 2003a 2003b). Saturated fats in other animal meats and foods appear to carry more risk than fish, refined carbohydrates, other food classes representing the bulk of recommended daily calories. Fish diet is rich in unsaturated fatty acids (mono- and polyunsaturated fatty acids), good protein quality (45%-80%). In addition, the fish foods contain mercurial, arsenic etc contaminants and appear to have adverse effects. Several other epidemiological studies (Gordon et al. 1981) have shown no evidence that man who eat less fat live longer or have fewer myocardial infarctions (MI). Epidemiological evidence that fish fed population on low carbohydrate diet for 6 months had low fat and low risk of heart disease (Westman et al. 2002). Other group reported that fish protein was better than other cardioprotective dietary and omega-3 options (Bergeron et al. 1989, 1992). The relationship of fish muscle protein characteristics in different seasons including casein content and W-6/W-3 contents was evidenced with relationship of improved blood pressure and episodes of heart complications after fish eating. Our hypothesis was: can fish proteins are cardiac protecting? Recently, study reported protein content of H. fossilis (BLOCH) fish to evaluate the nutritive value of fish that has impact on fish eating community in Nagpur. In Western world and Southeast Asia, fish eating concept is emerging as affluent style of cardiac protection. We project that fish protein content analysis can highlight further potentials of fish eating in cardiac protection due to its good contents on omega fatty acids, fish oils and protein values in fish. Authors reported a short cross sectional study on patients with cardiac heart disease (arrhythmia, early myocardial infraction sign, high blood pressure) having treatment of betablocker and diuretics for last known 3-6 months. Patients were monitored and intervened with W-3/W-6 EPA/DHA rich oils or with fish eating for at least 100 Grams/100 Kg weight/day in last 1-3 Months. Cross sectional routine BP, Heart indicators, dietary intake of fish proteins and oil content was calculated. To further calculation of fish protein content,  


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electrophoretic mobility indices were screened in the H.fossilis. The casein content, oil content and heart indicators were correlated to establish the effect of fish protein and oil content as effective cardiac protection measures. Intervention of fish eating and withdrawal of chemotherapy improved the serum lipid profile (HDL-3), BP, EKG. After 3 months fish rich diet intervention showed further improved BP, lipid profile (HDL-3), EKG finding towards normal. However, during 3-6 months intervention effect was slower. The H.fossilis protein content analysis suggested several proteins on electrophoresis bands with possibility of other protein candidates in cardioprotection. Casein protein was distinct candidate. During seasonal protein patterns, male fish protein suggested April-June and female Dec-Jan period as high protein yield cardioprotective diet.

Cardioprotection by fish eating is a dilemma despite of known fish infection, environmental hazards. Fish breeding industries have significant role to serve infection–free, protein rich fish food through out the year by measures of spawning in different seasons specially resting phase of fish. Liver oil and proteins as combined have proven as best known cardiac protection non-chemotherapeutic measures. Our growing understanding of fish eating intervention with slow withdrawal of betablockers/diuretics has become routine clinical practice. In this report, major points are: 1. W-3/W-6 Fatty acid rich oils with fish casein protein projects the enhanced cardiac protection perhaps due to casein sparing effect and reduced free radicals enough to the level of further progress of cardiac injury; 2. fish breeding with spawning in rest phase shows impact on fish protein value; 3. cardiac protection is multifactorial and enhanced by combined fish liver oil omega fatty acids and amino acid composition in fish proteins. Fish high quality proteins with fish liver oils serve best cardiac protection.

Fish meat or muscle has good protein quality. Salmon, halibut, tuna are major fish foods. Casein, Taurine are major fish proteins. In following sections, we focus on fish protein contents and their role in hypertension and heart disease.

Essential hypertension is associated with higher lipoperoxidation and imbalanced antioxidant status (means oxidative stress) with increased RBC enzymes (Yuan et al. 2002). Mechanisms are not well established yet but some experimental studies have established the modulation of HDL and reverse cholesterol transport, lipoprotein lipase activity in livers and action of actinopectin, ACE inhibitory peptides heart (Hersberger et al. 2005, O’Shea et al. 2010, De Lao et al. 2009, Jung et al. 2008, Siddique et al. 2004). Blocking
of AHR2 and ARNT1 gene expression was reported as cardioprotective (Antkiewicz et al. 2006).

3.1. One Amino Acid-one Fatty Acid Concept

One amino acid in fish protein may be alternative approach to keep high HDL, low (LDL+TG+C) lipids for cardioprotection. Author reported the benefits of casein protein in hypercholesterolemic hamsters. In gall stone bearing hamsters with high plasma cholesterol, casein (250 g/Kg wt) showed cholesterol lowering after 4 weeks. Cholesterol lowering effect against cholesterol saturation and cholelithiasis in hamsters was shown by using cytochrome P450 and cholesterol 7a Hydroxylase biomarker proteins. In casein fed animals, plasma lipids including total cholesterol, phospholipids, lipoproteins and biliary lipids were brought down in time fashion manner (Sharma et al. 2008). To prove the cardioprotective effect of casein, following studies suggest evidences in favor:

1) Researchers from Algeria compared fish protein consumption to casein in an animal model of hypertension. The fish protein diet lowered blood pressure and plasma total cholesterol compared with casein, leading researchers to conclude fish protein attenuated the development of hypertension (Yahia et al. 2003a). Casein intake changed the tissue antioxidant status in rats (Yahia et al. 2003b).

2) Dietary cod fish consumption rich in casein showed effect on muscle physiology by insulin induced activation of glutamate transfer protein and translocation characteristics in fish muscle (Tremblay et al. 2003)

3) Bergeron et al. 1989 reported the influence of fish proteins and compared to casein and soy protein for their effect on serum and liver lipids, and serum lipoprotein cholesterol levels in the rabbits. Investigators established the mechanism of cardioprotection by fish protein at molecular level as modulation of HDL and lipoprotein lipase activityCasein effect was closer with fish protein in cardioprotection and regulation of plasma lipids (Bergeron et al. 1992a, Jacques et al. 1995). Furthermore, interaction between fish proteins and lipids was responsible in regulation of serum and lipids in liver that gives benefit of cardioprotection (Bergeron et al. 1991). Fish protein actually showed effect in proportion of its incremental amounts in diet or factorial manner (Bergeron, 1992b). In following
section, individual amino acids are discussed their value in cardiac prevention.

**Taurine in Fish Proteins**

Taurine (T) was first noted as beneficial for stroke and cardiovascular diseases (CVD) prevention in genetic rat models, stroke-prone spontaneously hypertensive rats (SHRSP) (Yamori et al. 1984). The preventive mechanisms of T were ascribed to sympathetic modulation for reducing blood pressure (BP) and anti-inflammatory action. Recent epidemiological surveys revealed that taurine was effective in experimental arterio-lipidosis prone SHRSP selectively bred rats with higher reactive hypercholesterolemia. In fact, sulphur containing amino acids taurine, methionine showed attenuated effect on severe hypertension and stroke due to activation of 7α hydroxylase to accelerate cholesterol excretion into bile acids (Jerlich et al. 2000). Rats quickly developed arterial fat deposition and fatty liver which was attenuated by dietary Taurine supplementation (Yokogoshi et al. 1999). In fact, investigators suggested the role of rate limiting enzyme cholesterol 7α hydroxylase to bile synthesis to decrease cholesterol and inverse correlation between mRNA levels and enzyme gene CYP7A1 as contributor to increased serum triglycerides.

**Methionine, Cystein, Cystine in Fish Protein**

Sulphur containing amino acids are abundant in fish muscle. Their role in lowering lipid is not established but these amino acids play role in sarcoplasm in muscle cells and contractility (Ackman et al. 1995, Arino et al. 2003). Amino acid requirements in different age groups are specific as amino acid contents of fish foods stated below. In general amino acid requirement pattern is clear; methionine and cystine typically are most scarce in daily food. National Institute of Nutrition (ICMR) monitored the following requirements and suggested fish foods shown in Table 4 and Table 5 (NNMB report, 2007, FAO/WHO report).

*For adults*, methionine and cystine always are most scarce (100%). Even if only minimal amounts of amino acids are consumed, in 81% of foods methionine and cystine again are most scarce, in 19% tryptophan is scarce.

*For schoolchildren*, again methionine and cystine are most scarce in all foods except grains. In 47% of grains methionine and cystine are most scarce, but in 53% lysine is, depending on the applied amino acid requirements. However, nobody eats grains like bread and cornflakes only, again making

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methionine and cystine the scarcest amino acids in diets comprising lots of grains.

For younger children protein requirements match food-protein contents better, levelling relative availability; in 57% methionine and cystine are most scarce. In 25% phenylalanine and tyrosine are, in 13% tryptophan is, and in 5% of foods isoleucine is most scarce, depending on the applied amino acid requirements. But, again, methionine and cystine are the scarcest amino acids.

Table 5. Requirement of total amino acids available from fish foods in different age groups. Requirements as % daily values (%DV) are shown red for adults, yellow for schoolchildren and green in adolescents fed on fish food

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Cystine</th>
<th>Methionine</th>
<th>Phenylalanine</th>
<th>Tyrosine</th>
<th>Tryptophan</th>
<th>Isoleucine</th>
<th>Guanidine</th>
<th>Histidine</th>
<th>Creatine</th>
<th>Glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herring</td>
<td>269</td>
<td>167</td>
<td>149</td>
<td>389</td>
<td>231</td>
<td>231</td>
<td>167</td>
<td>216</td>
<td>233</td>
<td>180</td>
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<tr>
<td>Cod</td>
<td>256</td>
<td>198</td>
<td>162</td>
<td>396</td>
<td>233</td>
<td>228</td>
<td>162</td>
<td>216</td>
<td>223</td>
<td>215</td>
</tr>
<tr>
<td>Sardine</td>
<td>295</td>
<td>186</td>
<td>162</td>
<td>417</td>
<td>275</td>
<td>243</td>
<td>186</td>
<td>215</td>
<td>223</td>
<td>216</td>
</tr>
<tr>
<td>Mackerel</td>
<td>278</td>
<td>193</td>
<td>147</td>
<td>414</td>
<td>251</td>
<td>223</td>
<td>193</td>
<td>214</td>
<td>223</td>
<td>214</td>
</tr>
<tr>
<td>Tuna</td>
<td>316</td>
<td>233</td>
<td>216</td>
<td>482</td>
<td>269</td>
<td>262</td>
<td>233</td>
<td>226</td>
<td>223</td>
<td>224</td>
</tr>
<tr>
<td>Trout</td>
<td>301</td>
<td>222</td>
<td>164</td>
<td>429</td>
<td>258</td>
<td>260</td>
<td>222</td>
<td>221</td>
<td>223</td>
<td>222</td>
</tr>
<tr>
<td>Salmon</td>
<td>281</td>
<td>164</td>
<td>145</td>
<td>358</td>
<td>234</td>
<td>224</td>
<td>164</td>
<td>216</td>
<td>224</td>
<td>224</td>
</tr>
<tr>
<td>Brown shrimp</td>
<td>202</td>
<td>180</td>
<td>133</td>
<td>402</td>
<td>204</td>
<td>173</td>
<td>180</td>
<td>215</td>
<td>173</td>
<td>190</td>
</tr>
<tr>
<td>Mussel</td>
<td>254</td>
<td>191</td>
<td>191</td>
<td>353</td>
<td>219</td>
<td>214</td>
<td>191</td>
<td>217</td>
<td>191</td>
<td>190</td>
</tr>
</tbody>
</table>

Guanidine, Histidine, Creatine, Glycine

Creatine is considered as essential amino acid to keep creatine kinase enzyme CK-MB with other isozymes working in cardiac muscle contraction and it is also recommended for athletic activity (Food and Drug Administration, 1989, Haard, 1995). The mechanism of guanidine ME10092 is not known in cardiac prevention but its analogue CARIPORIDE is emerging to provide cardiac prevention by sodium-hydrogen exchange inhibition in mitochondria (Oliver et al. 2004, Mentzer et al. 2008). Histidine is converted in histamine in cooked fish but gives constipation problems (Arino et al. 2003, Food and Drug Administration, 1989).

Glycine is basically a novel anti-inflammatory, immunomodulatory cytoprotective agent and believed beneficial in cardiac prevention (Haard, 1995).
Squalene is bioactive isoprenoid substance present in shark liver oil. It is explored to neutralize the harmful effects of free radicals and improve membrane stabilizing properties. Squalene has been proven as safe dietary supplement without any side effect (Farvin et al. 2006, Thankappan et al. 2003).

3.2. Interventional Studies

Fish proteins showed similarity in casein for short-term metabolic effects of dietary interventions on various risk factors of CAD. Casein was given as supplements in form of beta casein A1 and A2 and effect was observed on plasma lipoproteins. Major effect was seen on HDL and triglycerides (Chin-Dusting et al. 2006, Veen et al. 2006, Nilausen et al. 1999). Studies provide little or no direct evidence about how fish proteins and amino acids taurine, methionine can prolong life or prevent or delay CAD events (Yamori et al. 2009). These studies also suffer from a major design flaw that only recently has been accepted in cardiovascular medicine: Metabolic or biochemical measures can provide mechanistic insights and are essential building blocks in therapeutic development, but they cannot reliably predict the effect of proposed interventions on clinical events. Authors describe metabolic and biochemical insight on fish protein composition and muscle physiological influence on heart physiology.

Cardiovascular Diseases and Alimentary Comparison Study CARDIAC trial was designed on biological markers of diet with hypertension and CVD mortality (Yamashita et al. 2006). CARDIAC (CVD and Alimentary Comparison) WHO-coordinated multi-center epidemiological survey on diets and CVD risks in 61 populations showed that twenty-four-hour urinary (24 hr) Taurine excretion was inversely related significantly with coronary heart disease mortality. Higher 24U-T excreters had significantly lower body mass index, systolic and diastolic BP, total cholesterol (T-Cho), and atherogenic index (AI: T-Cho/high density lipoprotein-cholesterol) than lower T excreters. T effects on CVD risks were intensified in individuals whose 24U-T and -magnesium (M) excretions were higher. Furthermore, higher Na excreters with higher heart rate whose BP were significantly higher than those with lower heart rate were divided into two groups by the mean of 24U-T, high and low T excreters. Since the former showed significantly lower BP than the latter, T may beneficially affect salt-sensitive BP rise. In other study on 61 populations

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including Guiyang, China and St. John’s, Newfoundland, Canada where in which the means of both 24U-T and -M were high and low, respectively. The former and the latter had low and high CVD risks, respectively (Yamori et al. 2006). Australian Aboriginals living at the coastal area in Victoria eat T- and M-rich bush and mainly sea foods and were free from CVD 200 years ago. Such diet pattern indicated that T- and/or M-containing seafood, vegetables, fruits, nuts, milk, etc was similar to prehistoric hunters’ and gatherers’ food should be good for CVD prevention. Now same population had highest CVD risks in those areas. In recent study, preventive effects of T good for health and longevity were first noted experimentally and later were proven epidemiologically in humans (Yamori et al 2006, 2009).

EXPEDITION study was an important, large, drug intervention study that tested the hypothesis that targeting an ischemia/reperfusion injury mechanism can result in a reduction in the incidence of MI after coronary bypass (CABG). The results of EXPEDITION revealed (1) compelling evidence that the incidence of intraoperative myocardial necrosis in patients undergoing CABG occurs more frequently than previously appreciated; (2) a reduction in the primary endpoint of all-cause death or nonfatal MI was achieved with cariporide (a guanidine analogue); and (3) the reduction in the primary endpoint was due almost exclusively to a reduction in nonfatal MI; however, this benefit was offset by a higher incidence of CVEs and mortality in patients receiving cariporide (Mentzer et al. 2008). It needs further research on other alternative amino acid or analogue with better outcome.

3.3. Nutrient Composition of Fish and Environment

There is some knowledge available to address whether fish protein composition can affect appetite or quality of life. High quality fish meal is a complete source of amino acids. It contains high levels of lysine and methionine, essential trace elements. Due to high nutrition value of fish meal, research for alternate sources of protein is continuing such as unsaturated fatty acid rich oils, legumes, by-product meats, brewer by-products with high nutritive value or available essential amino acids. The protein quality of fish is evaluated by freshness of flesh by volatile nitrogen content, intact proteins. Methods of assessing quality fall into three categories: 1. Inspection; 2. Chemical analysis; 3. Biological evaluation. Chemical tests of protein quality includes for: acid corrected pepsin digestible protein (AOAC and Torry methods), protein extraction methods by electrophoresis and SDS-PAGE,
multienzyme digestible proteins, total volatile basic nitrogen, lysine, sulphydryl groups and disulphide bonds. Biological tests are multienzyme and Torry pepsin solubility tests. Protein data in fish after PAGE electrophoresis is shown in Table 6.

In collaborative study, electrophoresis SDS-PAGE on Excel 15% homogeneous gels was easy and cheap silver staining method was better option than urea IEF method to identify fish species and distinguish different proteins in salmon, halibut and tuna fish food extracts (Etienne et al. 2000). Species differentiation was easy in different fish family by spot analysis as shown in Figure 2. For details of the technique, readers are referred to read original paper (Etienne et al. 2000).

Biogenic amines (cadaverine, putrescine, histamine, tyramine) are used to assess fish food quality (Chen et al. 2007) as shown in Table 7. Thiamine content was used as indicator of high quality as function of thermal processing conditions in canned salmon fish industry (Quitral et al. 2006). Temperature and food processing method used are two major factors in changing fish protein specially composition of methionine, cystein, taurine in fish food (Lipka et al. 1993).

**Table 6. Protein content of different fish are shown by using methods urea immunoelectrophoresis and SDS-PAGE methods**

<table>
<thead>
<tr>
<th>Fish species (common name)</th>
<th>Protein content (mg/g flesh)</th>
<th>Urea IEF</th>
<th>SDS-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncorhynchus gorbuscha (pink salmon)</td>
<td>182.4</td>
<td>215.6</td>
<td></td>
</tr>
<tr>
<td>Oncorhynchus keta (chum salmon)</td>
<td>125.8</td>
<td>135.2</td>
<td></td>
</tr>
<tr>
<td>Oncorhynchus mykiss (rainbow trout)</td>
<td>170.5</td>
<td>153.2</td>
<td></td>
</tr>
<tr>
<td>Salmo trutta (sea trout)</td>
<td>170.3</td>
<td>170.3</td>
<td></td>
</tr>
<tr>
<td>Salmo salar (Atlantic salmon)</td>
<td>208.1</td>
<td>167.1</td>
<td></td>
</tr>
<tr>
<td>Hippoglossus hippocoglossus (halibut)</td>
<td>124.8</td>
<td>136.9</td>
<td></td>
</tr>
<tr>
<td>Reinhardtius hippoglossoides (Greenland halibut)</td>
<td>171.2</td>
<td>155.6</td>
<td></td>
</tr>
<tr>
<td>Thunnus alalunga (albacore)</td>
<td>185.9</td>
<td>197.3</td>
<td></td>
</tr>
<tr>
<td>Thunnus albacores (yellow fin tuna)</td>
<td>148.8</td>
<td>173.9</td>
<td></td>
</tr>
<tr>
<td>Katsuwonis pelamis (skipjack tuna)</td>
<td>139.6</td>
<td>179.6</td>
<td></td>
</tr>
</tbody>
</table>

Source: Modified with permission from original reference Etienne et al. 2000.
Figure 2. SDS-PAGE. Extracts of raw (references R1-R21) and cooked (samples C1-C10) fish muscle were run on Excel gel homogeneous 15%. M) pI calibration proteins. The cathode is at the top of the gel. Reproduced with permission from reference (Etienne et al. 2000).

Table 7. Biogenic amine content in fish meals

<table>
<thead>
<tr>
<th>Fish</th>
<th>Histamine mg/100g¹</th>
<th>Putrescine mg/100 g¹</th>
<th>cadaverine mg/100 g¹</th>
<th>Tyramine mg/100 g¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>sardine</td>
<td>5.1</td>
<td>33.2</td>
<td>41.6</td>
<td>4.9</td>
</tr>
<tr>
<td>tuna</td>
<td>17.9</td>
<td>95.7</td>
<td>159.0</td>
<td>36.6</td>
</tr>
</tbody>
</table>

¹expressed as a concentration of the dry matter.
Table 8. Public online resources on fish diet and proteins for cardiovascular protection

http://linkinghub.elsevier.com/retrieve/pii/S004553504010173
http://www.pulsas.com/CARDIOL/home.htm
www.banglajol.info/index.php/JARD/article/viewPDFInterstitial/762/800
http://linkinghub.elsevier.com/retrieve/pii/S1050464801903742
http://linkinghub.elsevier.com/retrieve/pii/S1050464807000186
http://ohioline.osu.edu/sc172/sc172_17.html
http://aem.asm.org/cgi/content/abstract/74/11/3551
http://findarticles.com/p/articles/mi_m0887/is_6_20/ai_75818427

Temperature around the year in different months plays a significant role in fish breeding and fish growth.

The fish protein content, amino acid composition and growth hormone action all are dependent on dietary composition of fish meal and its quality. In recent study, Shriniwas et al. 2008 reported effect on temperature on fish liver proteins identifiable in H. fossalis and C. catla as shown in Figure 3 and protein contents on PAGE-electrophoresis in catala fish (a common human fish food in Nagpur, Central India).

Investigators established better SDS-PAGE electrophoresis method (see Appendix 1).

Figure 3. SDS-PAGE electrophoresis is shown for two fish species H. fossalis (A) and C.catla (B) muscle proteins.

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4. Effect of Fish Dietary Proteins on Coronary Artery Disease

In a randomized, single blind, controlled intervention trial, Singh et al. 200 Indo-Mediterranean dietary 24 weeks trial on 59 patients with high blood pressure and subsequent preliminary study Bharati D Shrinivas et al. 2008, both demonstrated effects of fish supplementation on blood pressures and blood lipids and nitric oxide in patients with hypertension and dyslipidemia. Epidemiological studies and intervention trials indicated that fish intake can decrease cardiovascular events in patients with high risk of coronary artery disease (CAD). It was evidence that total or whole fish intake can also decrease blood pressures and blood lipids. The beneficial effects of fish intake may be due to long chain w-3(EPA and DHA) fatty acids and amino acids in the fish proteins. Investigators examined the effects of mild fish intake on cardiovascular risk factors, in patients with hypertension and dyslipidemia. The effects of Indian fish (30g/day) and AHA-step 1 diet were compared for a period of 24 weeks in patients with mild hypertension (140/90-160/110mmHg) and dyslipidemia. All patients with known hypertension, with either low HDL (<40mg/dl) or high triglycerides (>150mg/dl) or hypercholesterolemia (T-C >200mg/dl), were randomly divided into fish intake group (Gr A, n=30) and control group (Gr.B, n=29) by the dietitian for computer generated data after an observation period of one week. Mean age (57.1±9.5 vs 58.5±8.7 years), body mass index (25.2±2.5 vs 24.6±2.6 kg/m²) and waist circumferences (89.8±8.8 vs 90.8±6.8 cm) were comparable at entry to the study. Mean systolic (155.6±11.5 vs 153.7±13.4 mmHg) and diastolic (100.4±8.8 vs 101.0±7.2 mmHg) blood pressures, in fish intake group and control group, were comparable, at entry to the study. However, after 24 weeks, there was a significant fall in both mean systolic (146.3±9.5 vs 152.5±12.0 mmHg, P<0.03) and diastolic (92.4±9.8 vs 98.6±7.6 mmHg, P<0.05) blood pressures in the intervention group compared to control group, respectively. Mean concentrations of blood glucose (126.8±35.1 vs 120.5±31.8 mg/dl) were comparable at entry to the study. Plasma levels of antioxidant vitamins E (20.5 ± 3.2 vs 21.5±3.3 pmol/L), and C (19.2±3.3 vs 19.6±3.4 pmol/L) were comparable at the entry to the study. After 24 weeks, plasma levels of vitamin E (28.6±3.8 vs 21.6±3.1 pmol/L) and C (25.4±4.0 vs 19.2±3.4 pmol/L) showed significant increase and TBARS and MDA (0.88±0.26 vs 1.52 ±0.31pm/dl) showed significant (p<0.02) decrease in the fish intake group compared to control group, respectively. HDL-C and triglycerides showed no
significant differences before entry to the study. However, after 24 weeks of treatment with fish, HDL-C (40.5±7.2 vs 35.2±8.5 mg/dl, p<0.01) showed a significant increase. T-C/HDL-C ratio (4.6±1.0 vs 5.6±1.1, mg/dl, p<0.05) and LDL cholesterol (120±12 vs 132±15, mg/dl, P<0.04), showed a significant decrease, while T-C and TG showed nonsignificant decline, in the intervention group compared to control group. Serum concentration of nitrite which is an indicator of nitric oxide were comparable at baseline (0.57±0.12 vs 0.63±0.16 µmol/L). After 24 weeks, there was a significant increase in nitrite levels in the intervention group compared to control group (0.93±0.21 vs 0.71±0.13 µmol/L) respectively. Increase in nitrite is an indicator of nitric oxide which is known to decrease endothelial function and blood pressures. Investigators indicated that mild fish intake can decrease blood pressures, modulate oxidative stress and increase HDL-C, T-C/HDL-C ratio and nitric oxide in patients with high risk of CAD.

4.1. Indian Fish Proteins in Lipid Lowering: One Amino Acid-One Fatty Acid Concept

Although the benefits of specific lipid-lowering drugs are unquestioned, the relation between these drug effects and the potential of diet to improve outcomes is unclear. Fish diet is emerging as cardioprotective food. Fish protein, amino acid and omega 3/6 Eicosapentanoic/Docosahexanoic fatty acids (EPA/DHA) rich fish oils are considered to reduce total blood fat LDL (Bad), cholesterol and simultaneously raise the HDL (Good) cholesterol. Eating fish (muscles) 30 gm/day reduces the chances of coronary heart disease by more than 70%. The fish proteins along with omega-3/omega-6 (EPA/DHA) polyunsaturated fatty acid content protects the heart by keeping HDL3 ratio high and rapid conversion of cholesterol to its products by Cholesterol oxidase. Shrinivas et al. 2008 analyzed diets with fish protein contents, omega 3/omega 6 (EPA/DHA) contents and essential amino acid composition and correlated diets with their lipid lowering effect. Idea was that fish muscle proteins and essential amino acid composition provide better cardiac protection along with omega 3/omega 6 fatty acids against sudden death from cardiovascular diseases. Investigators proposed possibility of ‘one amino acid-one fatty acid concept’ that fish proteins and amino acids may be alternative approach to keep high HDL, low (LDL+TG+C) lipids, and prevent the formation of blood clots to avoid the build up of plaque in the coronary and cardiovascular arteries specially in patients showing sensitivity to omega

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fatty acids. In preliminary experiment, different fish proteins, oil composition, amino acids were analyzed and compared. Borderline essential hypertensive patients with cardiac problem (High BP with risk of heart attack) were suggested to take 30 gm/day fish in diet (to keep up recommended omega 3/omega 6 (EPA/DHA) content) over six months. After follow-up, fish oil content, fish protein content, fish amino acid contents were correlated with heart indicators to establish the effect of fish protein and oil content on cardiovascular diseases. Simultaneously, the biochemical analysis of less known Indian fish protein content and protein composition were analyzed by electrophoretic mobility. In following section, we describe fish protein composition, omega fatty acids and benefits in cardiovascular prevention.

The source of Indian fish is important in fish protein quality (see Table 9). The amino acid composition and fish protein content, omega 3/omega 6 (EPA/DHA) content differ in fish (see Table 10). The progressive increased lipids lead to coronary heart disease and hyperlipidemia as main cause of death in most fast growing urban cities. Several previous studies report that fish protein, fish amino acid content were determinants to lower the blood lipids with possibility of cardiac prevention if taken in specific quantity not exceeding fat energy 10% intake. The protein content of fish Heteropneustes fossilis, Catla catla suggested specific proteins of size 35, 41, 67 and 94 kDalton as SDS-PAGE electrophoretic bands (see Figure 3). The fish grown during December and January months possibly served as better cardiac protector fish food and served as high yield cardioprotective proteins. On an average, fish diet brings down BP by -8/2.5mmHg Systolic/Diastolic pressure. However, strong belief is in favor of fish proteins playing significant role in Okinawa community. Fish proteins and omega 3/omega 6 (EPA/DHA) appear as effective measure to have keep fat energy intake below 10% and prevent coronary heart disease and stroke by keeping minimum hypertension, B.P. and hypercholesterolemia. The main player in lipid lowering is HDL-3 molecule composed of Apolipoprotein E2 and apo-A moieties bound with major cholesterol esters. Its main component LP-A apolipoprotein has been established as cholesterol scavenger to keep low total lipid content (LDL+TG+free cholesterol). However, the link between HDL-3 synthesis and effect of fish protein intake is not established. An alternate possibility appears of specific amino acid for specific fatty acid during synthesis of HDL-3 and Lp-A protein synthesis. If it is true, it amounts to support the fish diet intake and its effect on BP and lipid lowering. The fish proteins are effective diet component in lipid lowering. The amino acid-fatty acid combination may be effective tool to synthesize HDL-3 protein-lipid composition in favor of
keeping cholesterol esters rich Apo-protein moieties in HDL molecule as scavenger of cholesterol.

Different fish have different protein composition and its nutrition value. The temperature and fish feed further play important role in fish protein quality in muscle growth over months. The other additional fact can be attributed due to calcium mediated tropomyosin-actin activity in muscle contraction at low temperatures in December-January months. However much remains unknown if fish muscle and its protein composition differ during fish growth in different months of year. Also it is not established if fish eaten in different months of the year has different cardioprotection.

**Table 9. Fish protein and energy contents**

<table>
<thead>
<tr>
<th>Indian Fish</th>
<th>Protein gm%</th>
<th>Energy Cals/gm</th>
<th>BP(mmHg) -Sys/Dia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hilsa</td>
<td>21.8</td>
<td>273</td>
<td>-10/2.5</td>
</tr>
<tr>
<td>Rohu</td>
<td>16.6</td>
<td>97</td>
<td>-6/2</td>
</tr>
<tr>
<td>Catla</td>
<td>19.5</td>
<td>111</td>
<td>-9.5/3</td>
</tr>
<tr>
<td>Sardine</td>
<td>21.0</td>
<td>101</td>
<td>-5/3</td>
</tr>
<tr>
<td>Surmai</td>
<td>19.9</td>
<td>92</td>
<td>---</td>
</tr>
<tr>
<td>Mackerel</td>
<td>18.9</td>
<td>93</td>
<td>-10/2.5</td>
</tr>
<tr>
<td>Black Pomfret</td>
<td>20.3</td>
<td>111</td>
<td>---</td>
</tr>
<tr>
<td>White Pomfret</td>
<td>17.3</td>
<td>87</td>
<td>---</td>
</tr>
</tbody>
</table>

**Table 10. Omega 3/6 fatty acid content of popular fish and EAA (%) in fish proteins**

<table>
<thead>
<tr>
<th>Omega 3/6 PUFA</th>
<th>Qty gm</th>
<th>Amino acid</th>
<th>mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sardine</td>
<td>0.98-1.70</td>
<td>Lysine</td>
<td>8.8</td>
</tr>
<tr>
<td>Trout</td>
<td>0.98-0.84</td>
<td>Tryptophan</td>
<td>1.0</td>
</tr>
<tr>
<td>Anchovy</td>
<td>1.4</td>
<td>Histidine</td>
<td>2.0</td>
</tr>
<tr>
<td>Herring</td>
<td>1.2-1.8</td>
<td>Phenylalanine</td>
<td>3.9</td>
</tr>
<tr>
<td>Catfish</td>
<td>0.3-0.4</td>
<td>Leucine</td>
<td>8.4</td>
</tr>
<tr>
<td>Crab</td>
<td>0.35</td>
<td>Isoleucine</td>
<td>6.0</td>
</tr>
<tr>
<td>Lobster</td>
<td>0.07-0.41</td>
<td>Threonine</td>
<td>4.6</td>
</tr>
<tr>
<td>Mackerel</td>
<td>0.34-1.57</td>
<td>Methionine/cystine</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Valine</td>
<td>6.0</td>
</tr>
</tbody>
</table>

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5. **Effect of Fish Proteins on Atherosclerosis**

Randomized, controlled trials have been conducted to know the effects of fish dietary interventions alone as a strategy to arrest the progression of CAD as shown in table 4. The lifestyle Heart Trial randomly allocated 48 patients to either a very-low-fat diet (10% of daily energy intake) and intensive lifestyle intervention (exercise, stress management, smoking cessation, group counseling) or usual care (25). After 1 year, the experimental group showed more favorable changes in angina frequency and angiographic stenosis. After 5 years, the experimental group still had more favorable changes in low coronary stenoses and fewer cardiac events. (26)

In the St. Thomas’ Atherosclerosis Regression Study (STARS), 90 men were randomized to one of three arms: usual care; a low-fat, low-cholesterol diet, high in omega-6 and omega-3 fatty acids and high fiber (similar to AHA diet recommendations); or this diet plus cholestyramine. Patients in the fish diet-only group had significant improvements in several angiographic variables and fewer cardiac events or interventions compared with the usual-care group. Fish intake (2 meals containing fish meat weekly) may offer some promise in the secondary prevention of CAD. Fish is a functional food which is rich in n-3 fatty acids, coenzyme Q10 and selenium which are protective against CAD.

6. **Prevention of Coronary Artery Disease: Fish Diet**

There is a strong necessity for fish dietary research to eschew the type of data that would no longer be accepted to recommend a drug for prevention or treatment of CAD. In contrast to drugs, the basic fish diet will not change in future firm biological basis, ability to reduce one or more risk factors for CAD with better clinical outcomes or epidemiological data in favor of safe and feasible accepted fish diet over a lifetime. Advanced understanding of trial methods can overturn many erroneous principles or public policy "Does a program of dietary advice that can be followed by a typical person lead to fewer cardiovascular events?" sounds effectiveness or pragmatic type to answer the public-policy question. 

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CONCLUSION

This is appropriate time to apply to fish protein diet research. We believe that indications or claims made for weight loss or health improvement via fish diet - whether made by authors, the government, or associations - must be supported by 3 types of evidence: 1. proof that the diet provides essential nutrients in actual patients; 2. efficacy studies; and 3. randomized, controlled trials with clinical events as endpoints.

APPENDIX 1. SAMPLE PREPARATION AND SDS-PAGE ELECTROPHORESIS

The muscle was homogenized in lyses buffer separately containing 50 mM tris-HCL, pH 7.5, 50mM MgCl₂, 1mM EDTA, 1% Triton X-100 and 1mM PMSF (Phenyl methyl sulphonyl fluoride) as protease inhibitor. The homogenized tissues were centrifuged and supernatants were taken as a protein sample. These protein samples were stored at -20°C and used for gel electrophoresis.

Chemicals:

1) Acrylamide stock solution (30%)
   Acrylamide  29.2 Gm.
   Bis Acrylamide  0.8 Gm.
   Double Distilled Water (DDW)  upto 100ml.
   (Stored in refrigerator in the dark)

2) Running gel buffer (1.5 M, Tris HCl, pH 8.8):
   Tris  18.17 Gm
   D.D.W.  upto 100ml

3) Stacking Gel buffer (0.05 M, Tris HCl, pH 6.8):
   Tris  1.5 Gm
   D.D.W.  Upto 25ml

4) 10% SDS:
   SDS  10 Gm
   D.D.W.  upto 100ml

5) 10% APS (Ammonium per sulphate):
   APS  0.1gm
   D.D.W.  upto 10ml
6) Treatment buffer (pH 6.8)
   Tris 2.5ml (pH 6.8)
   SDS 4.0ml
   Glycerol 2.0ml
   β-Mercaptoethanol 1.0ml
   D.D.W. upto 10ml
   Bromophenol blue (pinch)

7) Tank buffer
   Tris 3.0 Gm
   Glycine 14.4 Gm
   SDS 10.0 ml
   D.D.W. upto 1000ml

8) Stain stalk (1% Coomassie brilliant blue R\textsubscript{250}):
   Coomassie brilliant blue 2 Gm
   D.D.W. upto 200ml

9) Working Stain:
   Stain stock 12.5ml
   Methanol 50ml
   Glacial acetic acid (GAA) 10ml
   D.D.W. upto 100ml

10) Destaining solution (50% Methanol, 10% acetic acid):
    Methanol 500ml
    GAA 100ml
    D.D.W. upto 1000ml

11) Preparation of separating gel (15%):
    30% Acrylamide (stock) 10ml
    Tris (pH 8.8) 5.0ml
    D.D.W. 4.6ml
    Ammonium per sulphate 200μl
    SDS 200μl
    TEMED 8.0μl

12) Preparation of staking gel
    30% Acrylamide (stock) 1.0ml
    Stacking gel buffer (pH 6.8) 750ml
    D.D.W. 4.1ml
    Ammonium per sulphate 60μl
    SDS 60μl
    TEMED 6μl
Procedure: The 15% Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was prepared on vertical gel (16X14cm) system (Bangalore Genei, India) in between two glass plates using 1mm spacer up to desired level. Stacking gel was poured in the vacant space. The comb of 1mm well was inserted in the stacking gel. After solidification of gel, the comb was taken out.

Meanwhile the protein sample was added with an equal volume of sample buffer (0.1M Tris-HCL, pH 6.8; 10% glycerol; 1% sodium dodecyl sulphate, 0.02% bromophenol blue), treated with 1% β-mercaptoethanol and denatured at 98°C for 3 min. The gel plates was attached to the electrophoretic chamber. The upper and lower tanks of chamber were filled with tank buffer. 50µl of sample was taken and mixed with 50µl of treatment buffer and denatured. 30µl of sample was loaded in each well with a micropipette. A lane containing molecular weight marker (Bangalore Genei, India) was included in a well and 50mA current was supplied to electrophoretic chamber. After running the samples the gel was then stained with Coomassie Brilliant Blue R-250 (SRL – India) and destained.

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Chapter 4

QUALITATIVE AND QUANTITATIVE CHARACTERISTICS OF LOW-DENSITY LIPOPROTEINS IN ATHEROSCLEROSIS-RELATED DISEASES

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ABSTRACT

Elevated serum low-density lipoprotein cholesterol (LDL-cholesterol) concentration is firmly established as a risk factor for atherosclerosis and cardiovascular disease (CVD). Yet, a number of CVD patients have LDL-cholesterol levels within the recommended range, suggesting the need for advanced lipid testing to determine residual risk. In this manner, further improvement of risk assessment might be accomplished through a more detailed insight into qualitative and

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quantitative characteristics of LDL particles. Plasma LDL population comprise complex spectrum of particles of different size, density and lipid composition. There is now ample of evidence that certain LDL subclasses, particularly small, dense LDL particles, are superior to LDL-cholesterol in terms of CVD risk prediction. Furthermore, elevation of small, dense LDL particles is also observed in various atherosclerosis-related conditions, such as end-stage renal disease or ischemic stroke. Whereas the measurement of LDL-cholesterol concentrations has proven clinical utility, the usefulness of LDL particles characterisation in clinical practice needs to be further explored. Understanding of structural complexity of LDL particles and their functional consequences might improve prevention and prognosis of atherosclerosis-related diseases, leading toward more specialized therapeutic approaches. Therefore, the questions of whom, when and how to assess small, dense LDL particles still remain open.

**INTRODUCTION**

Prevention of atherosclerosis development is one of major challenges for contemporary medicine and science. Over the last century the knowledge of the complex molecular mechanisms involved in atherogenesis has been greatly enhanced and available evidence suggests that dyslipidemia is deeply involved in the development of atherosclerosis and its associated complications, such as cardiovascular disease (CVD).

The role of low-density lipoproteins (LDL) in atherosclerotic process has been well documented through many clinical and experimental studies. High LDL-cholesterol concentration has been recognised as a main lipid risk factor for CVD, while the maintenance of its optimal level is an imperative of modern cardiovascular medicine. However, CVD risk prediction, if judged only via LDL-cholesterol levels, may misclassify considerable number of patients with elevated risk. It is often underappreciated that measuring lipids is really a surrogate for measuring the lipoproteins that actually carry lipids in circulation and act in atherogenesis. Even if this surrogate relationship between plasma lipid and lipoproteins has been described more than forty years ago [1], contemporary laboratory monitoring of dyslipidemia has been barely changed ever since.

Heterogenic nature of plasma lipoproteins was recognized in the early 1950's. Starting from this pioneer study of Gofman and Lindgren [2] until today, the knowledge on lipoprotein diversity has been steadily accumulating. Years of investigations revealed several molecular mechanisms responsible for...
such remarkable heterogeneity. Also, it has been confirmed that different qualitative and quantitative characteristics of LDL subfractions considerably influence degree of atherogenicity of this lipoprotein. Besides, these studies also exposed certain risk groups in the general population. Current investigations are focused to establish whether prognostic information derived from LDL-cholesterol concentrations might be improved by additional evaluation of qualitative and quantitative characteristics of LDL subclasses.

**QUALITATIVE AND QUANTITATIVE CHARACTERISTICS OF LDL PARTICLES**

As a general rule, LDL particles are described as lipoproteins within the density limits of 1.019-1.063 kg/L and size ranging from 20 to 30 nm. The size and density are determined by the amount and content of lipids within the particles. The hydrophobic core contains more cholesterol esters than triglycerides and is surrounded by a hydrophilic shell of free (unesterified) cholesterol and phospholipids. Structural integrity of the particles is maintained by apolipoprotein B-100 (apoB) [3]. Each LDL particle contains only one apoB molecule and is incapable of exchanging it with other lipoproteins due to the nature of lipid/protein interactions on the surface.

LDL plasma population is composed of heterogeneous particles, differing in size, density and lipid content [4], which can be separated by a variety of analytical techniques. For instance, by ultracentrifugal separation, it is possible to resolve up to seven distinct subfractions [5]. However, the most convenient approach is a classification into four major subclasses (LDL I-IV). LDL I and LDL II subclasses comprise largest, but less dense particles, whereas LDL III and LDL IV are presented by small, dense particles. If judged only by size, LDL plasma population can be divided into small (22.0-25.5 nm), intermediate (25.6-26.5 nm) and large (26.6-28.5 nm) particles [6]. From the clinical standpoint, the most important are small, dense LDL particles (≤ 25.5 nm).

A remarkable LDL particles diversity arises from the changes in the amount of their precursors, as well as in the activities of the enzymes involved in lipoprotein metabolism. Generally, it appears that generation of small, dense LDL particles could be a consequence of: overproduction of very-low-density lipoproteins (VLDL) [7], reduced lipoprotein-lipase and increased hepatic-lipase activities [8], but also due to remodeling processes mediated by cholesterol-ester transfer protein (CETP) [9]. More likely, production of small,
dense LDL particles involves all of previously mentioned mechanisms. It has been suggested that the VLDL-LDL delipidation cascade consists of parallel metabolic pathways responsible for the formation of different LDL subclasses. Interestingly, the largest VLDL particles have been identified as the precursors of the smallest and densest LDL subclasses [9]. The role of lipolytic enzymes in modulation of LDL particle size has been also confirmed [10]. Activity of hepatic lipase, the key enzyme involved in the hydrolysis of triglycerides in LDL particles, in cooperation with CETP significantly contributes to the increase of small, dense LDL particles. Thus, serum triglyceride concentration seems to be an important modulator of LDL subclass distribution [11], since CETP-mediated transfer of triglycerides from VLDL to LDL in hypertriglyceridemic state ultimately leads to the formation of smaller, triglyceride-rich LDL particles [8].

Austin and Krauss [12] employed mathematical model to convert densitometric profiles of LDL subclasses into Gaussian curves and revealed that plasma LDL particles possess bimodal distribution. Based on this typical appearance, the authors suggested classification in two distinct phenotypes. Accordingly, predominance of large, floating LDL I and LDL II particles (>25.5 nm) was designated as LDL A phenotype, whereas the predominance of small, dense LDL III and LDL IV particles (≤ 25.5 nm) refers to LDL B phenotype [12]. The expression of LDL B phenotype is genetically determined (heritability range is 35-45%) and based on an autosomal dominant or codominant model with varying polygenic effects [13]. As a result, the prevalence of LDL B phenotype can reach up to 35% in healthy adult men, while the frequency is 5-10% in young men and 15-25% in postmenopausal women [14]. Epidemiological studies revealed numerous environmental factors that may influence the expression of this phenotype in asymptomatic population, including abdominal obesity and metabolic syndrome [14], dietary habits [15], alcohol intake [16] and use of hormone replacement therapy or oral contraceptives [17].

Plasma LDL-cholesterol concentration has proven clinical utility and is traditionally used as a surrogate measure for circulating LDL particles. However, based on all previous observations, it is obvious that LDL particles composition may vary in terms of cholesterol content. Hence, plasma LDL-cholesterol concentration cannot provide sufficient information on quality and quantity of LDL particles.

Clinical application of LDL subclasses assessment is limited by the availability and cost of laboratory techniques. LDL particles characterisation requires either special equipment or a lengthy analytical time; therefore it is
still unsuitable for general clinical use. To date investigators have described several different approaches to separate LDL subclasses based on a variety of their physicochemical properties including particle density and size, charge and lipid composition (Table 1).

Table 1. The most commonly used separation techniques and nomenclatures for LDL subclasses

<table>
<thead>
<tr>
<th>Gradient gel electrophoresis</th>
<th>Ultracentrifugation</th>
<th>NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Size (nm)</td>
<td>Name</td>
</tr>
<tr>
<td>LDL I</td>
<td>27.2-28.5</td>
<td>LDL 1</td>
</tr>
<tr>
<td>LDL IIA</td>
<td>26.5-27.2</td>
<td>LDL 2</td>
</tr>
<tr>
<td>LDL IIB</td>
<td>25.5-26.5</td>
<td>LDL 3</td>
</tr>
<tr>
<td>LDL IIIA</td>
<td>24.7-25.5</td>
<td>LDL 4</td>
</tr>
<tr>
<td>LDL IIIB</td>
<td>24.2-24.7</td>
<td>LDL 5</td>
</tr>
<tr>
<td>LDL IVA</td>
<td>23.3-24.2</td>
<td>LDL 6</td>
</tr>
<tr>
<td>LDL IVB</td>
<td>22.0-23.3</td>
<td>LDL 7</td>
</tr>
</tbody>
</table>

Density gradient ultracentrifugation represents the original procedure and is still in use for research, as well as for development and validation of subsequent methods. Although numerous authors consider ultracentrifugation as a “gold standard” procedure for lipoprotein subclasses separation, this technique is less suitable for clinical and epidemiological studies, but is still irreplaceable for preparative purposes. Nondenaturing polyacrylamide gradient gel electrophoresis separates LDL particles on the basis of their charge and size and is the most widely used method. The most recently developed techniques employ the principles of nuclear magnetic resonance spectroscopy (NMR) [18] and ion mobility [19]. Mentioned techniques allow assessment of complete plasma LDL profile, such as particles size, number and concentration. On the other hand, such sophisticated methodologies are very expensive, which limits their wider applicability.

Another drawback has to be emphasised – none of the mentioned techniques is fully interchangeable. Furthermore, due to variety of methodologies and non-uniform nomenclature, an adequate comparison of the results is limited. For instance, Ensign and colleagues [20] recently evaluated correlations between the results of leading methods for LDL subclasses separation based on electrophoretic mobility, ultracentrifugation and NMR and showed a considerable diversity of results - complete agreement among
techniques was obtained in 8% of analysed samples [20]. Therefore, any of current techniques cannot be highlighted as superior than others. Each year, several publications present novel technology or improved modifications of existing methods, but additional studies are needed to adequately assess comparability among them. Hopefully, this will identify a reference method, but also the most convenient technique for application in routine laboratory practice.

**ATHEROGENIC PROPERTIES OF SMALL, DENSE LDL PARTICLES**

The hypothesis of central role of LDL in atherogenesis survived decades of research. However, after discovering that LDL comprises a mixture of particles with different qualitative characteristics, numerous in vitro studies have investigated how such heterogeneity influences the aterogenicity. The results consistently indicated the same: the greatest atherogenic potential belongs to small, dense LDL particles. Over the years, the mechanisms through which small, dense LDL particles achieve such effects have been explained (Table 2).

<table>
<thead>
<tr>
<th>Cause</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced affinity for LDL receptors</td>
<td>Longer retention in circulation</td>
</tr>
<tr>
<td>Smaller size of the particle</td>
<td>Easier penetration to arterial intima</td>
</tr>
<tr>
<td>Enhanced binding to proteoglycans</td>
<td>Longer retention in subendothelium</td>
</tr>
<tr>
<td>Reduced content of antioxidants</td>
<td>Accelerated oxidation</td>
</tr>
<tr>
<td>Increased affinity for scavanger receptors</td>
<td>Prompt takeover by macrophages</td>
</tr>
</tbody>
</table>

The common denominators for all these processes are increased number of particles and conformational changes in the structure of apoB.

Small, dense LDL particles have a lower affinity for receptors in the liver [21], due to changes in the part of apoB molecule crucial for the binding [22]. In addition, it was reported that conformational changes in other structural components of apoB, although not directly involved in ligand-receptor interactions, also contribute to longer retention of these particles in circulation [23]. On the other hand, small, dense LDL particles have increased affinity for...
scavenger receptors [24], which can further stimulate foam cell formation and atherogenesis. Also, their smaller size allows easier penetration into subendothelial space [25], whereas reduced amount of sialic acid on their surface enhances binding to proteoglycans, thereby increasing the residence time in subendothelial space [26]. Finally, small, dense LDL particles are more susceptible to oxidation [27], as a consequence of imbalanced amounts of polyunsaturated fatty acids, cholesterol and antioxidants [28, 29]. Bearing in mind high concentration of prooxidants in the subendothelial space, it is reasonable to expect that small, dense LDL particles, with already compromised antioxidant protection, can easily become oxidized. Once formed, oxidized LDL displays its proinflammatory, immunogenic, apoptotic and cytotoxic actions [30]. The spectrum of proinflammatory activities involves hemoattractant effects on circulating monocytes, induction of adhesive molecules expression on endothelial cells and proliferation of monocytes into macrophages [31].

Figure 1. Small, dense LDL particles as a feature of atherogenic lipoprotein phenotype in atherosclerosis-related diseases.
Krauss and colleagues [4] were the first to demonstrate that patients after myocardial infarction have increased small, dense LDL particles. Based on the results of following studies, now is appreciated that subjects with small, dense LDL particles have 3-7 times higher risk of developing CVD [13]. However, it is still not clarified whether small, dense LDL particles are risk factors per se or the previously mentioned relation with CVD is just a consequence of broader pathophysiological processes in which they participate [32].

**SMALL, DENSE LDL IN Atherosclerosis-related Diseases**

Novel evidence indicates that the net effect of dyslipidemia in atherogenesis go beyond plasma LDL-cholesterol concentration [33]. More comprehensive understanding offers the cluster of lipid disturbances named “atherogenic lipoprotein phenotype” or “atherogenic triad” [34]. This term implies concomitant presence of three the most frequent lipid abnormalities in patients with atherosclerosis: increased triglyceride, decreased HDL-cholesterol concentrations and presence of small, dense LDL particles (Figure 1) and its broader application started at the end of 20th century.

Atherogenic lipoprotein phenotype is closely associated with abdominal obesity and insulin resistance and is appreciated as one of the components of the metabolic syndrome [35]. Its clinical importance has been firmly documented, because majority of patients with atherosclerosis-related diseases have this characteristic trait (Figure 1). According to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) [36], each particular component of this phenotype is atherogenic, but their individual contribution cannot be easily determined. Therefore, some authors readily consider atherogenic lipoprotein phenotype as a whole as a risk factor [37-39].

Development of CVD is a complex process involving oxidative stress, inflammation and dyslipidemia [40]. Based on our previous data in asymptomatic population and in patients with clinically verified atherosclerosis [41-47], we further documented that all of the named risk factors could interact in this pathophysiological mechanism [48-52] and suggested a multimarker approach for the prediction of CVD [53-55]. It has been shown that small, dense LDL subclasses are abundant in patients with coronary atherosclerosis. Up to date, the waste majority of studies recognised small, dense LDL particles as independent predictors of coronary heart disease.
[7, 56-73]. On the other hand, the above-mentioned association of LDL particle size with triglyceride and HDL-cholesterol concentrations, as well as with other non-lipid risk factors, such as obesity or insulin resistance [34, 74-76], should not be ignored. These complex metabolic interrelationships further complicate attempts to define intrinsic atherogenic potential of small, dense LDL particles beyond increased LDL-cholesterol concentration. In the majority of aforementioned studies, multivariate logistic regression analysis failed to establish small, dense LDL particles as an independent risk factor for coronary heart disease. Yet, other cross-sectional and prospective studies demonstrated independent contribution of small, dense LDL in the disease development, even after adjustment for other traditional risk factors [7, 59, 61, 63, 67, 70, 71, 73]. In our own study [46], we found an independent association of diminished LDL size and coronary heart disease, regardless of other covariates. However, our further analysis of clinical accuracy revealed that small, dense LDL particles determination provides no additional power than the one obtained by routine lipid status measurement [46]. Taking all together, the question of authentic clinical significance of small, dense LDL particles in prediction of CVD is still under debate. All previously given facts strongly support the necessity of further investigations of LDL heterogeneity, especially if we appreciate that majority of existing studies are dealing with coronary heart disease, while there is a lack of information regarding LDL subclass distribution in other atherosclerosis-related conditions.

Growing evidence suggest that small, dense LDL particles could also be important in the development of ischemic stroke. Because of the high prevalence and mortality rates after stroke [77], effective prevention and treatment are tremendously important goals in clinical practice. Several risk factors have been established for stroke development, among them the most important being age, male sex, hypertension and other cardio- and peripheral-vascular co-morbidity conditions [78-80]. However, there is a lack of information regarding the significance of LDL subclasses determination in this category of patients. Available data showed the association of LDL particle size and subclasses distribution with the development of carotid atherosclerosis [81-86], but similarly as in coronary form, there is insufficient evidence to prove independent contribution of particular subclasses in the onset and progression of stroke. Still, Berneis et al. [73] have shown that LDL size is the most important lipid predictor of carotid intima media thickness, and this predictive potential was retained even after inclusion of all other traditional risk factors. In a Multi-Ethnic Study of Atherosclerosis (MESA) small, dense LDL particles were in strong positive association with intima
media thickness of carotid arteries in a mixed population of Caucasians, Chinese, African American and Hispanic population in the United States [87]. Such findings emphasise the importance of starting new studies which should offer more relevant evidence. Our study of LDL size and subclasses distribution in patients with ischemic stroke highlighted small, dense LDL as an independent predictor of both onset and consecutive short-term mortality [88]. These results suggest that LDL subclasses assessment could be useful not only in predicting the risk for the development, but also for identifying patients at higher risk of in-hospital mortality after stroke. In attempting to elucidate possible mechanism through which small, dense LDL particles participate in pathophysiology of stroke, one could rely on previously explained processes in patients with coronary atherosclerosis.

Chronic kidney disease is another condition associated with higher CVD risk [89]. It is also characterised by atherogenic dyslipidemia (Figure 1), mainly as a consequence of decreased removal of triglyceride-rich lipoproteins [89]. Bowden et al. [90] reported that lipoprotein abnormalities, especially altered LDL particle distribution, can be evident even in milder degree of renal impairment. Progressive renal failure is associated with considerable alterations in lipoprotein metabolism, while the greatest risk is observed in patients with end-stage renal disease [91]. However, in end-stage renal disease patients total cholesterol level does not account for the same risk of CVD as in the general population [92, 93]. Still, the relationship between lipoprotein abnormalities and CVD risk in this category of patients remains to be clarified.

A review of literature showed that the type of dyslipidemia in end-stage renal disease can be related to the choice of dialysis modality. Namely, the principal features of renal dyslipidemia in patients on peritoneal dialysis are elevated LDL-cholesterol and preponderance of small, dense LDL particles [94]. On the other side, haemodialysis patients often have total and LDL-cholesterol concentrations within recommended ranges, but elevated triglyceride and reduced HDL-cholesterol concentrations [94]. In accordance, Deighan and colleagues [95] analyzed LDL subfractions and hepatic lipase activity in 75 patients with end-stage renal disease (25 on haemodialysis, 25 on peritoneal dialysis and 25 predialysis patients) to assess the extent of small, dense LDL III subclasses formation in different stages of the disease. The authors [95] found that LDL III particles were increased in all the three patient groups as compared with controls, but the highest proportion was seen among those on peritoneal dialysis. In the same study, plasma triglyceride level and hepatic lipase activity were independent predictors of LDL III abundance [95]. Mekki et al. [96] recently reported that lipid abnormalities in patients...
undergoing haemodialysis are driven by reduced lipoprotein lipase and hepatic lipase activities, suggesting delayed catabolism of triglyceride-reach lipoproteins. Ambroch et al. [97] further demonstrated that LDL particles in patients with end-stage renal disease are triglyceride-enriched, cholesterol-depleted and smaller in size. In addition, such modified LDL particles were superior substrate for macrophages as compared with fibroblasts [97], indicating a tendency of intracellular accumulation and decreased uptake by the specific receptor pathway.

Results of Yeo et al. [98] indicated that small, dense LDL particles were not associated with CVD in end-stage renal disease patients with angiographically verified coronary atherosclerosis. More recently, Bowden and colleagues [99] published results of the study with more than thousand end-stage renal disease patients. The authors [99] investigated differences between serum lipid parameters and LDL particle number and size in terms of risk stratification when using NCEP ATP III guidelines. In the mentioned study, LDL particle size categorised more patients at increased risk when compared with LDL-cholesterol, non-HDL-cholesterol and triglyceride concentrations and the authors concluded that LDL particles characterisation can be used for advanced screening to predict CVD development [99]. At this point it should be stressed that, in spite of a clear improvement in cardiovascular prevention, the death rate among haemodialysis patients is still high compared to the general population and cardiac causes account for nearly half of overall mortality [100]. Therefore, it is reasonable to anticipate a certain contribution of alterations in LDL subclass distribution to the excess mortality in this category of patients. We recently performed 36 months follow-up study of 122 haemodialysis patients to assess potential predictive role of lipoprotein subclasses on lethal outcome [101]. Our results showed that smaller LDL particles were not more prevalent in the deceased when compared with the survived patients [101], a finding that, in certain manner, confirms previous results of Yeo et al. [98]. Nevertheless, further prospective studies should reveal the importance of LDL particles characterisation in attempting to improve survival of the patients until renal transplantation.

Renal transplant recipients also have an increased risk of CVD development [102]. Similarly to pre-transplantation period, the patients still suffer from dyslipidemia [103]. As documented by Rajman et al. [104], the presence of small, dense LDL particles is an early feature of the uremic dyslipidemia and might persists even after renal transplantation. Side effects of immunosuppressive medications represent significant sources of post-transplant dyslipidemia. It has been shown that cyclosporine administration is
more likely associated with dyslipidemia than the use of tacrolimus [105]. Recently, we have investigated heterogeneity of LDL particles and the influence of immunosuppressive drugs on their distribution in pediatric renal transplant recipients (unpublished data). We found that pediatric renal transplant recipients have significantly higher proportion of small, dense LDL particles than controls, as well as a shift towards smaller particles in patients treated with cyclosporine when compared with subjects on tacrolimus. In the study by Apanay et al. [106], patients treated with higher cyclosporine levels showed significantly higher LDL oxidability when compared to controls. Furthermore, Cofan et al. [107] noted that tacrolimus therapy resulted in a LDL oxidation profile similar to that in healthy controls. It appears that conversion from cyclosporine to tacrolimus might have beneficial effects on both LDL particles distribution and susceptibility to oxidation, thereby reducing LDL’s atherogenic potential. Overall, these findings made it obvious that pre-transplant condition or dialysis-specific alterations in LDL subclass distribution can be continued in post-transplant period, as it was documented by Rajman et al. [104]. It is also clear that traditional lipids and apolipoproteins measurements could not be sufficient for accurate and timely estimation of atherogenic dyslipidemia in end-stage renal disease, as well as for post-transplantation patients. The usefulness of advanced lipid testing could be particularly true for small, dense LDL particles estimation.

Based on all previously discussed data about the role of qualitative and quantitative characteristics of LDL subclasses in the development of atherosclerosis-related diseases, this chapter should also briefly address pharmacological influences on LDL particles size distribution. Among available hypolipemic agents, fibrates seem to possess the most prominent beneficial effect [108]. It has also been demonstrated that statins could reduce the proportion of small, dense LDL particles, but with significant variations among different members in this group [109-112]. Nowadays, research is extended toward exploring new therapeutic options beyond ordinarily prescribed hypolipemics [113, 114]. One could presume that future therapeutic approach might shift from prevailing use of statins towards drugs that, besides correction of LDL-cholesterol level, can efficiently improve disturbances in LDL subclasses distribution, too.

For the exclusive use of Elena Pivovarova
**PERSPECTIVES IN ROUTINE LABORATORY DIAGNOSTICS**

The basic criteria for routine use of each potential new CVD risk marker are firmly established [115]. First of all, it is required to satisfy exceptionally strict rules for the characteristics of analytical technique (regarding availability, simplicity and standardisation), as well as for laboratory costs. Next, the predictive power of a new marker has to be confirmed in prospective studies, whereby it needs to be stronger than prognostic value of existing traditional risk factors. Finally, it is equally important that new marker can be applied for risk prediction in all populations.

As it has been illustrated above, the function of small, dense LDL particles in the development of atherosclerosis is generally well understood. In spite of that, clinical significance of their assessment is still controversial. The key question that remains to be answered is whether specific LDL subclasses are independent risk factors for atherosclerosis-related diseases. If so, presence of small, dense LDL particles should not be neglected in general risk assessment. The opposing alternative is that relative risk attributable to small, dense LDL subclasses is merely a consequence of complex interactions with other traditional risk factors. In general, to indisputably determine independent association of small, dense LDL with CVD, confirmative findings of multiple logistic regression analysis are obligatory. Although many cross-sectional and prospective studies have dealt with this issue, their results are not homogenous.

In 2009, American Association for Clinical Chemistry (AACC) published recommendations for laboratory assessment of novel risk factors in primary prevention of CVD and stroke [116]. Analysing available data and following American College of Cardiology (ACC) and American Heart Association (AHA) guidelines for implementation of new diagnostic or therapeutic procedures [117], Wilson et al. [116] defined recommendations for determining LDL size and subclasses distribution. The authors concluded that, unlike for several other proposed CVD risk factors, none of the above mentioned criteria have been still satisfied in the case of LDL subclasses (Table 3).

In the introducing section of the above mentioned publication, it was emphasised that the recommendations were based on the results of prospective studies in healthy populations published until February 2005. Neither retrospective, nor anamnestic studies were analysed, except for estimation of marker's significance in secondary prevention [118].

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Table 3. Guidelines for the measurement of LDL subclasses in primary prevention

<table>
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<th>Recommendation</th>
<th>Description</th>
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<tr>
<td>Recommendation 1</td>
<td>Data analyses of existing studies are generally not adequate to show added benefit over standard risk assessment for primary prevention. There are insufficient data that measurement of lipoprotein subclasses over time is useful to evaluate the effects of treatments. Several methods are available to assess lipoprotein subclasses. Standardization is needed for this technology.</td>
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Analysis of Wilson and associates [116] did not include studies on clinical significance of small, dense LDL-cholesterol concentration (sdLDL-cholesterol), measured by a method based on selective precipitation in the presence of heparin and Mg$^{2+}$ ions [119]. Preliminary results on this new parameter were published in 2003 [119], but meanwhile the authors modified and improved the existing method [120], examined preanalytical variations [121, 122] and clinical significance [123-125]. This group further demonstrated that increased concentration of sdLDL-cholesterol is associated with the extent of coronary [123] and carotid atherosclerosis [126], but also that statin and fibrate therapy efficiently lowers sdLDL-cholesterol level in diabetics [127]. Having in mind the simplicity and availability of this proposed method, its value for prediction of the disease and evaluation of the therapy, it seems reasonable to expect that this parameter would eventually fulfill the ACC/AHA criteria [117] and would be recommended for routine use in primary prevention of CVD.

CONCLUSION

The distribution of LDL subclasses undoubtedly undergoes major changes in patients with atherosclerosis, but clinical significance of their assessment is still unresolved. Small, dense LDL particles seem to be important predictors of progression of atherosclerosis-related diseases and their predominance have been accepted as an emerging cardiovascular risk factor by the NCEP ATP III [36]. The studies discussed above suggest that screening for the presence of...
small, dense LDL may contribute in directing specific interventions for cardiovascular prevention. Based on current experiences, possible indications for LDL subfraction assessment can be summarized as follows: management of any form of atherosclerotic disease, diagnostic evaluation of primary and secondary dyslipidemias, as well as follow-up of asymptomatic individuals that are at increased risk for future development of atherosclerosis. Is the latest research on LDL particle heterogeneity sufficient enough to provide evidence that can ensure their implementation in clinical practice? This question is waiting to be answered in the upcoming NCEP ATP IV guidelines.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Education and Science, Republic of Serbia (Project No. 175035).

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Chapter 5

THE ROLE OF LIGAND-ACTIVATED TRANSCRIPTION FACTORS PPAR, LXR, CAR, AND PXR IN THE DEVELOPMENT OF METABOLIC SYNDROME

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ABSTRACT

It is known that the metabolic syndrome (MS) leads to serious cardiovascular disease which continues to be the number one cause of death in developed countries. MS is characterized by dyslipidemia, hypertension, glucose tolerance, and obesity. The MS distribution is growing catastrophically, but molecular mechanisms responsible for developments of complex impairments in MS still remain basically poorly investigated. The formation of complex MS symptoms suggests systemic impairments in lipid and carbohydrate metabolism; it appears that these impairments should have a common basis at the level of expression of appropriate genes. Expression of genes involved into lipid and carbohydrate metabolism is regulated by various transcription factors, including those referred to the superfamily of nuclear hormone receptors: peroxisome proliferator-activated receptors (PPAR), liver X
receptors (LXR), pregnane X receptors (PXR), and constitutive androstane receptors (CAR). PXR and CAR are mainly known as the sensors of xenobiotics. Since these transcription factors are ligand-activated, they represent perspective targets for pharmacological treatments. In the recent time, the search of natural and synthetic PPAR ligands is in intensive process. There is a cross-talk between signal transduction pathways of PPAR, LXR, PXR, and CAR; this suggests their integrated role in regulation of genes of lipid and carbohydrate metabolism. In our work at the model of rat strain with Inherited Stress-Induced Arterial Hypertension which demonstrates all signs of MS development it was shown that an increased content of triglyceride, VLDL and LDL cholesterol, a decreased content of HDL cholesterol, a high level of apoB-100, and a decreased level of apoA-I, increased body weight and blood glucose level are associated with increased DNA-binding activity of PPAR, LXR, PXR, and CAR in the liver; this suggests involvement of these transcription factors in the development of MS in ISIAH rats. We have also shown that stress can affects the DNA-binding activity of these transcription factors. In this review the role of ligand-activated transcription factors PPAR, LXR, CAR, PXR and the role of stress in the development of metabolic syndrome will be discussed.

**INTRODUCTION**

The metabolic syndrome (MS) is a complex of pathologies that increase the risk of cardiovascular diseases which still are the leading cause of mortality in industrial countries. It is known that MS is characterized by dyslipidemia, hypertension, glucose tolerance, and obesity.

In 1980th, Reaven (Reaven, 1988) proposed to pool such metabolic impairments under a common name of the X syndrome, which is now known as the metabolic syndrome.

Although the term “metabolic syndrome” is used for several years, only recently attempts to give clear definition of this term have been undertaken. According to the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) criteria of the metabolic syndrome include three or more impairments including visceral obesity, increase in blood triglycerides, decrease in blood HDL cholesterol, hypertension, and increased blood glucose (NCEP-ATP III, 2001).

NCEP ATP III has defined the criteria for MS in order to assist physicians in diagnosing patients at risk for coronary heart disease and diabetes mellitus type 2, are clustered metabolic risk factors (Grundy, 2005). NCEP ATP III
identified five easily measurable criteria for identifying patients with MS: the levels of triglycerides and fasting plasma glucose, HDL cholesterol, blood pressure and waist circumference (Table 1).

The prevalence of MS in the U. S. based on the data from National Health and Nutrition Examination Survey (NHANES) collected between 1999 and 2002 was 34.5% of population sample (n = 3601) of men and women over age 20. The prevalence increases with the age. Among men and women over 50 years MS distribution (according data 1999-2002) was approximately 50% (Ford, 2005).

Increasing prevalence of MS in most cases there is due to an increase in body weight. It is likely that the increase in body weight can change each of the metabolic risk factors, but there is evidence that the severity of each risk factor is under its own genetic control.

Some researchers believe that insulin resistance is the only major factor responsible for the metabolic syndrome (Reaven, 2004). However, regardless of root cause diagnosis of metabolic syndrome indicates an increased risk of cardiovascular disease. The magnitude of risk varies depending on the presence of risk factors and their severity (Grundy, 2005).

Insulin resistance is common among people with high triglycerides and low HDL cholesterol - two the most common risk factors among people diagnosed with MS (Ford, 2002). However, fasting hyperglycemia occurs only in 12% of people that meet diagnostic criteria for MS, because this is the last of the risk factors developing in patients with MS. A simple indicator of insulin resistance in people with MS is the ratio of fasting triglyceride concentration of HDL cholesterol. The value of triglyceride/HDL cholesterol = 3.5 points to insulin resistance as authentically as an increased level of fasting insulin (McLaughlin, 2005).

Another risk factor for MS is abdominal obesity (Grundy, 2005). Abdominal obesity contributes to increased insulin resistance, resulting in increased flux of free fatty acids from adipose tissue to the liver and insulin resistance in the muscles.

MS is associated with an increased relative risk of subsequent cardiovascular diseases by 65% and increases the risk of development of type 2 diabetes mellitus three times (Ford, 2005a).

The MS distribution is growing, but molecular mechanisms responsible for developments of complex impairments in MS still remain basically poorly investigated.

The development of complex MS symptoms suggests systemic impairments in lipid and carbohydrate metabolism; it appears that these
Impairments should have a common basis and it have to be at the level of expression of appropriate genes.

Expression of genes involved into lipid and carbohydrate metabolism is regulated by the number of transcription factors, including those referred to the superfamily of nuclear hormone receptors: peroxisome proliferator-activated receptors (PPAR) (Michalik, 2006; Lefebvre, 2006), liver X receptors (LXR) [5], pregnane X receptors (PXR), constitutive androstane receptors (CAR) [6].

Nuclear hormone receptors are ligand-activated transcription factors that regulate gene expression by interacting with specific DNA sequences upstream of their target genes. This sequence was termed the response element (RE). Nuclear hormone receptors regulate gene expression in response to a wide range of developmental, physiological, and environmental cues.

The PPAR, LXR, PXR, and CAR are activated after their interaction with corresponding ligands. PPAR, LXR, and CAR are localized in cytoplasm and after interaction with their ligands are translocated into the nucleus. PXR is localized in the nucleus where it is activated by its ligand. In the nucleus, after heterodimer formation with RXR protein (retinoid X receptor) nuclear receptors are specifically bound to regulatory sites of target genes and modulate their expression (Michalik, 2006). Expression of transcription factors and their iso-forms is tissue-and organ-specific.

**Peroxisome Proliferator-Activated Receptor (PPAR)**

PPAR has three isoforms: PPAR-alpha, PPAR-beta, and PPAR-gamma. PPAR-alpha is expressed predominantly in the liver, kidney and heart, and is primarily involved in fatty acid oxidation. PPAR-beta (delta) is abundantly and ubiquitously expressed. PPAR-gamma is most abundantly expressed in adipose tissue, with less expression in the colon and immune system. As well as in adipocytes and T cells, PPAR-gamma is also expressed in endothelial cells, vascular smooth muscle cells, and macrophages. Small quantities of PPAR-gamma1 are expressed in the liver; PPAR-gamma2 is expressed only in adipose tissue (Michalik, 2006).

PPARs were found to be mediators of pharmacologic agents that induce hepatocyte peroxisome proliferation. Peroxisomes are organelles that participate in fatty acid metabolism. Clofibrate analogues,
hypolipidemic agents that control plasma cholesterol and triglyceride levels, can induce proliferation of liver cell peroxisomes (Reddy, 1973; Reddy, 1974).

In addition, two lipid-lowering compounds structurally different from clofibrate, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) and 2-chloro-5-(3,5-dimethylpiperidino-sulfonyl)benzoic acid (tibric acid), also were found to stimulate hepatocyte peroxisome proliferation (Reddy, 1975). Although hypolipidemic drugs were demonstrated to activate peroxisome proliferation, these studies did not establish a mechanism. Subsequent studies identified a protein whereby peroxisome proliferators bind with affinity (Lalwani, 1987), and this protein was later identified as a member of the nuclear hormone-receptor superfamily that includes steroid, retinoid, and thyroid hormone receptors (Evans, 1988). The name peroxisome proliferator-activated receptor took origin from the cloning by Issemann et al. (Issemann, 1990) to identify possible endogenous mediators of peroxisome proliferation–induced gene transcription in rodent livers.

PPAR-alpha regulates liver and skeletal muscle lipid metabolism as well as glucose homeostasis. Acting as a molecular sensor of endogenous fatty acids (FAs) and their derivatives, this transcription factor regulates the expression of genes encoding enzymes and transport proteins controlling lipid homeostasis, thereby stimulating FA oxidation and improving lipoprotein metabolism. PPAR-alpha also exerts pleiotropic antiinflammatory and antiproliferative effects and prevents the proatherogenic effects of cholesterol accumulation in macrophages by stimulating cholesterol efflux (Lefebvre, 2006).

PPAR-beta regulates expression of genes involved into fatty acid catabolism (Michalik, 2006).
Table 1. The criteria for clinical diagnosis of metabolic syndrome

<table>
<thead>
<tr>
<th>Indices</th>
<th>Limiting values</th>
</tr>
</thead>
</table>
| Waist circumference            | Men: ≥ 102 cm (≥ 40 inches)  
                                  | Women: ≥ 88 cm (≥ 35 inches)                     |
| Increased triglyceride levels  | ≥ 1.7 mM/l (≥ 150 mg/dl)                             |
| Reduced HDL cholesterol        | Men: < 1.03 mmol/l (< 40 mg/dl)                      
                                  | Women: < 1.3 mmol/l (< 50 mg/dl)                 |
| High blood pressure            | Systolic blood pressure ≥ 130 mm Hg                  
                                  | or diastolic blood pressure ≥ 85 mm Hg           |
| Elevated fasting glucose       | ≥ 5.6 mmol/l (≥ 100 mg/dl)                           |

PPAR-gamma participates in expression of genes involved in lipid accumulation and also glucose metabolism (Michalik, 2006). Although PPAR-gamma was initially found to be critical for adipocyte differentiation and function, over time, PPARγ was discovered to play an important role in the cardiovascular system (Hamblin, 2009). PPAR-gamma is involved in the regulation of lipid metabolism, as ligand-dependent activation leads to an increase in genes that regulate fatty acid uptake and storage (Semple, 2005). PPAR-gamma plays a role in glucose homeostasis and insulin sensitivity (Forman, 1995). PPAR-gamma is mainly associated with adipose tissue, where it controls adipocyte differentiation and insulin sensitivity. It has been shown that PPAR-gamma can facilitate differentiation of fibroblasts into adipocytes (Chawla, 1994).

PPAR-alpha overexpression in the heart results in insulin resistance in heart and liver; its overexpression in muscles leads to glucose tolerance in muscles. PPAR-gamma overexpression in hepatocytes causes hepatic steatosis (fatty infiltration) due to triglyceride accumulation (Michalik, 2006). Hepatic steatosis is often associated with MS (Reddy, 2006).

It was shown that expression of PPAR-alpha and PPAR-gamma is increased in the vessels of hypertensive SHR (Spontaneously Hypertensive Rats) strain compared with the control WKY (Wistar-Kyoto) strain (Diep, 2001).

The endogenous ligands of PPAR are saturated fatty acids, native and oxidized unsaturated fatty acids, native and oxidized eicosanoids, prostaglandins, and prostacyclins.

Activators of PPAR-alpha (fibrates) and -gamma (thiazolidinediones) have been used clinically for a number of years in the treatment of hyperlipidaemia and to improve insulin sensitivity in diabetes. More recently,
PPAR activation has been found to confer additional benefits on endothelial function, inflammation and thrombosis, suggesting that PPAR agonists may be good candidates for the treatment of cardiovascular disease. In this regard, it has been demonstrated that PPAR activators are capable of reducing blood pressure and attenuating the development of atherosclerosis and cardiac hypertrophy (Robinson, 2009).

Cellular and animal models of PPAR-alpha help explain the clinical actions of fibrates, synthetic PPAR-alpha agonists used to treat dyslipidemia and reduce cardiovascular disease and its complications in patients with the metabolic syndrome. Although these preclinical studies cannot predict all of the effects of PPAR-alpha in humans, recent findings have revealed potential adverse effects of PPAR-alpha action, underlining the need for further study (Lefebvre, 2006).

**Liver X Receptor (LXR)**

There are two isoforms of LXR: LXR-alpha and LXR-beta. LXR-alpha is expressed most highly in the liver and to a lesser extent in the kidney, small intestine, spleen, and adrenal gland (Willy, 1995). In contrast to the restricted expression pattern of LXR-alpha, LXR-beta is ubiquitously expressed (Kainu, 1996). These two isoforms of LXR act as the regulators of lipid and glucose metabolism. LXRs have recently been suggested to play a novel role in the regulation of drug metabolism. CAR forms regulatory network with LXR in exerting its effect on lipid metabolism (Xiao, 2010).

LXRs have been shown to regulate the expression of a battery of metabolic genes, especially those involved in lipid metabolism (Xiao, 2010). LXRs function as cholesterol sensors, which protect mammals from cholesterol overload.

In the liver, excess of cholesterol is converted into bile acids and is removed from the cells, while the synthesis of cholesterol and lipoprotein capture decrease. Functional binding sites for LXR-alpha and LXR-beta were found in promoters of a number of genes encoding key enzymes, transporters, and regulators of these processes. LXR regulates expression of SREBP-1, the protein controlling expression of fatty acid synthase and other key genes involved into fatty acid biosynthesis (Herzog, 2007). Moreover, LXR may directly regulate expression of fatty acid synthase (Tobin, 2000). LXR also regulates expression of the gene encoding ABCA-1, the protein involved into apoA-I-mediated cholesterol transport from hepatocytes (Chawla, 2001).
It was shown that LXR is involved into regulation of glucose metabolism by modulating expression of genes encoding key enzymes of gluconeogenesis (phosphoenolpyruvatecarboxykinase and glucose-6-phosphatase) regulating blood glucose level (Cao, 2003).

The endogenous ligands of LXR are oxidized cholesterol derivatives. LXR regulate the nutrient metabolism pathways through their interactions with specific, naturally occurring oxysterols, including 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol and 24(S),25-epoxycholesterol (Lehmann, 1996; Peet, 1998).

LXRs were shown to function as sterol sensors protecting the cells from cholesterol overload by stimulating reverse cholesterol transport and activating its conversion to bile acids in the liver.

This finding has led to identification of LXR agonists as potent antiatherogenic agents in rodent models of atherosclerosis. However, first-generation LXR activators were also shown to stimulate lipogenesis via sterol regulatory element binding protein-1c leading to liver steatosis and hypertriglyceridemia. Despite their lipogenic action, LXR agonists possess antidiabetic properties. LXR activation normalizes glycemia and improves insulin sensitivity in rodent models of type 2 diabetes and insulin resistance. Antidiabetic action of LXR agonists is thought to result predominantly from suppression of hepatic gluconeogenesis. However, recent studies suggest that LXR activation may also enhance peripheral glucose uptake (Baranowski, 2008).

As regulators of metabolism, LXRs have been considered as potential drug targets by the pharmaceutical industry, and synthetic LXR ligands have been developed, which are widely used as tools in biomedical research. Synthetic LXR ligands include T0901317 and GW3965 (Baranowski, 2008).

**PREGNANE X RECEPTOR (PXR) AND CONSTITUTIVE ANDROSTANE RECEPTOR (CAR)**

PXR and CAR are two closely related and liver-enriched nuclear hormone receptors. PXR and CAR are expressed predominantly in the liver and intestines where their target genes are also located (di Masi, 2009).

PXR and CAR are mainly known as the sensors of xenobiotics and play a key role in xenobiotic detection and response. The xenobiotic response represents a complex group of chemical reactions aimed to inactivate and

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eliminate foreign chemicals. Xenobiotic response is also active against endogenous products of metabolism.

Recent results suggest that PXR and CAR also have important endobiotic roles in energy metabolism by affecting the metabolism of fatty acids, lipids and glucose. PXR and CAR exert their effects on energy metabolism through direct gene regulation or through crosstalk with other transcriptional regulators (Wada, 2009).

CAR and PXR modulate beta-oxidation of fatty acids, PXR controls expression of transcription factors and enzymes involved into lipogenesis (Moreau, 2008) and also influence on transcription of the scavenger receptor CD36 gene (Zhou, 2006). At the same time CAR and PXR influence on transcription factors and cofactors, involved into regulation of genes encoding enzymes of gluconeogenesis in the liver (Moreau, 2008).

CAR and PXR are transcription factors activated by a variety of endogenous and exogenous ligands. Both CAR and PXR elevate the expression of the detoxification machinery in the presence of endogenous and exogenous ligands, regulating the expression of Phase I and II metabolizing enzymes and of phase III transporters. Prescription drugs are included in the list of xenobiotic recognized by CAR and PXR. Among others, statins represent a good example of CAR- and PXR-mediated drug metabolism. Indeed statins, the most effective and prescribed cholesterol-lowering drugs, are target of xenobiotic response mediated by CAR and PXR (Marino, 2011).

The PXR has also recently been shown to play an endobiotic role by impacting lipid homeostasis (Zhou, 2006; Zhou, 2008). Expression of an activated PXR in the livers of transgenic mice resulted in an increased hepatic deposit of triglycerides. This PXR-mediated lipid accumulation was independent of the activation of the lipogenic transcriptional factor sterol regulatory element-binding protein 1c (SREBP-1c) and its primary lipogenic target enzymes, including fatty acid synthase (FAS) and acetyl CoA carboxylase 1 (ACC-1). Instead, the lipid accumulation in transgenic mice was associated with an increased expression of the free fatty acid transporter CD36 and several accessory lipogenic enzymes, such as stearoyl CoA desaturase-1 (SCD-1) and long-chain free fatty acid elongase (FAE).

The activation of PXR was also associated with an inhibition of pro-β-oxidative genes, such as peroxisome proliferator-activated receptor α (PPARα) and thiolase, and an upregulation of PPARγ, a positive regulator of CD36. The crossregulation of CD36 by PXR and PPARγ suggests that this fatty acid transporter may function as a common target of orphan nuclear receptors in their regulation of lipid homeostasis (Zhou, 2008).
Unlike other nuclear receptors, such as the steroid receptors (e.g., estrogen receptors-α and -β), which interact selectively with their physiological ligands, PXR ligands are structurally diverse and include prescription drugs, herbal medicines, dietary supplements, environmental pollutants and endobiotics (Kliewer, 2002).

Many PXR ligands have been identified among prescription drugs, and include the antibiotics rifampicin (RIF), clotrimazole and ritonavir; the antineoplastic drugs cyclophosphamide, taxol and tamoxifen; the endocrine drugs cyproterone acetate and RU486; the anti-inflammatory agent dexamethasone; the anti-type 2 diabetes drug troglitazone; the antihypertensive drugs nifedipine and spironolactone; and the sedatives glutethimide and phenobarbital (Willson, 2002). Among dietary supplements, vitamins K2 and E have been established as weak PXR activators (Tabb, 2003; Landes, 2003). Several groups also reported that a number of environmental pollutants are PXR ligands, such as organochlorine pesticides and polybrominated diphenyl ether flame retardants (Coumoul, 2002). In addition, some endobiotics were identified as PXR ligands, including certain bile acids, bile acid precursors and estrogens (Mnif, 2007; Goodwin, 2003).

CARs play roles in regulating expression of CYP genes in response to exogenous xenobiotics and endogenous lipid compounds. There is overlap between CAR and PXR target genes (Maglich, 2002). For example, PXR regulates the expression of both CYP2B6 and CYP3A4, whereas CAR preferentially regulates CYP2B6 as a consequence of its weaker binding to the PXR response element in the CYP3A4 promoter (Faucette, 2006). Other examples of CAR-regulated genes include CYP2C8, CYP2C9, and CYP2C19, phase II conjugation enzymes, such as UDP-glucuronosyltransferase UGT1A1, sulfotransferase Sult2a1, and glutathione S-transferases Gsta1 (Maglich, 2002). CAR has also been shown to regulate the repression of enzymes involved in gluconeogenesis, such as phosphoenolpyruvatecarboxykinase 1 (PEPCK1), and beta-oxidation enzymes, such as carnitinepalmitoyltransferase 1 (Tien, 2006). In contrast to PXR, CAR is constitutively active (Timsit, 2007).

CAR can repress the genes that encode enzymes involved in signal transduction, fatty acid oxidation, and/or energy metabolism, e.g. phosphoenolpyruvatecarboxykinase 1, enoyl coenzyme A isomerase, and carnitinepalmitoyltransferase 1 (Ueda, 2002).

Original ligands identified for CAR include the testosterone metabolites 5α-androstan-3α-ol (androstanol) and 5α-androst-16-en-3α-ol (androstenol) (Forman, 1998). Unlike classical NRs that are ligand activated or deactivated, CAR activity could be affected by both ligand-dependent and -independent
The Role of Ligand-Activated Transcription Factors…

pathways (Li, 2010). CAR has become best known for its ability to regulate induction of the \textit{CYP2B} gene family by phenobarbital (PB) and PB-like inducers [e.g. chlorpromazine, phenytoin, dichlorodiphenyltrichloroethane, 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and polychlorinated biphenyls], thereby revealing its function as a xenobiotic-sensing nuclear receptor (Sueyoshi, 1999).

CAR has the potential to impact numerous signaling pathways via the genes it modulates directly and by its interference with other nuclear receptor signaling pathways. This creates a unique integrative mechanism to modulate the metabolism of not only xenobiotic substances but also endogenously produced steroids and dietary factors. Unlike its closest relative PXR, the function of which relies solely on ligand binding, CAR functions ligand independently and can be regulated by both direct ligand binding and indirect activation processes (Swales, 2004).

**MS MODELING**

A high percentage of MS morbidity determines much attention to MS modeling, mechanisms of MS development, and new approaches to MS treatment. Although, several inbred, congenic and transgenic rat strains modeling human MS are known, none of existing experimental models gives complete description of mechanisms underlying disease complexity in humans.

It is known that chronic stress causes systemic disturbances in lipid metabolism and cardiovascular functions and plays an important role in the development of MS in humans Chandola, 2006). A rat strain with inherited stress induced arterial hypertension (ISIAH rats) was obtained at the Institute of Cytology and Genetics (Siberian Branch of Russian Academy of Sciences). The ISIAH rat strain was selected from Wistar rats by the level of systolic arterial blood pressure under conditions of mild emotional stress. In ISIAH and Wistar rats basal levels of blood pressure are 150 - 160 and 120 mm Hg, respectively. Mild emotional stress, immobilization in a narrow cage for 30 min, causes a significant increase of blood pressure in ISIAH rats up to 200 mm Hg, whereas in WAG rats such stress insignificantly influences this parameter (Fedoseeva, 2011).

In our work it was shown that ISAH rat strain demonstrates all signs of MS development (Pivovarova, 2011). Hypertensive ISIAH rats have statistically lower HDL cholesterol and increased triglyceride level than the normotensive WAG rats (by 16.2 and 34.7%, respectively; Table 1). Such

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changes in the lipid spectrum are typical for MS and are atherogenic signs [19].

Analysis of cholesterol distribution in lipoprotein fractions (Table 2) also revealed the atherogenic tendency in ISIAH rats. These animals were characterized by increased cholesterol content in the athero-genic lipoprotein fractions (VLDL and LDL, by 46 and 31%, respectively) and decreased cholesterol content in the anti-atherogenic lipoprotein fractions: HDL₂ and HDL₃ (by 33 and 18%, respectively).

The level of apoA-I, the main HDL protein, was lower in blood of ISIAH rats (by 24%) as compared with WAG rats. The level of apoB-100, the main LDL protein, was higher (by 49.5%) in blood of ISIAH rats than in WAG rats (Table 2). The decreased level of apoA-I and the increased level of apoB-100 are also risk factors for development of cardiovascular diseases [19].

An average body mass of ISIAH rats was higher (by 8.5%) than that of age matched WAG rats. A basal glucose level in ISIAH rats was also higher (by 15%) than that of WAG rats (Table 3). Increased body weight and blood glucose level are characteristic signs of MS (NCEP-ATP, 2001).

According to literature data, a hypertensive strain of rats known as SHR (Spontaneously Hypertensive Rats) is characterized by increased LDL cholesterol content compared with the normotensive WKY (Wistar-Kyoto) strain (Yu, 1993). Another hypertensive rat strain known as Lyon hypertensive differs from the normotensive control (Lyon normotensive rats) by increased body weight and increased blood triglyceride content (Sassolas, 1981). Insulin resistance was demonstrated in several hypertensive animal models including SHR (Shimamoto, 2006).

### Table 2. Comparative study of hypertensive ISIAH and normotensive WAG rats: lipid spectrum, body mass, and glucose level

<table>
<thead>
<tr>
<th>Indices</th>
<th>ISIAH compared to WAG, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>no significant differences</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>decreased by 16.2%</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>increased by 34.7%</td>
</tr>
<tr>
<td>Body mass</td>
<td>increased by 18.5%</td>
</tr>
<tr>
<td>Basal glucose level</td>
<td>increased by 15%</td>
</tr>
</tbody>
</table>

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Table 3. Comparative study of hypertensive ISIAH and normotensive WAG rats: content of cholesterol in separate lipoprotein fractions and relative content of apoA-I and apoB-100

<table>
<thead>
<tr>
<th>Indices</th>
<th>ISIAH compared to WAG, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherogenic lipoprotein fractions</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>increased by 46%</td>
</tr>
<tr>
<td>DL</td>
<td>increased by 31%</td>
</tr>
<tr>
<td>ApoB-100</td>
<td>increased by 49.5%</td>
</tr>
<tr>
<td>Anti-atherogenic lipoprotein fractions</td>
<td></td>
</tr>
<tr>
<td>HDL₂</td>
<td>decreased by 33%</td>
</tr>
<tr>
<td>HDL₃</td>
<td>decreased by 18%</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>decreased by 24%</td>
</tr>
</tbody>
</table>

Table 4. Comparative study of hypertensive ISIAH and normotensive WAG rats: functional activity of transcription factors in the liver nuclear extracts

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>DNA-binding activity, ISIAH compared to WAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR</td>
<td>increased by 9.4-fold</td>
</tr>
<tr>
<td>LXR</td>
<td>increased by 3.9-fold</td>
</tr>
<tr>
<td>CAR</td>
<td>increased by 7.1-fold</td>
</tr>
<tr>
<td>PXR</td>
<td>increased by 4.8-fold</td>
</tr>
</tbody>
</table>

Thus, rats obtained during selection for increased blood pressure have some symptoms typical for MS. ISIAH rats used in this study differ from all other hypertensive strains: this strain was selected for enhanced response of blood pressure to the emotional stress. More pronounced MS symptoms observed in ISIAH rats suggest an essential role of the emotional stress in MS development (Pivovarova, 2011).

Table 5. The DNA-binding activity of the transcription factors in the liver nuclear extracts of hypertensive ISIAH and normotensive WAG rats after stress

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>DNA-binding activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ISIAH</td>
</tr>
<tr>
<td>PPAR</td>
<td>not changed</td>
</tr>
<tr>
<td>LXR</td>
<td>not changed</td>
</tr>
<tr>
<td>CAR</td>
<td>decreased</td>
</tr>
<tr>
<td>PXR</td>
<td>decreased</td>
</tr>
</tbody>
</table>

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POSSIBLE MECHANISMS OF MS DEVELOPMENT AND APPROACHES TO THE MS TREATMENT

The MS distribution is growing catastrophically, but mechanisms responsible for developments of complex impairments in MS still remain basically poorly investigated.

The formation of complex MS symptoms suggests systemic impairments in lipid and carbohydrate metabolism; it appears that these impairments should have a common basis at the level of expression of appropriate genes.

It was shown that ligand-activated transcription factors PPAR, LXR, PXR, and CAR participate in regulation of expression of genes involved into lipid and carbohydrate metabolism (Lefebvre, 2006; Herzog, 2007; Moreau, 2008; Van der Leij, 2007). All of this transcription factors are expressed in the liver.

The liver is the main organ controlling energy homeostasis of the body. The liver maintains triglyceride homeostasis by storing circulating fatty acids as triglycerides, oxidizing fatty acids and secreting triglycerides as VLDL. Under normal conditions, the major sources of energy to maintain the whole-body energy homeostasis are triglycerides converted from fatty acids and glucose produced in the liver. During starvation or exercise, fatty acids are released from adipocytes and glucose is produced in the liver to be used by skeletal muscle to meet the body’s energy requirements. Hepatic lipid metabolism involves the synthesis of lipids (lipogenesis), fatty acid oxidation (β-oxidation) and lipid secretion. Lipogenesis is defined as de novo fat synthesis and includes fatty acid synthesis and subsequent conversion of fatty acids to triglycerides in the liver and adipose tissue. Gluconeogenesis provides another major source of energy. Hepatic gluconeogenesis is tightly linked to fasting and starvation. Disruption of lipid and glucose metabolism in the liver might trigger various cardiovascular and metabolic diseases, such as atherosclerosis, type II diabetes, obesity and insulin resistance (Wada, 2009).

In our work at the model of rat strain with Inherited Stress-Induced Arterial Hypertension (ISIAH) which demonstrates all signs of MS functional activity of PPAR, LXR, PXR, and CAR in the liver has been evaluated by their ability to bind oligonucleotides, which imitate binding sites (response elements) in the regulatory regions of target genes (Pivovarova, 2011). The DNA-binding activity (functional activity) of transcription factors was determined by Electrophoretic Mobility Shift Assay (EMSA). Nuclear protein extracts were isolated from liver cells. Nuclear extracts were incubated with labeled oligonucleotides (600-1000 cpm per sample). Native electrophoresis
was performed in 4.5% PAAG. After electrophoresis gel was fixed, dried, and radioautographed.

The DNA binding activity of PPAR, LXR, PXR, and CAR was higher in liver nuclear extracts of ISIAH rats than in WAG rats. Quantitative densitometry of radioautographs has shown that in ISIAH rats the binding activity of PPAR, LXR, PXR, and CAR was higher by 9.4-, 3.9, 4.8, and 7.1-fold correspondingly as compared to WAG rats (Table 4).

Thus, observed signs of MS in ISIAH rats are associated with increased DNA-binding activity of PPAR, LXR, PXR, and CAR; this suggests involvement of these transcription factors in the development of MS in ISIAH rats (Pivovarova, 2011).

Since the stress plays a crucial role in MS development (Chandola T. et al., 2006), we investigated the influence of mild emotional stress on functional activity of PPAR, LXR, PXR, CAR in the liver.

Male rats (4-5 months old) of stress-sensitive hypertensive ISIAH and normotensive WAG (Wistar Albino Glaxo) strains were used in this study. The rats of both strains were divided into two parts (four groups). One part was put into stress by restriction of animals in narrow cylindrical cages. Another part was a reference intact group.

The DNA-binding activity of PPAR and LXR was increased in WAG rats after stress, whereas DNA-binding activity of these transcription factors did not change in ISIAH rats after stress. The DNA-binding activity of CAR and PXR was increased in WAG rats after stress, whereas DNA-binding activity of these transcription factors was decreased in ISIAH rats after stress (Table 5).

Our data suggests that the stress can influence DNA-binding activity of transcription factors PPAR, LXR, PXR, CAR which regulate expression of genes involved into lipid and carbohydrate metabolism.

These transcription factors are ligand-activated; there are a number of endogenous and exogenous ligands. Ligands for the transcription factors can be formed in the process of lipid metabolism. Lipoprotein lipase can hydrolyze lipoproteins, generating ligands for PPAR (Chawla, 2003). Oxidized LDL (ox-LDL) also can be the source of the ligands. It was shown that there is a correlation between the level of ox-LDL and the development of MS as a whole and its components such as abdominal obesity, hyperglycemia, and hypertriglyceridemia (Holvoet, 2008). It was also shown that ox-LDL can activate PPAR-alpha and PPAR-gamma (Taketa, 2008). Oxidized LDL trapped in the liver through scavenger receptor, where they are degraded (Mathieu, 2006); their oxidized lipid components can be ligands for...
transcription factors, affecting thus the network of genes involved in lipid and carbohydrate metabolism.

There is a cross-talk between signal transduction pathways of PPAR, LXR, PXR, and CAR (Woods, 2007); this suggests their integrated role in regulation of genes of lipid and carbohydrate metabolism.

In situation of an uncontrolled medication, as well as pollution of the environment the study of the role of transcription factors in the development of MS is especially important.

Thus, stress, nutrition, medicines, environment pollution via ligand-activated transcription factors can lead to the abnormalities in lipid and carbohydrate metabolism and MS development (Figure 1).

![Figure 1. The role of transcription factors PPAR, LXR, CAR, and PXR in MS development.](image)

Since PPAR, LXR, PXR, and CAR are ligand-activated receptors, they represent perspective targets for pharmacological treatments. At present, the search of natural and synthetic PPAR ligands is intensively performed (Michalik, 2006). Involvement of not only PPAR, but also LXR, PXR, and
CAR in the development of MS suggests that their signal transduction
pathways should be taken into consideration during design of pharmacological
preparations.

CONCLUSION

The development of complex MS symptoms suggests systemic
impairments in lipid and carbohydrate metabolism. It appears that these
impairments should have a common basis and it have to be at the level of
expression of appropriate genes. Expression of genes involved into lipid and
carbohydrate metabolism is regulated by ligand-activated transcription factors
including those referred to the superfamily of nuclear hormone receptors:
PPAR, LXR, PXR, and CAR. Since these transcription factors are ligand-
activated, they represent perspective targets for pharmacological treatments.
Complex studies of regulatory mechanisms, signaling pathways, and
transcription targets of PPAR, LXR, PXR, and CAR can significantly help
better understanding of mechanisms of MS development and provide valuable
information for development of appropriate pharmacological approaches for
MS therapy.

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ENDOTHELIAL LIPASE: ITS ROLE IN THE METABOLISM OF HDL-CHOLESTEROL, THE RELATIONSHIP TO CARDIOVASCULAR RISK AND THERAPEUTIC OPPORTUNITIES

D. Agapakis, S. Fotiadis, C. Savopoulos, and A. I. Hatzitolios
First Propedeutic Internal Medicine Department, AHEPA Hospital, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece

ABSTRACT

The endothelial lipase (EL) is an enzyme with lipolytic activity against phospholipids of high density lipoprotein-cholesterol (HDL) in plasma.

The EL is synthesized by vascular endothelial cells. In particular, it is synthesized mainly in heart, muscle, and adipose tissue and then transported to the luminal surface of endothelial cells where it hydrolyzes lipoprotein triglycerides and phospholipids. In addition, the endothelial-derived EL can be a direct regulator of HDL metabolism increasing the catabolism of HDL particles, thereby reducing the levels and the size of the tank and impacting recycling.

Both experimental in vitro and in vivo studies have revealed remarkable information with regard the physiological role of EL in inflammatory conditions and its potential role in modulating lipoprotein...
metabolism under inflammatory conditions, including atherosclerosis. EL also seems to have local actions in the vascular wall and the regulation of circulating lipoproteins, and it seems to be associated with established and novel cardiovascular risk factors like hypertension, diabetes, dyslipidemia, obesity, and leptin, which potentially contributes to the progress of atherosclerosis and therefore could be regarded as a biochemical marker of atherosclerosis.

The EL inhibition is considered to be an attractive intervention target in order to favorably modulate the atherosclerosis process by improving the HDL levels.

This article reviews the effect of EL on the metabolism of HDL-C, its association with cardiovascular risk factors and the potential perspective to arrest or even improves atherosclerosis by EL inhibition.

**INTRODUCTION**

From large multi-center clinical intervention trials of the last decade it has been demonstrated the inverse relationship between levels of cholesterol in high density lipoprotein (HDL) and cardiovascular risk confirming what has been observed in previous epidemiological and experimental studies, and thus further supporting the antiatherogenic role of the HDL [1-4]. It has also been found that low levels of HDL are the most important independent risk factor for morbidity and mortality in advanced heart failure, compared to total cholesterol (T-C) and low density lipoprotein (LDL) [5]. Moreover, greater predictive value in preventing cardiovascular risk is offered when HDL is included instead of using T-C or LDL by themselves [6].

Therefore HDL increase continues to be an attractive therapeutic challenge aiming to atherosclerosis prevention and novel treatments are attempted to be developed. With regard HDL action, therapeutic intervention focuses at different steps including the transport of cholesterol from peripheral tissues to the liver, inhibition of oxidation of LDL, arresting vascular inflammation and improving endothelial antithrombotic properties. Both the function and structure of HDL-particles contribute to atheroprotective actions. Among the factors that shape the metabolism of HDL, endothelial lipase (EL) is recognized as an important one.
**ENDOTHELIAL LIPASE: ITS ROLE IN THE METABOLISM OF HDL**

This is a new member of the lipase enzyme family, a glycoprotein 68 kDa encoded by a gene on chromosome LIPG 18q21.1, which was discovered in the relatively recently [8]. The EL is characterized by lipolytic activity of phospholipase A1 which has almost exclusively as a substrate phospholipids of HDL-particles which are hydrolyzed in position sn-1, in contrast to the lipoprotein, liver and pancreatic lipase which are predominantly triglyceride lipases [9]. It is produced in a unique way mainly by endothelial cells and to a lesser extent by macrophages and smooth muscle cells. The tissue expression is mainly in liver, thyroid, lungs, kidneys, testes and placenta. After production, the EL binds to proteoglycans on the endothelial surface initiating its local action [9]. Two potential lipid binding sites and four heparin or proteoglycans have been identified. EL promotes, the hydrolysis of phospholipids leading to the creation of small, dense HDL-particles which are structurally unstable, thus facilitating the release of lipid poor apolipoprotein (Apo) A-I.

Overexpression of EL in mice was associated with reduced levels of HDL and Apo A1 due to increased catabolism and excretion by the kidneys [10-11]. The hydrolysis of phospholipids result non-esterified fatty acids that are received and incorporated into cellular phospholipids and triglycerides increasing the total amount of cellular lipids, while is suppresses the rate of synthesis of fatty acids. The remaining residual cholesterol in HDL-particles is particularly sensitive to selective hepatic uptake, which is greatly increased, through receptor SR-B1 [10, 12]. On the contrary, in experimental models where EL activity was abolished, an increase in the quantity and size of HDL-particles was observed even under nutrition conditions with foods rich in cholesterol, which are highly atherogenic [13 -14].

Thus, the fluctuating lipolytic activity of EL remodels and modifies the phospholipid content of the HDL particles, through dose-related increase or decrease of catabolic rate [10, 13]. Besides the above enzymatic activities, the EL can also enhance cell attachment and processing of HDL-particles to the endothelium through heparinic sulfate proteoglycan assisted pathways. [15]. Enzymatic and non-enzymatic functions of EL appear to be interrelated synergistic.

In conclusion, in vitro and in vivo studies have documented that EL facilitates through two ways the catabolism of HDL, modifying its
concentration and composition, as an important negative regulator of HDL concentration [13, 16]. It seems therefore that EL might prove a new promising therapeutic target.

**THE ROLE OF EL IN REVERSE CHOLESTEROL TRANSPORT (RCT)**

The RCT is the key atheroprotective mechanism of HDL, including the efflux of cholesterol from intracellular reservoirs in peripheral cells and foam cells of atherosclerotic plaques and its transfer to the liver [17].

This function includes mainly the one-way efflux of free cholesterol from intracellular cholesterol pool from the atherosclerotic lesions of the macrophage foam cells, toward lipid poor Apo AI by ATP binding-cassette transporter 1 (ABCA1) where they are changed to nascent discoidal HDL.

A two-way flow of free cholesterol between cells and HDL via receptor SR-B1, whose ligands are rich in phospholipids lipoproteins [18-19]. Through the actions of lecithin cholesterol acyltransferase (LCAT) first and then of the protein transport of phospholipids (PLTP) are generated the mature spherical HDL-3 initially and successively the large HDL-2 particles which have antiatherogenic properties. Both forms of HDL are substrates for the hepatic and endothelial lipase. The large HDL-2 either interact with VLDL and LDL cholesterol ester exchanging triglycerides via the enzyme cholesterol ester transfer (CETP), or attribute esterified cholesterol in liver cells through hepatic receptor SR-B1 to be further metabolized and excreted as bile acids, while the same HDL-2 will be converted back to small HDL-3, which also have antiatherogenic properties [20]. Noteworthy that the ability of HDL particles to employ and pay lipids is inversely proportional to the amount of fat they contain.

The specific effect of EL on intracellular cholesterol efflux mediated by HDL is not supported by many studies and not always leads to clear conclusions. It is considered however reasonable to assume that the inhibition of EL strengthens the mechanism of RCT in vivo [21]. It has been suggested that depletion of phospholipids in HDL induced by the EL leads to decreased affinity of the first with the cell membrane and reduced ability to efflux cholesterol to HDL. Indeed, studies in vitro, shows that overexpression of EL was associated with changes in chemical composition and physical properties of HDL, which had resulted in reduced binding capacity of HDL to SR-B1.
receptor in peripheral cells and thereby decreased the selective free cellular cholesterol uptake through this receptor [22]. Moreover, the subsequent reduction in the nucleus of HDL-particles due to loss of phospholipids, results in their structural instability, as has been mentioned, and the released Apo AI will interact with the ABCA1-mediated cholesterol efflux. This was found in the experimental study of Qiu and Hill where overexpression of EL in macrophages altered the composition of phospholipids of cell membranes and produced Lyso-phosphatidylcholine (lyso-PC) that has the ability to stimulate the binding of Apo AI with the ABCA1 receptor resulting in increased efflux of cholesterol, through both enzyme and non-enzymatic mechanisms of EL. While the suppression of EL has the opposite effect [23]. These results suggest that EL can promote the inhibition of cholesterol efflux from macrophages. Similarly, Yancey et al. observed that overexpression of EL caused a 63% increase of cholesterol efflux through the ABCA1 receptor and reduced cholesterol efflux through the SR-B1 receptor by 90%, probably due to increased production of pre-beta HDL in serum [24]. Instead, in his study Gauster et al. didn’t find any increase of cholesterol efflux through ABCA1 under similar circumstances. In a recent and very important study of Braun JR et al. it was observed large increase in cholesterol efflux capacity of HDL in cases of shortage of (EL/−) mice [25]. These findings highlight the need for more studies on the in vivo effects of EL on RCT and that overexpression or lack of EL seem to change the degree of involvement of free cholesterol efflux pathways, via activation of different cellular receptors, presumably in an effort to maintain the homeostasis of cholesterol.

On the other hand, the EL acts in the second phase of RCT hepatic uptake of cholesterol ester in HepG2 cells via SR-B1 and tethering HDL-particles [12, 26]. In particular, it was found that the hepatic uptake of cholesterol requires the formation of HDL-particles by hepatic expression of EL [12]. Nevertheless, the total body cholesterol excretion, which is the last stage of RCT following hepatic metabolism and the formation of bile acids, remains unchanged in mice with EL/− despite the increasing concentration of HDL in plasma [21, 25].

In conclusion, it can be said that the inhibition of EL results in increased levels of HDL without interfering with the hepatic uptake of cholesterol, and this is a positive contribution to the process RCT with the subsequent reduction in atheroma in vascular lesions. However, additional clinical studies are needed in order to quantify the magnitude of cholesterol efflux capacity in conditions of changing levels of EL.
EL AS A RISK FACTOR FOR ATHEROSCLEROSIS

Several studies have supported the positive role of EL in the formation and evolution of the atherosclerotic lesion. Thus, it is found that the EL can be expressed in endothelial cells, smooth muscle cells of the intima and also in neo-vascular network of atherosclerotic lesions in coronary arteries, suggesting that it plays an active role even in early atherosclerotic lesions [11]. Presumably by the action of EL released free fatty acids and phospholipids are offered as a source of energy for the functioning of endothelial and smooth muscle cells of blood vessels [27]. Recently, Huang et al. found higher concentration of EL in plasma of people with atherosclerosis compared with healthy individuals [28]. The proatherogenic properties of EL are also established from studies where the overexpression of EL was found to enhance the adhesion of monocytes to the vascular wall, thereby promoting atherosclerosis [29-30]. On the other hand, the work of Ishida et al. examined atherosclerotic models in transgenic mice with functional deletion of EL (apo E−/−, LIPG−/−), and found a reduction in the size of atherosclerotic lesions, reduction of vascular infiltration of macrophages and increased levels of HDL [31]. Moreover, there are further data which support the association of EL with inflammatory processes. In the experimental study of Yasuda et al. it was demonstrated that the cytokines TNF-α and IL-1β induce the expression of mRNA of EL endothelial cells as enhancing the mechanical forces involved in vascular diseases [32-33]. Similar studies in vitro in macrophage cell cultures and in vivo also found positive relationship between pro-inflammatory gene and the expression of EL in vascular endothelium, which in turn affects the synthesis of cytokines sustaining a vicious cycle [30, 34]. Different results emerged from the work of Ko et al. in hyperlipidemic mouse apoE−/− and LDLR−/− where no change was observed in atherosclerosis, despite an increase in HDL as result of EL’s inactivation [35]. However, the experimental model chosen is extremely atherogenic and it is uncertain what kind of extrapolations can be made from this study.

On the other hand, the study of Ahmed et al. found that the intact activity of EL on HDL is essential for activation of the nuclear receptor PPAR, and this activation represents the main mechanism for the expression of anti-inflammatory action of HDL i.e inhibition of vascular adhesion of leukocytes [36]. However, activation of PPAR may only be an indicator of the intensity of the inflammatory process and the role of EL probably emphasizes the link between abnormal action and reactive response. Some anti-atherogenic properties of EL suggest the work of Brown et al where the lack of EL and
hepatic lipase resulted in the formation of small dense LDL [25]. In the same vein, the Broedl et al. showed that in lipidemic mice the overexpression of EL caused an increase in clearance of Apo B lipoproteins [31, 37]. While the lack of EL was associated with increased levels of VLDL / LDL as a result of mild lipolytic action of the TRG and increasing residence time of VLDL in plasma [35]. However, the impact on human’s EL on Apo B lipoproteins is limited and the reciprocal increase in anti-atherogenic and atherogenic lipoproteins in LIPG-/-, Apo E/- mice do not appear to be balanced, and it is associated with dramatic reduction of atherosclerotic volume by 70% tilting the balance towards the atheroprotective increased levels of HDL [31].

With regard other mechanisms of HDL atheroprotection, it has been shown that the EL promotes engagement and recruitment of non-oxidized and oxidized LDL in macrophages [23]. It has been shown, that the inhibition of EL enhances all atheroprotective mechanisms of HDL which have been shown both in vitro and vivo (efflux of cholesterol, anti-inflammatory, antioxidant, PON-1, PAF-AH, antithrombotic) [38].

Finally, infection increases the expression of EL and in this way modifies the lipopolysaccharide-neutralizing capacity of HDL (removing phospholipids). Also, mice EL -/- showed increased ability of HDL to inhibit the secretion of TNF-α which stimulates the lipopolysaccharide and injected HDL from these animals in wild mice infected with endotoxin showed a longer survival [38].

This behavior of HDL, as a key component of innate immunity should be considered since torcetrapib (inhibitor of CETP) led to a true increase of HDL but increased also the frequency of deaths secondary to infections [39].

In conclusion, the EL is involved in all pathophysiological mechanisms of atherosclerosis associated with low levels of HDL, while its targeted inhibition leads to atheroprotective HDL-particles and interesting immunological activities.

**EL CORRELATION WITH CARDIOVASCULAR DISEASE AND RISK FACTORS**

These features of the EL have been confirmed in clinical studies and have been linked to levels of EL with established and novel cardiovascular risk factors, the metabolic syndrome and the progress of atherosclerosis. Particularly in the study of Badellino et al. asymptomatic individuals with a

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family history of premature coronary heart disease, an increase in EL mass was positively correlated with all risk factors, i.e. obesity (waist circumference, body mass index > 30), hypertension, fasting glucose, smoking, lack of exercise, insulin resistance, the hypertriglyceridemia, the concentration of Apo B, the LDL in women, and calcification of the coronary artery which is a well known indicator of subclinical atherosclerosis [40]. A similar correlation has also been found between increased EL and increased visceral obesity and proatherogenic lipid profiles associated with it (T-C, VLDL, TRG, Apo B) [41]. Interestically, it appears that the EL is an alternative way of recruiting FFA from adipose tissue in mice LPL-/ with serious effects on the metabolism of lipoproteins [42]. Additionally, it has been shown that inflammatory markers such as hs-CRP, sTNFr2, IL-6, sICAM-1 and leptin correlated positively and directly with EL. Thus, overexpression of EL in obesity and metabolic syndrome may be explained by the proinflammatory state where inflammatory cytokines released by fat cells [34]. In contrast, negative correlation was found between EL and adiponectin, which is found at low levels in obesity and it is known to inhibit the TNF-a induced EL. This effect is achieved through inhibition of the nuclear factor-KB (NF-KB) a key initiator of secretion of EL [34]. The study of Yoko et al. identified a clear negative correlation between levels of EL with the levels of HDL, in patients who had developed cardiovascular disease [43]. On diabetic patients, the recent study by Sammy et al. showed that the EL is expressed in excess (Diabetes Mellitus can be regarded as a state of subclinical inflammation), although the impairment of cholesterol efflux mechanism was associated with very low HDL levels in these patients [44]. Also, a small study found EL in vitro to be proportional to the extracellular glucose concentration, but not in vivo [45]. While a larger study, found increased concentration of EL in diabetics who were under oral anti-diabetic treatment but not in insulin treated patients [46]. It appears that insulin alters the expression of EL. Also, a positive association of EL with CRP in diabetic and non-diabetic patients has been observed, but not in insulin treated patients [46]. Finally, a clinical study of Fujii et al. assessed the role of EL in dialysis patients and found correlation between EL, hypoalbuminemia and inflammation in these patients [47].

In conclusion, there is growing evidence that the EL is closely linked to the factors and markers of cardiovascular risk and plays an active role in shaping the profile of atherogenic dyslipidemia that characterizes the metabolic syndrome.
GENE POLYMORPHISMS OF EL AND HDL

The gene polymorphisms of LIPG are associated with functional changes in the action of EL and participate in shaping levels of HDL [48]. They can also play an important role in the extent of morbidity associated with elevated levels of EL and the therapeutic response to pharmacological interventions. For these reasons, these polymorphisms have been the subject of intensive research and the most common mononucleotide polymorphism 584 C/T produces a variety Thr111Le, is the focus of interest of several researchers. Ma et al. found a significant correlation between the correspondence of 584 C/T and HDL levels but no difference in the development of CHD [49]. Tang et al. in a Chinese population found that the T allele correspondence of the same mononucleotide polymorphism 584 C/T offers cardiovascular protection [50]. Unlike studies in Caucasian healthy populations showed no association of 584 C/T with HDL levels or CHD [51]. While in Japanese patients with AMI a positive correlation with the polymorphism was observed, regardless of HDL [52]. Also, the polymorphism 584 C/T was associated with a higher incidence of retinopathy in diabetic patients with lower levels of LDL, mild increase in HDL and arterial blood pressure too [53]. The diversification of the distribution of the gene variant of LIPG according to race and ethnicity and the differing impact of environment on the functional expression of EL in the populations studied (such as association with smoking, type of diet) produces inconsistent results of investigations and make it difficult to generalize the findings. More extensive investigations are necessary to confirm any correlation between the gene of EL with the levels, the type of HDL particles and cardiovascular disease.

FACTS AND CHALLENGES IN THE THERAPEUTIC TARGETING OF EL

These findings of EL and its inverse relationship with HDL shape the challenge to design new methods and therapies aimed at inhibiting EL as a tool to increase HDL and the subsequent reduction cardiovascular risk.

So far there are no specific drugs to inhibit selectively the expression of EL. There are available easy to use biochemical methods to determine the change in the activity of the enzyme [54-55]. From biotechnology and biochemistry are under development substances with inhibition activity. The

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sulfonylfuran urea 1 is one such substance potentially suitable to inhibit the EL in a selective manner and is under development [56]. Sulpharaphan (SFN) which is a naturally occurring isothiocyanate present in cruciferous vegetables, has important antioxidant and anti-inflammatory effects, inhibits EL expression through NF-κB in endothelial cells [57]. Still, the boronic acid may seem to be the basis for the design of new drugs since in vivo testing has shown to inhibit EL and to increase HDL by about 30% [58].

Similar efforts are also in the field of molecular pharmacology of the composition antisense nucleotides to the intervention and modification of gene expression of EL, while developing methods for future diagnostic and therapeutic approach (kits) in patients who develop morbidity related to the EL [59]. From already used lipid-lowering drugs, statins cause a small increase in levels of HDL. However, their contribution is important mainly because qualitatively alter the dysfunctional HDL-proatherogenic occurring in conditions of metabolic syndrome, diabetes, CHD, infections. Only two studies exist to assess the effect of statins on levels of EL. In the in vitro study of Qiu and Hill evaluated the effect of administration of atorvastatin and simvastatin in the expression of EL in macrophages in human cell cultures. Significant dose-dependent reduction of EL mRNA was found in macrophages via mechanisms of inhibition of HMG-CoA reductase and the NF-κB [60]. In a clinical study of Kojima Y et al. the administration for six months of pitavastatin in hypercholesterolaemic patients, reduced the levels of EL by 14%, increased HDL levels by 11% and significantly increased the size of HDL-particles by 12%, supporting the view of pleiotropic action of statins. Furthermore, in the same study it was found that Rho A is an important factor pathway in regulating the expression of EL in endothelial cells [43]. The effect of fibrates and nicotinic acid on the expression of EL there are currently no available data. However, it is known that fibrates are PPARa activators. The activation of these receptors causes increased expression of proprotein convertase PC 5/6 and furin, which in turn causes proteolytic cleavage of EL on the cell surface [61-62]. Thus, it seems logical that the beneficial effects of these drugs on HDL, through a specific regulatory pathway also include reducing the EL. The niacin is the most effective lipid lowering agent for increasing HDL, has properties of PPAR-gamma agonist and exhibits pleiotropic actions such as increased adiponectin and reduction of CRP. Finally, pioglitazone also acts as a PPAR-gamma activator and has the specificity to trigger factor IkBα in vitro and in vivo in experimental models. This factor is an inhibitor of NF-kB, which is positively correlated with the induction of expression of EL and the VCAM-1. Consequently, pioglitazone is...
expected to reduce the levels of EL [63]. Overall, it appears that the PPARs are involved in the pathophysiology of HDL homeostasis through the regulation of secretion of EL.

**CONCLUSION**

In conclusion, the EL acts as a regulator of HDL levels and is associated with an inverse relationship. It has proatherogenic properties, in particular vascular expression and may interfere with the atherosclerotic process through direct local action in the vascular wall and through the control of circulating lipoproteins. The EL is positively correlated with cardiovascular risk factors and could be considered a biochemical marker of inflammatory and atherosclerotic process. In clinical practice, it appears that certain statins, including simvastatin, atorvastatin and pitavastatin may partially inhibit this action of El and at present we look forward to novel treatments which will target EL inhibition.

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Chapter 7

THE ASSOCIATION BETWEEN DYSLIPIDEMIA AND NEUROPATHY – OWOLABI MO

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ABSTRACT

Microvascular damage is an important pathophysiologic mechanism underlying peripheral and autonomic neuropathy. Microvascular injury is mediated by various vascular risk factors, which are also components of the metabolic syndrome. There is increasing evidence for the role of dyslipidemia in the development and progression of diabetic peripheral and autonomic neuropathy.

Although small fiber neuropathy has been reported in non-diabetic patients with hypertriglyceridemia, the role of dyslipidemia in peripheral neuropathy is often examined in the context of other vascular risk factors which may confound the association. A number of long-term studies of large cohorts of patients with diabetes have been completed. In these studies over the course of seven or more years of follow-up, it is not glycemia but serum lipids, hypertension, and body mass index that are independently associated with the risk of developing diabetic neuropathy.

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There is emerging evidence that the aggregate effect of all the vascular risk factors may induce microvascular damage resulting in peripheral neuropathy. Oxidative stress provoked by oxidized LDL amongst other mechanisms may play a major role and constitute a therapeutic target. Statins and fibrates may play a role in the prevention and management of diabetic peripheral neuropathy.

**INTRODUCTION**

Peripheral and autonomic neuropathies are common causes of neurological disability. [1-3] Microvascular damage is an important pathophysiologic mechanism underlying these neuropathies. [2-4] Microvascular injury is mediated by various vascular risk factors, which are also components of the metabolic syndrome. [2, 3, 5, 6] There is increasing evidence for the role of dyslipidemia in the development and progression of diabetic peripheral and autonomic neuropathy. [2, 3, 5, 6] Although small fiber neuropathy has been reported in non-diabetic patients with isolated hypertriglyceridemia, the role of dyslipidemia in peripheral neuropathy is often examined in the context of other vascular risk factors which may confound the association. [5-10].

A number of long-term studies of large cohorts of patients with diabetes have been completed. In these studies over the course of seven or more years of follow-up, it is not glycemia but serum lipids, hypertension, and body mass index that are independently associated with the risk of developing diabetic neuropathy. [5-10] There is emerging evidence that the aggregate effect of all the vascular risk factors may induce microvascular damage resulting in peripheral neuropathy [2, 3]. In this review, the association between various types of dyslipidemia and neuropathy is examined.

**ROLE OF LIPID DISORDERS IN DIABETIC PERIPHERAL AND AUTONOMIC NEUROPATHIES**

Diabetic peripheral neuropathy (DPN) is the most common late complication of diabetes mellitus (DM), and some of its consequences include pain, falls, foot ulceration, amputation, sleep impairment, depression and impaired quality of life. [2, 3] Unfortunately, the pathogenesis of DPN is poorly understood and there is no effective treatment. [2, 3] Recent studies
have shown that chronic hyperglycemia measured by HbA\textsubscript{1c} is neither a consistent marker of DPN occurrence nor its severity. [2, 3] For instance, although the Diabetes Control Complications Trial (DCCT) reported a significant reduction in neuropathy in the intensively treated groups (with lower HbA\textsubscript{1c}), the Veterans Affairs Diabetes Trial, observed a non-significant increase in autonomic neuropathy in the intensive-therapy group (with lower HbA\textsubscript{1c}). [2, 3] In the Veterans Affairs Diabetes Trial, and a recent Swedish study, HbA\textsubscript{1c} had no significant effect on DPN. [2, 3] Also in the Veterans Affairs Cooperative Study in Type 2 DM study, a 2.07% reduction in HbA\textsubscript{1c} had no significant impact on diabetic peripheral or autonomic neuropathy. [2, 3]

Recent studies have shown that vascular risk factors apart from hyperglycemia are involved in the development and progression of diabetic neuropathies. [2, 3] The pathogenesis of DPN is complex and multi-factorial. Owolabi et al explored the relationship between total cardiovascular risk load (TCRL) using the United Kingdom Prospective Diabetes Study (UKPDS) cardiovascular risk engines, and the presence of DPN. In their cohort of patients with mean HbA1c of 6.9%, TCRL was a stronger statistical correlate and predictor of DPN than HbA\textsubscript{1c}. This may have implications for prevention and monitoring of DPN particularly in those with better glycemic control. [2]

In a further study of those with DPN, Owolabi et al demonstrated that TCRL had the strongest significant correlation to DPN severity (p =<0.001, rho = 0.285) with age and dyslipidemia (LDL/HDL ratio), which are also components of the TCRL, emerging as independent statistical predictors of DPN severity in multivariate analysis [2, 3].

**Pathophysiology of Diabetic Neuropathy: Beyond Hyperglycemia**

Until recently, it was thought that hyperglycemia was the driving force underlying the development of diabetic neuropathy. This was based originally on results from the DCCT Diabetes Control and Complications Trial (DCCT). In the DCCT, type 1 diabetic subjects receiving intensive therapy with an average glycosylated hemoglobin (HbA1c) of 7.2% had a reduced 60% cumulative incidence of diabetic neuropathy when compared to patients receiving conventional treatment (average HbA1c of 9.0%). [10]

However, the continuing longitudinal study of the DCCT, the Epidemiology of Diabetes Complications and Interventions Cohort (EDIC)
yielded unanticipated results 20 years later. Within a year of discontinuing the DCCT and beginning EDIC, the glycemic control in the two treatment groups equalized to an average HbA1c of 8%. All patients were examined annually for diabetic neuropathy; one decade later, patients from the intensive-DCCT cohort had a lower incidence of diabetic neuropathy compared to patients from the conventional-DCCT cohort, despite 10 years of convergent glycemic control. Although the underlying mechanism(s) of this result was not determined, the cohort with a higher incidence of DPN had more dyslipidemia than the conventional cohort. [10]

This interesting, unanticipated finding is further supported by the Eurodiab Trial, a longitudinal study of over 3,000 individuals with type 1 diabetes. [5, 6, 11] Of the 1,200 subjects who did not have diabetic neuropathy at baseline, hypertension, serum lipids and body mass index were each independently associated with the risk of developing diabetic neuropathy during a 7-year follow-up period. Of these risk factors, dyslipidemia was closely linked with the onset and progression of diabetic neuropathy. [10]

In support of these findings, Wiggins et al evaluated the mechanisms underlying diabetic neuropathy progression using indices of sural nerve morphometry obtained from two identical randomized, placebo-controlled clinical trials. They found elevated triglycerides to be the only clinical parameter that correlated with a loss of myelinated fiber density, independent of disease duration, age, diabetes control, or other variables. [12]

Dyslipidemia and Neuropathy: The Emerging Evidence

The emerging idea that dyslipidemia contributes to the development of diabetic neuropathy may explain the earlier incidence of diabetic neuropathy in individuals with type 2 compared to type 1 diabetes. Lipid profiles are commonly abnormal early in the course of type 2 diabetes in a temporal pattern that correlates with the presence of diabetic neuropathy. [10] In contrast, lipid profiles are nearly always normal in type 1 patients at the time of diabetes diagnosis. [8] Dyslipidemia develops later in the course of type 1 diabetes, and these abnormal lipid profiles coincide with the delayed onset and progression of diabetic neuropathy. [10]

Accumulating data from several large scale trials of patients with type 2 diabetes also point to early dyslipidemia as a major independent risk factor for the development of diabetic neuropathy. [10] In the United Kingdom Prospective Diabetes Study (UKPDS), newly diagnosed type 2 patients were

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randomized into either intensive treatment with an oral hypoglycemic agent or insulin or conventional treatment with diet. After 10 years, intensive treatment resulted in approximately 1% lower HbA1c vs. conventional treatment but there was no difference in the development of diabetic neuropathy between the two groups, which had similar lipid and blood pressure. [10]

This finding, at first unexpected in light of the earlier DCCT data, was supported by the Veterans Affairs Cooperative Study (VACS), which demonstrated no difference in the prevalence of diabetic neuropathy in type 2 diabetic patients over a 2-year period comparing standard and intensive glycemic control. [10] These results suggested that independent factors other than glycemic control are critical to the development of diabetic neuropathy. [8]

As with EDIC and Eurodiab, analysis of the UKPDS and VACS data points to dyslipidemia as a critical independent factor for the development of diabetic neuropathy. [8] Type 2 diabetes clusters with risk factors for coronary heart disease including obesity, hypertension, and dyslipidemia; individuals with two or more of these factors are diagnosed with the metabolic syndrome. In a cross-sectional study of type 2 diabetic subjects, those with metabolic syndrome were twice as likely to have diabetic neuropathy [13], and the driving factor was dyslipidemia. This supports the observation by Owolabi et al where aggregated vascular risk factors for stroke or coronary heart disease was the strongest correlate of the presence as well as the severity of DPN. [2, 3]

In a European study of 85 type 2 diabetic patients with at least two additional metabolic syndrome parameters, the prevalence of microvascular complications, including diabetic neuropathy, increased with each additional parameter present [13]; abnormalities in (low-density lipoprotein) LDL profiles were more closely related to diabetic neuropathy than hyperglycemia. [2, 3, 10]

Finally, prospective studies of patients with idiopathic neuropathy, confirm a higher prevalence of hyperlipidemia than impaired glucose tolerance or hypertension, suggesting that dyslipidemia is an essential factor underlying nerve injury. Collectively, this evolving and exciting literature links dyslipidemia to the development and progression of diabetic neuropathy. [2, 3, 10]

Neuropathy may develop as a result of endothelial dysfunction, which can cause a reduction in neuronal blood flow. Endoneural capillary density is reduced and correlates with decreased density of myelinated fibres in neuropathic diabetic patients. [2, 3, 10, 14] These effects may impair the
function of motor, sensory and autonomic neurons in patients with diabetes. [2, 3, 8, 14] Endothelial injury resulting from multiple vascular risk factors particularly dyslipidemia seem to pay a major role. [2, 3, 8, 14]

Indeed, intensive treatment of the cardiovascular risk factors associated with diabetes, including hyperglycemia, dyslipidemia and hypertension, has been shown to reduce the risk of microvascular complications including diabetic neuropathy. [2, 3, 8, 14]

Prospective studies tended to show a positive association between lipid levels and diabetic microangiopathy but results are inconsistent with regard to which lipid fraction is most predictive. [8] Whereas hypertrygliceridemia is an independent risk factor for neuropathy, evidence is mounting on the possible role of modified LDL-cholesterol in DPN.

The Role of Modified Lipids in the Pathophysiology of Diabetic Neuropathy

Using cell culture and mouse models of diabetic neuropathy, it has been suggested that oxidized LDLs (oxLDLs) are one notable ‘lipid factor’ responsible for nervous system injury (10) oxLDLs are critically involved in endothelial cell dysfunction, evident from the large body of literature implicating oxLDLs in atherosclerotic lesion formation [10] oxLDL is strongly cytotoxic, which may explain the areas of necrosis detected within atherosclerotic lesions. [10] In man, oxLDL is a highly analytic marker for macrovascular disease, including stroke and myocardial infarction [10]. In patients with types 1 and 2 diabetes, serum levels of oxLDL in proportion to total LDL particles are associated with diabetic neuropathy. [10] oxLDLs cause apoptotic injury and death in both endothelial cells and neurons. [10] In endothelial cells, oxLDLs induce multiple events associated with apoptotic injury, including Bid degradation, cytochrome c release, and caspase-3 activation. [15, 16] In neurons, oxLDLs induce DNA fragmentation, increase reactive oxygen species (ROS) and activate a caspase-3-dependent death mechanism. [15, 16] These data suggest that, in diabetes, neurons are exposed to both glucose and oxLDL whichindependently increase ROS which in conjunction with hypertension inflicts endothelial and neuronal injury (Figure 1). [8, 10, 15, 16]
HYPERTRIGLYCERIDEMIA AND PERIPHERAL NEUROPATHY

Apart from oxidized LDL, hypertriglyceridemia may also be important in the development of neuropathy. The relationship between hypertriglyceridemia and neuropathy has been explored in several case series. Mc Manis in 1994 described a group of 6 patients with moderately elevated cholesterol levels and exceedingly high serum triglyceride levels. Each had mild, slowly progressive neuropathy characterized by pain in the feet, without proximal extension or involvement of the hands. [17] Weakness and autonomic symptoms and signs were absent. No other cause of peripheral neuropathy was found. In one patient, symptoms resolved with correction of hypertriglyceridemia. [17] Despite the possible confounding effect of elevated cholesterol, this observation suggests that marked increases in serum triglycerides may cause length-dependent painful small-fiber neuropathy.

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In a prospective study, Drory et al. evaluated 16 patients with marked hypertriglyceridemia without other causes of neuropathy, using nerve conduction and autonomic function tests. [18] Six subjects (37%) showed mild signs of asymptomatic polyneuropathy. They concluded that hypertriglyceridemia may be associated with a mild subclinical axonal polyneuropathy. [18]

This association was further explored in a larger study by [7] Kassem et al. in 2005. They investigated 24 patients with a high triglyceride level (>300mg/dl) without neurological complaints. They observed that over 2/3 of the patients had significant delays in the sural and median nerve distal latencies. In addition, over half of the patients had a significant decrease in the motor nerve conduction velocities while about 1/3 had a decrease in the sensory nerve conduction velocities. The amplitudes were relatively spared. This pattern does not fulfil the criteria for small fibre or axonal neuropathy.

Nevertheless, collectively these studies suggest that severe hypertriglyceridemia is associated with peripheral neuropathy. This conclusion is further supported by a recent case report [19] in which a patient with extremely high serum triglyceride (> 10,000 mg/dl) presented with severe peripheral neuropathy. However larger studies are warranted to fully characterize the clinical and neurophysiological presentation of hypertriglyceridemia-induced neuropathy.

**ROLE OF LIPID DISORDERS IN HIV-ASSOCIATED NEUROPATHIES**

In HIV populations that are aging due to improved longevity with combination antiretroviral therapy, both hypertriglyceridemia and sensory neuropathy have become increasingly common. Banerjee et al explored the relationship between hypertriglyceridemia and sensory neuropathy using a comparative study design in 436 HIV patients. [20] They observed that among HIV-positive patients, those with triglyceride levels ≥ 244 mg/dl were more likely to have sensory neuropathy than those with levels ≤ 142 mg/dl independent of other known risk factors (adjusted odds ratio 2.7, 95% confidence interval 1.4-5.5). [20] This observation further strengthens the link between hypertriglyceridemia and peripheral neuropathy.
ABETALIPOPROTEINEMIA AND NEUROPATHY

Rare disorder of lipid metabolism may also be associated with neuropathy. Abetalipoproteinemia, or Bassen-Kornzweig syndrome, is a rare autosomal recessive disorder with impaired absorption of fat and fat-soluble vitamins from food. It is caused by a deficiency of apolipoprotein B-48 and B-100, which are used in the synthesis and exportation of chylomicrons and VLDL respectively. [21] The association between abetalipoproteinemia and neuropathy has been explored only in few case reports.

In a case series of three sisters with abetalipoproteinemia, clinically, a progressive sensory neuropathy was found. [21] There was a diminution in the amplitude of sensory action potentials and a slight-to-moderate slowing in maximum sensory conduction velocity, initially most marked in distal portions of the nerves. Motor conduction was normal, although EMG indicated subclinical signs of partial chronic denervation. The sural nerves showed a decreased number of large fibers and evidence of paranodal demyelination and regeneration of unmyelinated fibers was found. [21] This pattern suggests a sensorimotor neuropathy.

TANGIER’S DISEASE AND NEUROPATHY

Tangiers disease is also known as familial alpha lipoprotein deficiency (analphalipoproteinemia). There are a few case reports describing the presence of chronic neuropathy in patients with this disease. This neuropathy is characterised by lipid accumulation in and subsequent damage of Schwann cells of myelinated and unmyelinated fibres. [22, 23] Rarely, there is suggestion of damage to the vasa nervorum with progressive sensorimotor distal neuropathy. [24]

DYSLIPIDEMIA AND AUTONOMIC NEUROPATHIES

The role of isolated dyslipidemia in autonomic neuropathies is not well characterized. However, as highlighted above, several studies have shown dyslipidemia to be an independent predictor of autonomic neuropathy in diabetes mellitus. Indeed Voulgari et al. reported an odds ratio of 1.35 for developing cardiac autonomic neuropathy in those with elevated LDL-
cholesterol, and an odds ratio of 1.30 in those with elevated triglycerides. However these findings require further confirmation in prospective large scale studies. [25]

STATINS, FIBRATES AND NEUROPATHY

If the idea that dyslipidemia contributes to the development of diabetic peripheral and autonomic neuropathy is true, lipid lowering drugs may be beneficial in the treatment of diabetic neuropathy.

Fenofibrate is a Peroxisome Proliferator-Activated Receptor-alpha PPARα agonist that lowers plasma lipids by improving their removal by the liver and improving fatty acid metabolism. In genetic dyslipidemia in mice, including ApoE knockout, leptin deficient, and LDL receptor knockout mice, fenofibrate improved the lipid profile and increased high-density lipoprotein. These lipid improvements correlate with prevention of insulin resistance and atherosclerosis. The Fremantle Diabetes Study was an observational investigation of 1,237 patients with type 2 diabetes. The data suggest that therapy with a statin or fibrate protects against diabetic peripheral sensory neuropathy, but calls for confirmatory evidence via a randomized clinical trial. [10]

Clinical evidence supporting the use of statins to prevent neuropathy is limited, although studies in animals have shown promising results. Studies in diabetic rats showed that rosuvastatin treatment improves nerve conduction velocity (NCV) and restores perception to thermal pain. Recent preclinical studies with rosuvastatin have also demonstrated improvements in NCV in a streptozotocin-induced rat model of diabetes, as well as improvements in hyperalgesia. [10]

The clinical benefits of statins on erectile dysfunction (ED) have not been established. There are some data to suggest that statins may improve nocturnal penile tumescence. Preclinical studies with rosuvastatin would tend to support a beneficial effect in ED. Studies are required to confirm whether this benefit of statin treatment is translated to diabetic patients. [8]

On the flipside of the coin, there is some evidence that statins may induce neuropathy. Lo described three patients who developed small fibre neuropathy after 1 month of statin therapy with clinical resolution upon prompt drug withdrawal. [26] All patients showed abnormal sympathetic skin responses (SSR) in comparison with controls. SSRs returned to normal in tandem with
clinical improvement. One patient redeveloped small and large fibre neuropathy when the similar drug was re-administered. [26]

Therefore more properly designed long-term randomised controlled clinical trials with exhaustive risk-benefit analysis are warranted to determine the role of statins, fibrates and ezetimibe in the treatment and prevention of neuropathies.

**CONCLUSION**

While hyperglycemia may play a key role in diabetic neuropathies in poorly controlled diabetes, dyslipidemia and other vascular risk factors become more important with improved glycemic control. [2, 3, 10] Therefore, aggregated vascular risk load assessment is the preferred integrated marker for diabetic neuropathy. This needs to be further confirmed in prospective studies. [2, 3] There is inconclusive evidence regarding the type of lipid disorder with the strongest association to diabetic neuropathy. This needs to be explored in large scale multicentre prospective studies.

The specific clinical and neurophysiological patterns of the different lipid disorders associated with neuropathy require better characterization. Furthermore, the underlying molecular mechanisms need to be well studied and outlined for further drug refinement and subcellular targeting [2, 3].

**REFERENCES**


The Association between Dyslipidemia and Neuropathy


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Chapter 8

**FENOFIBRATE-MEDIATED ANTI-ATHEROGENIC ACTIVITY: LONG-TERM FOLLOW-UP STUDY OF RHEUMATOID ARTHRITIS PATIENTS BY COMPARING WITH STATIN THERAPY**

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**ABSTRACT**

**Objectives**

Fenofibrate exerts multiple pleiotropic effects as a selective peroxisome proliferator-activated receptor alpha (PPAR-α) agonist in addition to its well-characterized lipid-lowering effects.

Fenofibrate suppressed the production of pro-inflammatory cytokines through the inhibition of NFκB pathway and ubiquitin-proteasome system, leading to the decrease in the incidence of atherosclerosis and type II diabetes mellitus (DM) that are tightly associated with chronic inflammation.

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Anti-inflammatory and anti-oxidant activities of fenofibrate have been studied in the chronic inflammatory diseases such as rheumatoid arthritis (RA) with a higher risk of atherosclerosis-related death.

Methods

In this chapter, our long-term follow-up study on the anti-inflammatory and anti-oxidative activities of fenofibrate in RA were compared with the parallel study of statins therapy and control concerning to the changes in the serum levels of lipid profile and inflammatory parameters including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), matrix metalloproteinase-3 (MMP-3), pentosidine and homocysteine.

Results

Both serum lipid profile and the most inflammatory parameters such as CRP, ESR and MMP-3 remained basically stable in all the 3 groups during the 18 months study. Although serum pentosidine and homocysteine levels elevated significantly in the control group without lipid-lowering agents, the two parameters did not change significantly in fenofibrate and statins groups. Prednisolone dosage decreased significantly in all the 3 groups.

Conclusions

Lipid-lowering agents such as fenofibrate and statins may play a role as anti-atherogenic and anti-oxidant agents in the chronic inflammatory disease like RA.

ABBREVIATIONS

AGE: advanced glycation end-product,
CRP: C-reactive protein,
DAS: disease activity score,
DM: diabtes mellitus,
DMARD: disease-modifying anti-rheumatic drug,
INTRODUCTION

The average life-span of the patients suffering from a variety of rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) has been decreased compared with the general population despite of the rapid improvement of disease control [1, 2, 3, 4, 5]. The major causes of death in the rheumatic diseases include infections of various kinds that are presumably associated with accelerated aging resulted from immunological abnormalities and immunosuppressive therapy inherent to the diseases [6]. These are followed by cardiovascular accidents associated with atherosclerosis-related diseases such as myocardial and cerebral infarctions [7, 8, 9].

Dyslipidemia, a risk factor for atherosclerosis, has been suspected to be an adverse event of steroid therapy in RA and SLE [10, 11, 12]. However, the anti-atherogenic activity of steroid has been described [13, 14]. Other risk factors for atherosclerosis include homocysteine and pentosidine: a family of advanced glycation end products (AGEs) generated under oxidative stress and aging [9, 15, 16, 17, 18, 19, 20].

The Trial of Atorvastatin in Rheumatoid Arthritis (TARA Study), comparing atorvastatin, a lipid-lowering statin, to a placebo was conducted by McCarey et al. in 2004. Six-month administration of atorvastatin significantly

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improved the Disease Activity Score 28 (DAS28), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and number of swollen joints. The anti-inflammatory effects of atorvastatin thus received special attention [21]. A report in Nature also detailed the clinical anti-inflammatory activity of fenofibrate, a lipid-lowering fibrate agonist of peroxisome proliferator-activated receptor α (PPAR-α) [22]. We thus anticipated that lipid-lowering agents with pleiotropic effects may serve also as anti-inflammatory agents in RA.

We compared the lipid-lowering activity and anti-inflammatory activity of fenofibrate and statins administered in the patients with RA complicated with dyslipidemia. In the previous communication, fenofibrate showed more anti-inflammatory and anti-dyslipidemic activities than stains during a relatively short period [23]. Thus, we have planned to study the long term effects on the other risk factors for atherosclerosis: serum homocysteine and pentosidine in the RA with dyslipidemia treated by fenofibrate or statins.

Table 1. Patients’ background

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fenofibrate</th>
<th>Statins</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients (male/female)</td>
<td>48 (17/31)</td>
<td>38 (8/30)</td>
<td>19 (6/13)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.8±15.4</td>
<td>64.0±9.1*</td>
<td>63.2±12.2</td>
</tr>
<tr>
<td>Complications</td>
<td>4 (Sjs=1, OA=2, Gout=1)</td>
<td>7 (Sjs=4, OA=2, Gout=1)</td>
<td>2 (Sjs=1, OA=1)</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.87±1.55</td>
<td>1.01±1.63</td>
<td>0.49±0.49</td>
</tr>
<tr>
<td>MMP-3 (ng/mL)</td>
<td>199.0±203.9</td>
<td>210.1±236.4</td>
<td>208.8±249.0</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>28.7±19.9</td>
<td>36.3±32.5</td>
<td>36.5±14.5*</td>
</tr>
</tbody>
</table>

Mean±SD.

* p<0.05 significant compared with control by Fisher’s exact test.

CRP C-reactive protein, MMP-3 matrix metalloproteinase 3, ESR erythrocyte sedimentation rate, Sjs Sjögren syndrome, OA osteoarthritis, MTX methotrexate, DMARD disease modifying antirheumatic drug.
MATERIALS AND METHODS

A total of 105 RA patients complicated with dyslipidemia were randomly assigned to the fenofibrate group (Lipidil®, Kaken Pharmaceutical Co., Ltd., 200 mg/day, n = 38), the statins group (n = 19) and control group without lipid-lowering agents (n=48). Statins used included atorvastatin (Lipitor:n=16), pitavastatin (Livalo:n=2) and pravastatin (Mevalotin:n=1). Diagnosis of dyslipidemia was made if one of the following criteria was met: total cholesterol (TC) ≥ 220 mg/dL, low-density lipoprotein-cholesterol (LDL-C) ≥ 140 mg/dL, high-density lipoprotein-cholesterol (HDL-C) < 40 mg/dL or triglycerides (TG) ≥ 150 mg/dL, as previously described [23].

The baseline blood chemistry (serum lipids profile, homocysteine, and pentosidine) was compared with those obtained 18 months after the start of the study. Homocysteine (nmol/ml) was examined by HPLC [24] and pentosidine (ug/ml) was assayed by ELISA as previously described [25].

The effects on RA activity were assessed by comparing the serum levels of CRP, ESR, MMP-3 and the dosage of prednisolone (PSL). An informed consent form, which had been formally approved by the Ethical Committee of Toh University of Yokohama, was used to explain the purpose of this study and the drugs to be administered to the patients.

The numerical results are expressed as mean ± S.D. For the statistical analysis, a Fisher’s exact test was used to assess the patients’ background, Wilcoxon signed rank test for the comparison between the baseline and post-treatment data, Mann-Whitney U test for intergroup comparison. Values below p < 0.05 were regarded as significant.

RESULTS

Patients’ Background

Patients’ data are shown in Table 1 and Figure 1. Each group included a few patients complicated with Sjogren syndrome (Sjs), osteoarthritis (OA) or gout as indicated in Table 1. Mean±SD age was 64.0±9.1 years old in the fenofibrate group (M=8,F=30), 63.2±12.2 years old in the statin group (M=6, F=13) and 57.8±15.4 years old in control group (M=17, F=31). Although there were no significant differences in sex distribution among 3 groups, the age in fenofibrate group was significantly older than control group (p<0.05). The
disease activity assessed by CRP (mg/dl), ESR (mm/h) and MMP-3 (ng/ml) was similar between 3 groups except that ESR in the statins group was significantly higher than control group (p<0.05).

Among disease-modifying anti-rheumatic drug (DMARD) users, methotrexate (MTX) was prescribed to 10 of 38 patients (26.3%) in the fenofibrate group, 7 of 19 (36.8%) in the statins group and 21 of 48 (43.8%) in the control group. In Japan, the dosage of MTX has been officially regulated below 8 mg/week during the present study. In addition, all the MTX users were supplemented with folate. No biologic users were in the statins group (control vs statins; p<0.05), but 3 of 38 (7.9%) in the fenofibrate and 9 of 48 (18.8%) in the control group. Steroid (prednisolone <10mg/day) was similarly prescribed to 32 of 48 (66.7%) in control group, 23 of 38 (60.5%) in fenofibrate and 9 of 19 (47.4%) in statins groups.

Figure 1. Changes in serum lipid levels.

Mean±SD. Open circles represent control. Closed circles represent fenofibrate. Open triangles represent statins.* p<0.05, ** p<0.01, *** p<0.001 significant compared with control by Wilcoxon signed rank test. TC total cholesterol, LDL-C low-density lipoprotein-cholesterol, TG triglycerides, HDL-C high-density lipoprotein-cholesterol.
Baseline HDL-C was similar between 3 groups, but baseline TC, LDL-C and TG in control group were significantly lower than fenofibrate group (p<0.001, p<0.001 and <0.05) and statins group (p<0.01, p<0.01 and <0.05). The baseline lipid profile (TC, HDL-C, LDL-C, and TG) was comparable between fenofibrate and statins groups as shown in Figure 1.

No adverse episode including changes or addition of other DMARDs was recorded during the study.

**Improvement in Serum Lipid Profiles**

As shown in Figure 1, lipid profile in fenofibrate group improved after 18 months (TC: from 219.7±34.6 to 208.4±25.6, HDL-C: from 68.8±20.0 to 70.1±22.1, LDL-C: from 130.7±26.3 to 120.0±21.0, and TG: from 121.2±54.0 to 105.4±47.1), but the changes did not reach a statistical significance. In both control and statins groups, the lipid profile basically remained unchanged.

Mean±SD. Open circles represent control. Closed circles represent fenofibrate. Open triangles represent statins.** p<0.01, *** p<0.001, Wilcoxon signed rank test versus baseline.

Figure 2. Changes in prednisolone (PSL) dosage.
Improvement of Inflammatory Activity

The dosage of PSL decreased significantly in all the three groups after the 18 months compared with the baseline (fenofibrate group <0.001, control group <0.001, and statins group <0.01) (Figure 2). Among 3 groups, the reduction rate of PSL was more pronounced, but not statistically significant in fenofibrate group (-62.1±33.9%) than control (-47.6±48%) and statins groups (-44.4±37.5%). Although the CRP and ESR levels insignificantly decreased from 1.01±1.63 to 0.57±1.01 mg/dl and from 38.3±32.5 to 31.8±24.7 mm/h in the fenofibrate group, the 2 parameter levels in statins and control groups remained unchanged. MMP-3 level insignificantly decreased in all the groups.

Changes in Serum Pentosidine and Homocysteine Levels

Both pentosidine and homocysteine levels increase with normal aging and inflammation [16, 19, 20]. As indicated in Figure 3, serum Δpentosidine (97.7±198.9%, p<0.01) and Δhomocysteine (10.6±31.4%, p<0.05) increased significantly after 18 months in control group without lipid-lowering treatment. In contrast, Δpentosidine did not change significantly in fenofibrate (44.3 ±233.4%) and statins (47.4±187.6%) groups.

![Graph](image)

Figure 3. % changes in Δpentosidine and Δhomocysteine.

Mean±SD.* p<0.05, ** p<0.01, Wilcoxon signed rank test versus baseline. * p<0.05, Mann-Whitney U test versus control.
Δpentosidine in statins group decreased significantly compared with the control (p<0.05). Pentosidine both in fenofibrate (from 0.09±0.10 to 0.09±0.13) and statins (from 0.12±0.12 to 0.13±0.16) groups was unchanged, while pentosidine in control group (0.11±0.12 to 0.15±0.16) increased significantly compared with fenofibrate group (p<0.05) after 18 months treatment.

Also, Δhomocysteine remained unchanged in fenofibrate (11.4±35.5%) and statins (2.7±19.8%) groups (Figure 3). However, homocysteine level increased insignificantly in 3 groups.

Figure 4 showed the contribution of MTX to serum homocysteine level. Δhomocysteine in normal control group insignificantly elevated irrespective of MTX therapy. However, Δhomocysteine in both fenofibrate and statins groups insignificantly decreased by MTX usage.

Serum pentosidine level did not change significantly between with and without MTX in all the 3 groups (data not shown). Thus, low dose MTX did not affect the serum levels of homocysteine and pentosidine significantly.
DISCUSSION

Both elevation of serum homocysteine and pentosidine were suppressed by the use of fenofibrate or statins compared with control group without lipid-lowering agents, though the lipid-lowering agents including fenofibrate and statins insignificantly improved serum lipid levels after the 18 months treatment.

Homocysteine: a traditional risk factor for cardiovascular diseases, has been increased with normal aging and cardiovascular diseases that may be associated with chronic inflammation [26, 27, 28, 29]. Although an increase in homocysteine level in general population with dyslipidemia by fenofibrate has been frequently reported [30, 31, 32], this may not the case in Japanese RA patients as was shown in the present study.

Serum homocysteine was reported to be elevated in RA treated with higher dose (25~40mg/week) MTX [33] or even by low-dose MTX [34], though concomitant folate supplementation may decrease the plasma homocysteine level [34] and most anti-RA drugs (NSAIDs, steroids, biologics, leflunomide, COX-2 inhibitors) may not affect major adverse effects on cardiovascular risk factors [35]. In the present study, 3~40% patients were treated with low dose MTX in combination with folate supplementation that may not affect homocysteine metabolism, as was shown in Figure 4.

Homocysteine may be produced by inflammatory mechanism through NFκB pathway that may be blocked through PPARα (fenofibrate) and/or statins [22, 36].

Pentosidine: a member of AGEs and a biomarker of chronic inflammation has been reported to elevate with normal aging and in RA [37, 38, 39, 40, 41]. Aging has been proposed as mediated by systemic, chronic, low grade inflammation [6] and RA is a typical of diseases mediated by a systemic, chronic inflammation. The serumpentosidine level may be reflection of chronic inflammation of unknown etiology. And most anti-inflammatory /anti-rheumatic treatment for RA decreased serum pentosidine [41, 42, 43, 44].

There may be possible reasons why anti-dyslipidemic activity of both fenofibrate and statins in the present long-term study was not clear in spite of the previous report that fenofibrate improved the most lipid levels significantly in our short-term study [23]. 1) the dyslipidemia assessed by lipid profile in Japanese population was basically mild probably due to the traditional diet [45, 46] and the contribution of lipid-lowering agents to the treatment may be sometimes difficult to assess. 2) To treat chronic inflammatory diseases such
as RA, a combination of several drugs with different mechanisms of action that may affect lipid metabolism has been routinely used [9, 10, 14].

In conclusion, anti-inflammatory therapy including anti-RA drugs, fenofibrate and statins may be useful to prevent inflammation-associated cardiovascular diseases with dyslipidemia, hyperhomocysteinemia and elevated serum pentosidine.

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Fenofibrate-Mediated Anti-Atherogenic Activity


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